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Phosphorylation of GATA-1 increases its DNA-binding affinity and is correlated with induction of human K562 erythroleukaemia cells

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ABSTRACT

We have investigated by electrophoretic mobility shift assay (EMSA) the level of GATA-1 DNA-binding activity in nuclear extracts prepared from the human erythroleukaemic cell line, K562, after erythroid induction by hemin, sodium butyrate (NaB) or Trichostatin A or treatment with *N*-acetylcysteine (NAC). Relative to extract from untreated cells, GATA-1 binding activity increased markedly in all cases. However, immunoblot analysis revealed unchanged levels of GATA-1 protein after induction. Incubation of induced but not uninduced K562 extracts with phosphatase prior to EMSA weakened the binding activity, suggesting that the increase in GATA-1 binding following induction of K562 cells was a consequence of phosphorylation. When the mouse erythroleukaemic cell line MEL was induced with dimethylsulphoxide (DMSO), NaB or NAC, GATA-1 binding activity fell with DMSO, rose significantly with NaB and remained at about the same level in NAC-induced cells. In this case immunoblotting revealed that GATA-1 protein levels were in accord with the EMSA data. The DNA-binding activities of induced and uninduced MEL cell nuclear extracts were decreased by incubation with phosphatase, showing that phosphorylation and DNA binding of GATA-1 are already optimal in these cells. The DNA-binding activity of affinity-purified GATA-1 from MEL cells was also reduced by phosphatase treatment, showing that phosphorylation/dephosphorylation is directly affecting the factor. Furthermore, when a comparison was made by EMSA of nuclear extracts prepared from K562 and MEL cells untreated or incubated with okadaic acid, a phosphatase inhibitor, GATA-1 binding was seen to increase with K562 cells, whereas with MEL cells there was no change in GATA-1 binding. Overall the results suggest that the level of GATA-1 phosphorylation increases after the induction of K562, but not MEL cells, where GATA-1 is already highly phosphorylated. Furthermore, phosphorylation increases the binding affinity of GATA-1 for a canonical binding site.

INTRODUCTION

GATA-1 is the first described member of a family of transcription factors which share a high degree of homology in the amino acid sequence of the two zinc finger DNA binding domains and a common consensus DNA binding sequence, A/TGATA(AG/GC) (1–4). GATA-1 was originally thought to be erythroid specific (5), but is now known to be present not only in megakaryocytes, mast cells and haematopoietic progenitor cells (6,7), but also in mouse testis (8). The binding motif for this protein has been found in *cis* elements of all erythroid expressed genes studied. It has been demonstrated that GATA-1 plays a major role in erythroid development by targeted gene disruption of the GATA-1 gene in embryonic stem (ES) cells. Expression of this factor is essential for cells to complete the programme of erythroid differentiation, with GATA-1^{-} cells being blocked at the proerythroblast stage (9). Further studies with transgenic mice harbouring a GATA-1 'knockdown' mutation, which leads to decreased levels of GATA-1, has shown that erythroid maturation is dependent upon the concentration of GATA-1 (10). Furthermore, in addition to GATA-1, GATA-2 is expressed in mammalian haematopoietic progenitor cells and the mRNA for GATA-2 declines as GATA-1 increases during erythroid differentiation (11). This finding has led to the suggestion that erythroid differentiation is regulated by a precise quantitative balance in the levels of GATA-1 and GATA-2 (12).

Both human and murine erythroleukaemia cell lines have been used extensively to study aspects of erythroid development. MEL cells, a murine transformed proerythroblast cell line (13), can be induced to terminally differentiate with dimethylsulphoxide (DMSO) (14), leading to a 10- to 50-fold increase in the level of adult α- and β-globins (15), which is partly the result of transcriptional activation (16). Another commonly used cell line, K562, is a human bipotential erythroleukaemia cell line that expresses erythroid markers and can be further differentiated along the erythroid pathway by treatment with hemin to give increased expression of embryonic and fetal globins (17,18) as a consequence of enhanced transcription of the globin genes (19,20).

The sites of phosphorylation of GATA-1 have been mapped in uninduced and DMSO-induced MEL cells, to show that six serine residues at the N-terminus are phosphorylated in uninduced cells

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and a seventh, near to the C-terminal boundary of the DNA-binding domain, becomes phosphorylated after induction (21). However, no role for phosphorylation of GATA-1, either in influencing DNA binding or in transcriptional activation was found (21). Conversely, it was reported that the DNA binding of baculovirus-expressed human GATA-1 was sensitive to treatment by alkaline phosphatase, and the phosphotryptic peptide composition was similar to that of GATA-1 purified from K562 cells (22). Moreover, studies on numerous transcription factors have shown that their activity is modulated by *in vivo* phosphorylation, the post-translational modification capable of producing rapid modulations of protein activity in response to changes in metabolic activity, environmental conditions or hormonal signals (23). The effect on the activity of individual transcription factors is variable, but includes changes in cellular localization, DