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Regulation of GATA-2 Phosphorylation by Mitogen-activated Protein Kinase and Interleukin-3*

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GATA-2 is a member of a family of transcription factors which bind a common DNA sequence motif (WGATAR) through an evolutionarily conserved zinc finger domain. An essential role for GATA-2 in the development of hematopoietic stem cells has recently been shown in gene targeting experiments in mice. Here we show that GATA-2 exists in hematopoietic progenitor cells as a phosphoprotein. Stimulation of progenitors with interleukin-3 (IL-3) results in enhanced phosphorylation of GATA-2 which occurs within 5 min. IL-3 is known to signal in part through mitogen-activated protein (MAP) kinase, and evidence for MAP kinase signaling in the control of GATA-2 phosphorylation was obtained by genetically manipulating the MAP kinase pathway in COS cells using either constitutively activating or interfering mutants of MAP kinase kinase. Furthermore, using an interfering mutant of MAP kinase kinase, we directly demonstrated a critical role for the MAP kinase pathway in the IL-3-dependent phosphorylation of GATA-2 in hematopoietic progenitor cells. Finally, *in vitro* phosphorylation experiments using recombinant GATA-2 raise the possibility that MAP kinase itself may phosphorylate GATA-2. Our results provide evidence for phosphorylation via the MAP kinase pathway constituting a cytoplasmic link between GATA-2 and growth factor receptors and are consistent with the hypothesis that GATA-2 is involved in the growth factor responsiveness and proliferation control of hematopoietic progenitor cells.

Hematopoietic stem cells are characterized by both their ability to self-renew and their capacity to undergo terminal differentiation down one of at least eight different lineage pathways. Cells in which the balance between self-renewal and differentiation is constitutively dysregulated in favor of self-renewal are thought to be more prone to leukemogenesis (1). The mechanisms controlling the choice between self-renewal and differentiation are not fully understood, but seem likely to involve receptor-mediated signals producing changes in the functional balance of cellular transcription factors or complexes. In this context, elucidating the signal transduction pathways that link growth factor receptors at the cell surface to transcription factors in the nucleus, is germane to a full understanding of the mechanisms underlying both the normal regulation of hematopoietic stem cells and their pathology.

We have been focusing on the GATA family of transcription factors which are emerging as key regulators of hematopoietic cell fate. GATA factors are characterized by their ability to bind a common DNA sequence motif (WGATAR) by virtue of an evolutionarily conserved C_2C_2 zinc finger DNA binding domain (2). To date, four GATA factors have been described (GATA-1–4); of these only three (GATA-1, -2, and 3) are expressed in hematopoietic cells, where their pattern of expression is complex and may show some minor species variation. GATA-1, originally cloned from erythroid cells (3, 4), has been shown to be expressed also in megakaryocytes and mast cells (5, 6). In gene targeting experiments, loss of GATA-1 function results in a block in erythroid differentiation at the proerythroblast stage (7, 8). GATA-3 expression in mammalian hematopoietic cells is restricted to T-lymphocytes (9, 10), although it is interesting to note that GATA-3 was originally cloned from chicken erythroid cells where it is expressed at a low level (11). Ectopic expression of GATA-2 in chicken erythroid progenitor cells blocks differentiation potential (12). GATA-2 is expressed not only in early erythroid cells but also in multipotent hematopoietic progenitor cells (13–16). Recently, gene targeting experiments in mouse embryonic stem cells have demonstrated that GATA-$2^{-/-}$ mice have a severe deficit in all hematopoietic lineages, suggesting a critical role for GATA-2 in the biology of the progenitor cells of definitive hematopoiesis (17).

A large number of transcription factors have been shown to exist within cells as phosphoproteins. The functional consequences of phosphorylation vary but include regulation of intracellular localization, DNA binding, and transcriptional regulation (18). Only recently have the protein kinase cascades involved in growth factor-induced phosphorylation events begun to be elucidated. Many receptor tyrosine kinases and cytokine receptors, including interleukin-3 (IL-3) which is a key cytokine regulator of progenitor cell self-renewal (19, 20), have been shown to activate the ras-MAP kinase ((mitogen-activated protein kinase), also known as ERK (extracellular signal-regulated kinase)) pathway (21–24). MAP kinases (MAPKs)[1] are one element in a series of kinases that serve to connect the nucleus with cytosolic and plasma membrane events. The idea that phosphorylation of transcription factors by MAPKs might provide the cytoplasmic link between receptor-mediated events and changes in gene expression in the nucleus is supported by the observation that activated MAPKs can enter the nucleus (25, 26). MAPK substrates include cytosolic phospholipase A_2

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; GMSA, gel mobility shift assay; FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; CAT, chloramphenicol acetyltransferase; GST, glutathione *S*-transferase; MBP, myelin basic protein; SLF, steel factor.

(27) and a number of transcription factors, *e.g.* c-*myc* (28), c-*jun* (29), and Elk-1 (30).

Until recently, little has been known about the phosphorylation status of the GATA family of transcription factors. A very recent report (31) demonstrates that GATA-1 exists as a phosphoprotein in erythroid cells with phosphorylation occurring on serine residues. Both the function of this serine phosphorylation and whether it is regulated by signaling remain unknown. The goal of the studies presented here was to determine if GATA-2 exists as a phosphoprotein within the hematopoietic progenitor cell compartment and, if so, whether this phosphorylation is linked to signal transduction pathways controlling the proliferation status of cells. In this report we present evidence that within hematopoietic progenitor cells, GATA-2 is phosphorylated through the MAPK signal transduction pathway in response to IL-3.

MATERIALS AND METHODS

GMSA and Supershift Assays—Nuclear protein extracts were prepared as described (32), with the omission of the final dialysis step. Gel retardation assays were performed by mixing 1 μl (approximately 1.5 μg of total protein) of nuclear extract with 0.1 ng of a radiolabeled GATA-containing oligonucleotide, in the presence of 2 μg of poly(dI-dC)·poly(dI-dC) as nonspecific competitor DNA. The mixture was incubated in binding buffer (20 mM HEPES, pH 7.9, 50 mM NaCl, 1 mM $MgCl_2$, 4% Ficoll) for 20 min at room temperature before electrophoresis at 250 V through a 4% polyacrylamide gel, prepared and run in 0.25 × TBE buffer. For the antibody supershift experiments, a rat anti-GATA-1 monoclonal antibody (N6, Santa Cruz Biotechnology Inc.) and anti-GATA-2 polyclonal antibody (a kind gift of S. H. Orkin) were added to the incubation last. The sequence of the double-stranded GATA-containing oligonucleotide used (derived from the mouse α1-globin promoter) was 5′-GAGGTGATCCAGGCAACTGATAAGGATTCCCA-3′.

Cell Labeling and Immunoprecipitation—The HL-60 cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 4 mM L-glutamine, and antibiotics. 416B cells were maintained in Fischer's medium supplemented with 20% horse serum, 4 mM L-glutamine, and antibiotics. The IL-3-dependent pro-B cell lines, BA/F3 (33) and Bcl-2-BA/F3 (BA/F3 expressing the human Bcl-2 gene product) (34), were maintained in RPMI 1640 supplemented with 10% FCS and 2% mIL-3 conditioned medium. All cell lines were grown at 37 °C in the presence of 5% CO_2, in a highly humidified incubator. Cell labeling was performed as follows. Cells were incubated in phosphate-free medium containing dialyzed FCS and [^{32}P]orthophosphate (1 mCi/ml, Amersham Corp.) or in methionine/cysteine-free medium containing dialyzed FCS and [^{35}S]methionine/cysteine (100 μCi/ml, Amersham) 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 phenylmethylsulfonyl fluoride. After incubating for 1 h at 4 °C, an equal volume of RIPA buffer without NaCl was added to the extracts followed by centrifugation at 17,000 × *g* for 20 min. The GATA-2 protein was then isolated from the supernatants by immunoprecipitation using 5 μg of agarose-conjugated monoclonal anti-GATA-2 antibody (Santa Cruz Biotechnology Inc.). The immunoprecipitates were washed 5 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, fractionated by SDS-PAGE, then transferred to PVDF membrane (Bio-Rad) prior to autoradiography.

PAGE and Immunoblotting—Standard Laemmli-type SDS-PAGE gels were made using an acrylamide mixture that contained a 29:1 ratio of acrylamide to *N,N′*-methylenebisacrylamide (bisacrylamide). SDS-PAGE gels used to separate phosphorylated from nonphosphorylated GATA-2 and MAPK contained an acrylamide/bisacrylamide ratio of 30:0.165. For Western analysis, gels were electroblotted onto PVDF membranes using a semidry transfer apparatus. Three different rabbit polyclonal antisera were used to detect GATA-2; one (a kind gift of S. H. Orkin) was raised against the N terminus of murine GATA-2, the other two antibodies (kind gifts of R. K. Patient and D. Bertwhistle) were raised against peptides derived from different regions of *Xenopus* GATA-2 and cross-reacted with human and murine GATA-2. Transfected MAPK was detected using monoclonal antibody 9E10 (35), which is directed against the *myc* tag present in the *myc*-ERK2 expression vector. Endogenous MAPK was detected using polyclonal rabbit antiserum 122 (36).

Transient Expression in COS Cells—COS cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS. Transfection was carried out using the DEAE-dextran method, as described previously (37). Cells were allowed to recover for 24 h prior to a 24-h period of serum starvation. After 24 h, the culture medium was aspirated and replaced with phosphate-free DMEM without serum. When required, EGF was added just before harvesting at a concentration of 10 ng/ml for 7 min. After rinsing twice with cold DMEM, the cells were harvested and lysed in S buffer (20 mM Tris-HCl, pH 8.0, 40 mM sodium pyrophosphate, 50 mM sodium fluoride, 5 mM $MgCl_2$, 100 μM sodium orthovanadate, 10 mM EGTA) including 2× detergents and protease inhibitors (2% Triton X-100, 1% sodium deoxycholate, 40 μg/ml leupeptin, 40 μg/ml aprotinin, 6 mM phenylmethylsulfonyl fluoride). After centrifugation, the supernatants were diluted using 1 volume of S buffer without detergents and used in immunoprecipitation or Western blot analyses.

The conditions for co-transfection of the chloramphenicol acetyltransferase (CAT) reporter with the CA-MAPKK and pMT2-GATA-2 expression vectors were the same as described above. The reporter plasmid, $p\alpha^D6$, which comprises concatamerized GATA-motif oligomers linked to an α^D-globin promoter driving a CAT reporter gene (38), was a kind gift of G. Felsenfeld. Cell extracts for CAT assays were prepared and CAT assays were performed, as described previously (30). The total amount of DNA transfected within each experiment was kept constant with the use of carrier plasmid DNA. Following autoradiography, the regions of the thin layer chromatography plate containing labeled chloramphenicol or its acetylated forms were individually cut out and counted by liquid scintillation to determine the conversion percentage.

In Vitro Phosphorylation Assays—The coding region of the human GATA-2 cDNA (amino acids 1–480) was isolated from pMT2-GATA-2 by restriction endonuclease digestion with *NcoI*, 3′-end-filled by Klenow fragment, and cloned into the *Sma*I site of pGEX-2T (Pharmacia Biotechnology Inc.) containing glutathione *S*-transferase (GST). The integrity of the final construct, pGEX-2T-GATA-2, was confirmed by sequencing. Expression of GST-GATA-2 in *Escherichia coli* (DE3) was induced for 3 h with 0.1 mM isopropylthiogalactoside and the GST-GATA-2 fusion protein purified using a GST column (Amersham) according to the manufacturer's instructions. Two μg of each GST fusion protein or 15 μg of myelin basic protein (MBP) was incubated with thiophosphorylated MAPK (ERK2/$p42^{mapk}$) in a buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 μM ATP, and 0.5 μCi of [γ-^{32}P]ATP in a 40 μl reaction volume. The phosphorylation reaction was performed at 30 °C and terminated after 30 min by the addition of Laemmli's sample buffer. The phosphorylated proteins were analyzed by SDS-PAGE and transferred to PVDF membrane prior to autoradiography, phosphoamino acid analysis, or phosphopeptide mapping.

Phosphoamino Acid Analysis—Labeled protein excised from PVDF membrane was hydrolyzed in 5.7 M HCl for 1 h at 110 °C, dried by Speed Vac, and applied to thin layer electrophoresis at pH 1.9 with control phosphoserine, phosphothreonine, and phosphotyrosine. The plate was dried and the position of each phosphoamino acid was detected by ninhydrin before exposing to X-Omat film.

Phosphopeptide Mapping—To generate a tryptic digest, the Immobilon fragment containing the labeled protein was incubated with 1 mg/ml tosylphenylalanyl chloromethyl ketone-treated trypsin for 18 h at 37 °C followed by oxidization by performic acid. Phosphopeptide mapping was performed by two-dimensional separation on cellulose thin layer plates. The first dimension was electrophoresis at pH 2.1. The second dimension was chromatography in the vertical direction using 1-butanol:pyridine:acetic acid:water (75:50:15:60, v/v).

RESULTS

GATA-2 Is Phosphorylated in Hematopoietic Progenitor Cells—As a cellular model for the study of transcription factor GATA-2 in multipotential progenitors we initially made use of the multimyeloid progenitor cell line 416B which was originally derived from long term culture of mouse bone marrow cells (39). The GATA profile of 416B cells at the protein level was assessed by gel mobility shift assay (GMSA) using a GATA-containing oligomer derived from the mouse α-globin gene promoter. These data are presented in Fig. 1*A* and show that 416B cells contain GATA binding activity. Antibody supershift analysis using an anti-GATA-1 monoclonal antibody and an anti-GATA-2 specific polyclonal antiserum demonstrated that the predominant GATA activity in these cells is attributable to

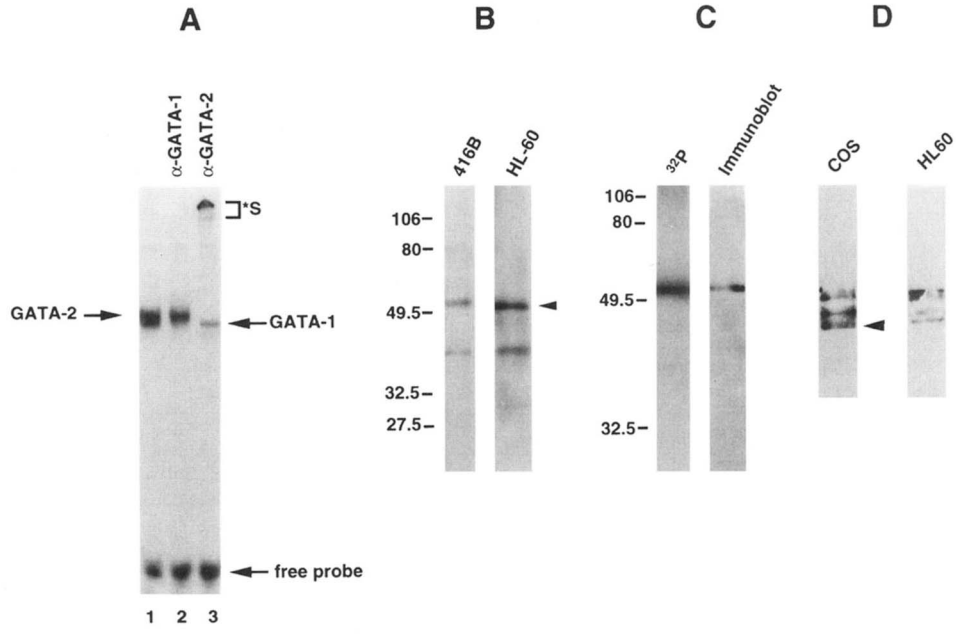

FIG. 1. **Phosphorylation of GATA-2 in hematopoietic progenitor cells.** *A*, a 416B nuclear protein extract was assayed by GMSA using a radiolabeled GATA motif-containing oligomer derived from the mouse α-globin promoter. The positions of GATA-1 and GATA-2 protein-DNA complexes (*arrow* in the figure) were confirmed by the addition of anti-GATA-1 and anti-GATA-2 antibodies; the GATA-2 supershift (*bracketed* in the figure) comprises a discrete band as well as slow migrating complexes which remain in the region of the slot (**S*). *B*, immunoprecipitations of [^{32}P]orthophosphate-labeled murine 416B and human HL-60 cells were performed using an agarose-conjugated, anti-GATA-2 monoclonal antibody. The expected migration position for authentic human GATA-2 protein is indicated by an *arrowhead*. The identity of the faster migrating bands observed in both cell lines (approximately 38 kDa) remains obscure. The *numbers* next to the autoradiographs indicate relative molecular mass in kilodaltons. *C*, the *left lane* shows data obtained using an agarose-conjugated anti-GATA-2 monoclonal antibody for immunoprecipitation of [^{32}P]orthophosphate-labeled COS cells which had been transfected with a human GATA-2 expression vector (pMT2-GATA-2). The *right lane* shows immunoblot analysis of the same material using anti-GATA-2 polyclonal antisera. The SDS-PAGE fractionated material was transferred to PVDF membrane prior to autoradiography and ECL-based Western analysis. The *numbers* next to the autoradiographs indicate relative molecular mass in kilodaltons. *D, left lane*, material, immunoprecipitated from pMT2-GATA-2-transfected COS cells using an agarose-conjugated anti-GATA-2 monoclonal antibody, was fractionated on modified SDS-polyacrylamide gels that contain a low amount of bisacrylamide, Western blotted using anti-GATA-2 polyclonal antisera, and visualized using ECL. *Right panel*, 5 mg of HL-60 whole cell lysate was immunoprecipitated with an anti-GATA-2 monoclonal antibody, fractionated by modified SDS-PAGE, and immunoblotted with anti-GATA-2 polyclonal antisera.

GATA-2. To determine whether GATA-2 is phosphorylated *in vivo*, we labeled logarithmically growing 416B cells using [^{32}P]orthophosphate. GATA-2 was immunoprecipitated from labeled cell extracts using an anti-GATA-2 specific monoclonal antibody and analyzed by SDS-PAGE and subsequent autoradiography. These results are shown in Fig. 1*B*. In 416B cells, the anti-GATA-2 monoclonal antibody immunoprecipitated two major ^{32}P-labeled proteins of 54 kDa and 38 kDa (Fig. 1*B*). We found a pattern of bands similar to that observed in murine 416B cells when labeled extracts from human HL-60 cells were immunoprecipitated with the same anti-GATA-2 antibody; HL-60 cells are considered to represent a transformed promyelocytic progenitor cell and can be chemically induced to undergo macrophage or granulocyte differentiation. The identity of the 54-kDa band as GATA-2 was confirmed in two independent ways. Firstly, immunoprecipitation of lysates derived from ^{32}P-labeled COS cells transfected with a human GATA-2 cDNA expression vector (murine GATA-2 cDNA clones have not been reported) yielded a similarly sized product (Fig. 1*C*). Secondly, Western blot analysis of the 416B, HL-60, and GATA-2 transfected COS cell immunoprecipitates using three different rabbit anti-GATA-2 polyclonal antisera confirmed the 54-kDa band as GATA-2. In these experiments, the 38-kDa band was not reactive with any of the three different polyclonal antisera used; a typical result obtained from immunoblotting of GATA-2 transfected COS cells is shown in Fig. 1*C*. Since the polyclonal antibodies used were raised against peptides derived from different portions of the GATA-2 protein, it seems unlikely that the unreactive 38-kDa protein is either a degradation product of GATA-2 or a GATA-2 isoform. It may therefore represent a co-immunoprecipitated phosphoprotein partner of GATA-2.

Taken together, these data demonstrate that GATA-2 exists as a phosphoprotein in proliferating hematopoietic progenitor cells. In considering the significance of such a result, it is important to determine what proportion of the total GATA-2 in these cells exists in the phosphorylated form. Using polyacrylamide gels with a low percentage of bisacrylamide, the phosphorylated and unphosphorylated forms of GATA-2 could be resolved (Fig. 1*D*). Analysis of the phosphorylation status of these differently migrating forms of GATA-2 by both ^{32}P labeling and alkaline phosphatase treatment (data not shown) demonstrated that the fastest migrating band represents the unphosphorylated GATA-2 (*arrow* in Fig. 1*D*). Using this gel system, over 80% of GATA-2 was found to be phosphorylated in exponentially growing HL-60 cells (Fig. 1*D*).

Regulation of GATA-2 Phosphorylation by IL-3—Within the hematopoietic system, soluble or stromal matrix-associated growth factors play a key role in regulating the self-renewal and differentiation of stem cells. One of these, IL-3, can support the proliferation of murine multipotent stem cells and the development of different classes of lineage-restricted progenitor cells. In recent years, a number of murine hematopoietic progenitor cell lines have been derived which are critically dependent on IL-3 for their continued growth and survival; when IL-3 is withdrawn, these cells die by apoptosis. BA/F3 is an IL-3-dependent murine hematopoietic progenitor cell line initially thought to be representative of the pro-B cell compartment (33), although recent evidence suggests that it may have erythroid if not multilineage potential. The presence of GATA-2 mRNA in these cells has recently been reported (40) and by

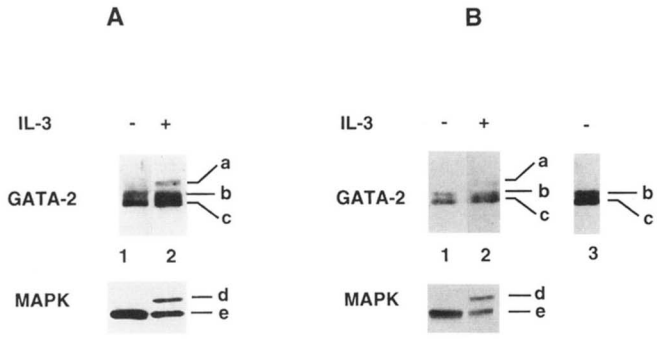

FIG. 2. **Stimulation of GATA-2 phosphorylation by exposure to IL-3.** IL-3-dependent murine hematopoietic progenitor cells were assayed for the phosphorylation status of endogenous GATA-2 and MAPK under conditions of IL-3 deprivation and IL-3 restimulation. The different phosphorylated forms of GATA-2 are labeled *a*, *b*, and *c*, while *d* and *e* represent the phosphorylated (activated) and unphosphorylated forms of MAPK. For analysis of GATA-2 phosphorylation, cell lysates were immunoprecipitated with an agarose-conjugated anti-GATA-2 monoclonal antibody prior to SDS-PAGE and subsequent Western blotting using an anti-GATA-2 polyclonal antiserum. For analysis of MAPK activity, cell lysates were directly Western blotted using anti-ERK-2 antibody. *Panel A*, analysis of BA/F3 cells deprived of IL-3 for 6 h (*lane 1*) and then stimulated with IL-3 for 5 min (*lane 2*). *Panel B*, analysis of Bcl-2-BA/F3 cells deprived of IL-3 for 24 h (*lane 1*) and then stimulated with IL-3 for 5 min (*lane 2*); *lane 3* is a longer exposure of *lane 1*.

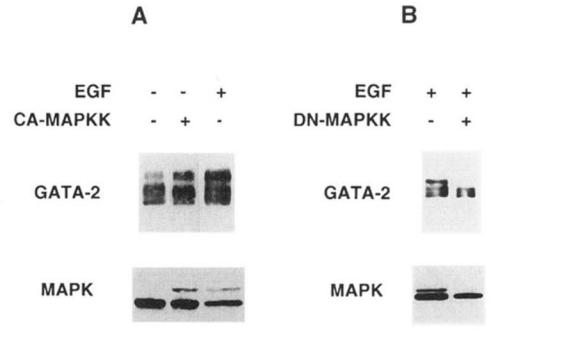

FIG. 3. **Manipulation of the MAPK pathway in COS cells.** COS cells were co-transfected with human GATA-2 and *myc*-tagged MAPK expression vectors. MAPK activity in these cells was modulated either by stimulation with EGF or by co-transfection of either constitutively activated or dominant negative MAPKK. *A*, COS cells transfected with both pMT2-GATA-2 (5 μg) and *myc*-ERK2 (3 μg) as well as 5 μg of either constitutively active MAPKK (*CA-MAPKK*) expression vector or the vector alone. *B*, COS cells transfected with pMT2-GATA-2 (3 μg) and *myc*-ERK2 (1 μg) as well as 7 μg of either a dominant negative MAPKK (*DN-MAPKK*) expression vector or the empty vector. 24 h post-transfection, the culture medium was replaced with serum-free DMEM, and the cells were incubated for an additional 24 h. EGF was then added to the indicated samples for 7 min at a concentration of 10 ng/ml. The cells were lysed in S buffer, and GATA-2 was immunoprecipitated with the anti-GATA-2 monoclonal antibody, fractionated by modified SDS-PAGE and immunoblotted using anti-GATA-2 polyclonal antiserum; these results are presented in the *upper panels* of *A* and *B*. An equivalent amount of lysate was analyzed for MAPK activity by direct Western blotting using antibody 9E10 directed against the *myc* tag; the results of this analysis are presented in the corresponding *lower panels* of *A* and *B*.

GMSA we have confirmed that GATA-2 is indeed present as a DNA binding protein in these cells (data not shown). We used BA/F3 cells to ask whether the phosphorylation of GATA-2 is regulated by IL-3. We compared the phosphorylation status of GATA-2 in IL-3-deprived *versus* IL-3-stimulated BA/F3 cells. BA/F3 cells were deprived of IL-3 for 6 h and then re-exposed to IL-3 for 5 min. GATA-2 protein was immunoprecipitated using anti-GATA-2 monoclonal antibodies, fractionated on low-bis polyacrylamide gels, and then Western blotted using anti-GATA-2 polyclonal antiserum (Fig. 2*A*). The pattern of bands obtained is slightly different from that seen in Fig. 1*D*; this may reflect either differences in GATA-2 phosphorylation states between BA/F3 and HL-60 or differences in mobility between human and mouse GATA-2. In the IL-3-deprived BA/F3 cells (*lane 1*), the predominant form of GATA-2 is the fast migrating form (labeled *c* in Fig. 2*A*). Stimulation of the IL-3-deprived cells for 5 min with IL-3 results in enhanced phosphorylation of GATA-2 (Fig. 2*A*, *lane 2*).

Although the phosphorylation of GATA-2 appears to be enhanced as a result of IL-3 stimulation, phosphorylated forms of GATA-2 are clearly still present in IL-3-deprived cells. This may reflect the fact that the period of IL-3 deprivation was only 6 h; increasing the period of IL-3 deprivation in BA/F3 cells is not possible since 6 h is the maximum time that these cells can be kept in the absence of IL-3 before initiating an apoptotic program. However, the onset of apoptosis as a result of IL-3 withdrawal is considerably delayed in BA/F3 cells which express the human Bcl-2 gene product (Bcl-2-BA/F3) (34). In these cells, after 24 h in the absence of IL-3, the b-form of GATA-2 was clearly still present (Fig. 2*B*, *lane 1*). In contrast, there was no evidence of the a-form of GATA-2 (Fig. 2*B*, *lane 1*) even after longer exposures of the film (see *lane 3*); the a-form appeared rapidly after stimulation with IL-3 (Fig. 2*B*, *lane 2*).

IL-3 is known to signal in part through the MAPK pathway (41, 42). Like GATA-2 phosphorylation, MAPK activation occurs rapidly in response to IL-3 signaling. We therefore analyzed the status of activation of MAPK in our BA/F3 and Bcl-2-BA/F3 lysates; the phosphorylated and nonphosphorylated forms of MAPK (labeled *d* and *e*, respectively, in the *lower panels* of Fig. 2) can be separated by PAGE where the phosphorylated form migrates more slowly (43, 44). The results show the absence of phosphorylated MAPK in IL-3-deprived cells and its rapid appearance after 5 min of re-exposure to IL-3.

Control of GATA-2 Phosphorylation by the MAPK Pathway—Our results in BA/F3 cells demonstrate that IL-3 signaling modulates GATA-2 phosphorylation and further raise the possibility that the MAPK pathway may be involved in this process. We initially tested this hypothesis by manipulating the MAPK pathway using the COS cell transient transfection system. COS cells contain no endogenous GATA-2 activity (45). MAPK activity in COS cells can be suppressed by serum starvation and then rapidly stimulated by a brief exposure of the cells to epidermal growth factor (EGF). MAPK activity can also be modulated genetically using functional mutants of MAP kinase kinase (MAPKK) (46) whereby glutamic acid substitutions at Ser-217 and Ser-221 residues produce a constitutively active MAPKK mutant that activates MAPK by specific phosphorylation of threonine and tyrosine residues (44, 46). MAPKK molecules in which these same serines have been mutated to alanine residues act as interfering, or dominant negative, mutants which block MAPK activation (44, 46). COS cells were transfected with GATA-2 and MAPK expression vectors and serum-deprived for 24 h. MAPK activity was modulated both by EGF stimulation and through co-transfection of constitutively activated MAPKK (CA-MAPKK) or dominant negative (DN-MAPKK) mutants of MAPKK. The results obtained with the CA-MAPKK mutant are presented in Fig. 3*A* and show that co-transfection of this mutant causes a relative increase in the abundance of the slower migrating phosphorylated forms of GATA-2. The effect caused by this mutant mimics that observed in response to EGF stimulation. The lower panel shows that the increased GATA-2 phosphorylation observed in these experiments correlates well with the levels of phosphorylated MAPK present in the cells. Further evidence for the role of the MAPK pathway in the control of GATA-2 phosphorylation was obtained using the interfering MAPKK mutant

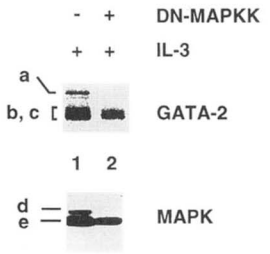

FIG. 4. **An interfering MAPKK mutant abrogates the IL-3-dependent GATA-2 phosphorylation in hematopoietic progenitor cells.** ^{35}S-Labeled parental BA/F3 cells (*lane 1*) and BAF3/DN-MAPKK cells (*lane 2*) were deprived of IL-3 for 6 h and then stimulated with IL-3 for 5 min. Cell lysates were immunoprecipitated with an agarose-conjugated anti-GATA-2 monoclonal antibody followed by SDS-PAGE. Analysis of MAPK activity was performed as described in Fig. 2.

(DN-MAPKK) which acts as a dominant negative *in vivo*. These results are presented in Fig. 3*B* and show that, in the presence of the DN-MAPKK expression vector, EGF stimulation did not result in the production of the uppermost phosphorylated forms of GATA-2 (*upper panel*). The *lower panel* shows that the DN-MAPKK expression vector successfully suppressed MAPK activity in these experiments.

These data obtained in COS cells provide strong evidence for the involvement of the MAPK pathway in the regulation of GATA-2 phosphorylation. We next addressed the critical issue as to whether the MAPK pathway actually mediates the IL-3-dependent phosphorylation of GATA-2 in hematopoietic progenitor cells. BA/F3 cells were stably co-electroporated with eukaryotic expression vectors containing the DN-MAPKK mutant (pEF-DN-MAPKK) and a selective puromycin resistance gene (pBABE-puro); DN-MAPKK expression in the stable transfectants was confirmed by Western blotting (data not shown). The phosphorylation of GATA-2 in response to IL-3 stimulation in these DN-MAPKK transfected BA/F3 cells (BAF3/DN-MAPKK) was compared to that of parental BA/F3 cells. BA/F3 and BAF3/DN-MAPKK cells were labeled with [^{35}S]methionine/cysteine and deprived of IL-3 for 6 h prior to re-exposure to IL-3 for 5 min. GATA-2, immunoprecipitated from lysates of these cells, was analyzed by SDS-PAGE; these results are presented in Fig. 4. The uppermost phosphorylated form of GATA-2 (form a) is not evident in IL-3-stimulated BAF3/DN-MAPKK cells; the successful impairment of the MAPK pathway under these conditions of IL-3 stimulation was demonstrated by the analysis of MAPK phosphorylation presented in the lower panels. These results thus provide clear evidence for the involvement of the MAPK pathway in the IL-3-dependent phosphorylation of GATA-2 in hematopoietic progenitor cells.

GATA-2 Is Phosphorylated by MAPK in Vitro—Our results clearly identify the activation of MAPK as one of the control points for GATA-2 phosphorylation but do not distinguish whether MAPK phosphorylates GATA-2 directly or brings about GATA-2 phosphorylation in an indirect fashion via additional intermediate kinases. We addressed this question by performing *in vitro* phosphorylation and two-dimensional phosphopeptide mapping experiments. A full-length human GATA-2 cDNA clone was fused at its initiator methionine onto GST in an *E. coli* expression vector. GST-GATA-2 was used in an *in vitro* kinase assay using purified MAPK which had been chemically activated by thiophosphorylation. No protein phosphorylation was observed when the MAPK was incubated with [γ-^{32}P]ATP in the absence of substrate, demonstrating that the purified kinase was not significantly contaminated with exogenous substrates (Fig. 5*A*, *lane 1*). Addition of GST-GATA-2 fusion protein to the incubation resulted in marked phosphorylation of the fusion protein (Fig. 5*A*, *lane 3*), while addition of GST alone did not generate any phosphorylation (data not shown). The efficiency of phosphorylation of GATA-2 by MAPK was ascertained by comparison with a control kinase reaction using MBP as a substrate (Fig. 5*A*, *lane 2*). When normalized for protein concentration, the efficiency of *in vitro* phosphorylation of GST-GATA-2 by MAPK ranged from 25–40% that of MBP. Phosphoamino acid analysis of MAPK-labeled GST-GATA-2 protein revealed that GATA-2 was phosphorylated on threonine and serine residues, with threonine phosphorylation predominating (Fig. 5*B*). Phosphoamino acid analysis of GATA-2 derived from transfected COS cells, however, showed serine phosphorylation to predominate over threonine phosphorylation (data not shown). A comparison of the two-dimensional phosphopeptide maps obtained from GST-GATA-2 phosphorylated by MAPK *in vitro* and GATA-2 phosphorylated *in vivo* in COS cells expressing CA-MAPKK is shown in Fig. 5*C*. Several phosphopeptides are generated by tryptic cleavage of GST-GATA-2 labeled by MAPK *in vitro*. Four of these, b, c, d, and e, are clearly present in the two-dimensional map of GATA-2 labeled in COS cells; phosphopeptide a may also be present in both maps. Phosphopeptide f, which is clear in the *in vitro* map, is not readily apparent in the *in vivo* map; this phosphopeptide may result from the presence of the GST-tag in the *in vitro* material, partial proteolytic digestion, or spurious *in vitro* phosphorylation possibly due to inappropriate or incomplete renaturation of GST-GATA-2. There are also phosphopeptides present in the COS cell fingerprint that are absent from the *in vitro* fingerprint, showing that some sites are not directly phosphorylated by MAPK.

Analysis of Transactivation Potential of GATA-2—Using the COS cell system we next asked whether the alteration of GATA-2 phosphorylation brought about by activation of the MAPK pathway modulated the transactivation potential of GATA-2. We compared the ability of GATA-2 to transactivate a GATA-dependent CAT reporter gene in the presence or absence of co-transfected CA-MAPKK. These results are presented in Fig. 6; *panel A* shows representative data obtained in one of the seven independent transfection experiments performed. The collective data from these seven independent experiments is presented graphically in *panel B*. Interestingly, transfection of CA-MAPKK alone produced a small and GATA-independent stimulation in CAT activity. Co-transfection of the CA-MAPKK and GATA-2 produced no significant alteration in CAT activity compared to the transfection of GATA-2 alone. Thus, we conclude that co-transfection of the CA-MAPKK mutant did not significantly alter the transactivation potential of GATA-2 in COS cells.

DISCUSSION

In this report we have shown that GATA-2 is phosphorylated in proliferating hematopoietic progenitor cells. Interestingly, several phosphorylated forms of GATA-2 were found; some existing stably in quiescent cells, others more characteristic of mitogen (IL-3) stimulated cells. These results obtained in hematopoietic cells were further supported by mitogen (EGF) stimulation experiments conducted in GATA-2 transfected COS cells. The mitogen-associated phosphorylated forms of GATA-2 appear to be associated with activation of the MAPK pathway. This is particularly clear in our COS cell experiments where the use of a constitutively activated mutant of MAPKK demonstrated that activation of the MAPK pathway *in vivo* is sufficient for enhanced GATA-2 phosphorylation; that activation of this pathway is necessary for this event was unequivocally demonstrated using an interfering MAPKK mutant which acts as a dominant negative *in vivo*. Furthermore, using this interfering MAPKK mutant, we have directly demonstrated a

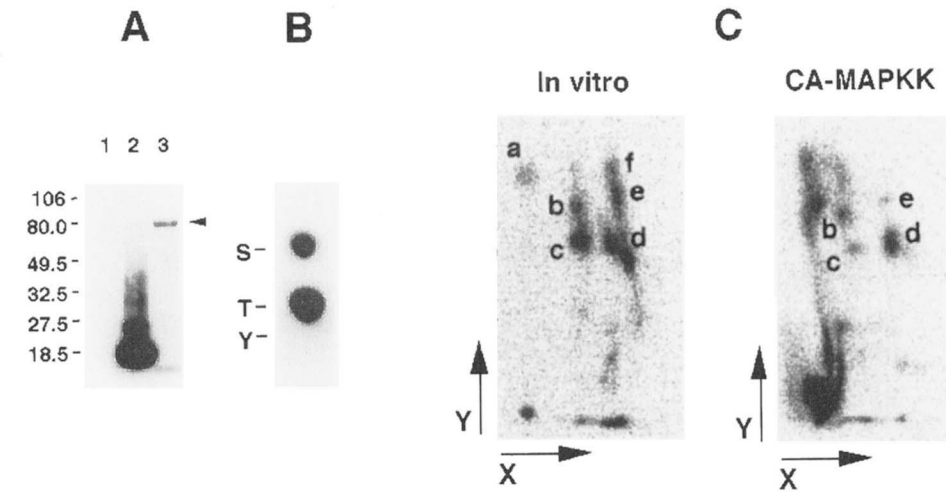

FIG. 5. **Phosphorylation of GATA-2 by MAPK *in vitro*.** *A, in vitro* kinase assays performed using chemically activated (thiophosphorylated) MAPK. The lanes contain the following reaction substrates: *1*, no substrate; *2*, 15 μg of MBP; *3*, 2 μg of GST-GATA-2 fusion protein. The quantity and quality of MBP and GST-GATA-2 used in these *in vitro* reactions was confirmed by Coomassie Blue staining. The reaction products were fractionated on a 10% polyacrylamide gel; *numbers* are in kilodaltons, and the phosphorylated GATA-2 fusion protein is indicated by an *arrowhead*. *B*, phosphoamino acid analysis of GST-GATA-2 phosphorylated *in vitro* by MAPK. The positions of serine (*S*), threonine (*T*), and tyrosine (*Y*) are marked. *C*, phosphopeptide fingerprints of GST-GATA-2 labeled by MAPK *in vitro* (*left panel*) and phospholabeled GATA-2 immunoprecipitated from COS cells co-transfected with CA-MAPKK (*right panel*). The horizontal dimension (*X*) was electrophoresis, and the vertical dimension (*Y*) was chromatography. The data were collected by the Molecular Dynamics PhosphorImager™.

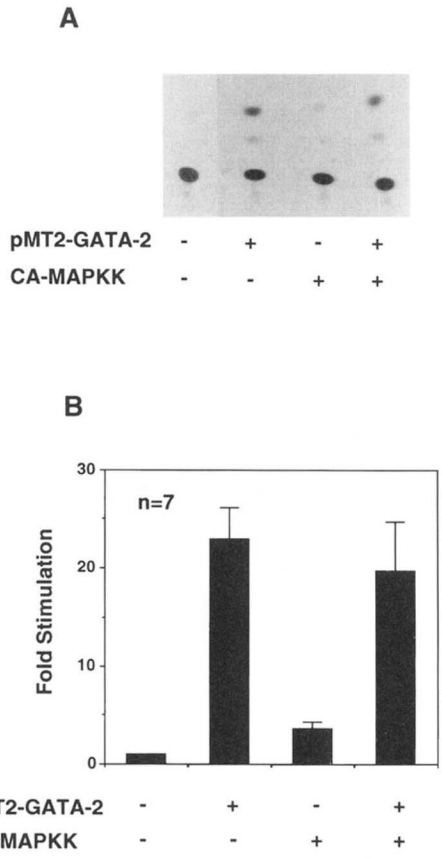

FIG. 6. **Analysis of transactivation potential of GATA-2.** COS cells were transfected with a GATA-dependent CAT reporter gene along with the combinations of GATA-2 and CA-MAPKK expression vectors indicated in the figure. *A*, representative data obtained in one of the seven experiments performed. *B*, the collective data from all seven experiments performed are presented graphically. CAT activities were quantitated by scintillation counting or 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 the standard errors indicated by the *vertical bars*.

critical role for the MAPK pathway in the regulation of GATA-2 phosphorylation in response to IL-3 stimulation in hematopoietic progenitor cells. Taken together, our results are consistent with the hypothesis that GATA-2 is involved in the growth factor responsiveness and proliferation control of hematopoietic progenitor cells. The implications of our results are particularly interesting in light of the recently reported phenotype of the GATA-2 knockout (17). In these mice, definitive hematopoiesis is profoundly impaired with the loss of virtually all hematopoietic lineages suggesting a major defect at the stem or early progenitor cell level. Colony formation assays conducted *in vitro* with GATA-$2^{-/-}$ ES cells suggest an impaired responsiveness to certain cytokines, leading the authors to speculate that GATA-2 regulates genes controlling the growth factor responsiveness of early hematopoietic cells. Our data raise the possibility that GATA-2 itself may directly mediate the cellular response to growth factor through alterations in its phosphorylation status.

Our results also raise a secondary, although interesting, question: does MAPK bring about GATA-2 phosphorylation in a direct or an indirect fashion? Both our *in vitro* phosphorylation data and our two-dimensional phosphopeptide mapping experiments argue that MAPK may directly phosphorylate GATA-2 *in vivo*. The GATA-2 protein has at least 14 potential sites for MAPK phosphorylation; systematic mutagenesis of these positions is currently underway in our laboratory. The notion that GATA-2 is directly phosphorylated by MAPK is an attractive one since MAPKs translocate to the nucleus upon activation and are therefore ideally suited to act as a convergence point that integrates and transduces diverse cytokine receptor-mediated signals to transcription factors. It will now be interesting to determine if stimulation by other cytokines results in GATA-2 phosphorylation, either through MAPK-dependent or independent pathways. A strong candidate cytokine in this regard is the *steel* factor (SLF) (47). Like IL-3, SLF also activates the MAPK pathway (49, 50), and, interestingly, *in vitro* differentiation experiments with GATA-$2^{-/-}$ ES cells reveal a profound deficiency in the generation of SLF-dependent colonies (17). In our own preliminary experiments using the human factor-dependent cell line TF-1 (48), we have found that both IL-3 and SLF effect rapid phosphorylation of GATA-2 (and

also MAPK) in factor-deprived cells. These preliminary experiments lend further support to the notion that phosphorylation of GATA-2 in response to ligand binding is relevant for hematopoietic progenitor cell expansion and is closely associated with MAPK activation.

Another member of the GATA family of transcription factors, namely GATA-1, has recently been shown to exist as a phosphoprotein in erythroid cells. GATA-1 is phosphorylated exclusively on serine residues, but the cellular mechanisms regulating this phosphorylation are not yet known. Systematic mutations of these serine residues do not appear to alter the transactivation function of GATA-1 as judged by reporter gene assays conducted in COS cells, nor do they alter the DNA binding ability of GATA-1 proteins expressed in COS cells. Consistent with the results obtained with GATA-1, we observed no significant change in the transactivation potential of GATA-2 when co-transfected with the CA-MAPKK into COS cells. We suspect that cell fate could prove a more sensitive and relevant assay than transient transfection systems. The expression of GATA factors overlaps at certain stages of hematopoietic cell development (2) and the different GATA members display subtly different binding site preferences (49, 50). Phosphorylation may modulate both the binding site preferences and the transactivation ability of GATA factors in the context of a native transcriptional complex. Modulations such as these could dramatically alter the cellular transcriptional program elicited by GATA factors and thus ultimately regulate proliferation *versus* differentiation decisions. Testing such a model will require expression of GATA phosphorylation mutants in stem cell populations which can undergo proliferation and differentiation in response to cytokine-induced signaling. GATA-2$^{-/-}$ ES cells offer one such cellular model; the IL-3-dependent cell line FDCP-mix A4 which can undergo multilineage differentiation in response to both stromal components and hematopoietic growth factors may provide another (51).

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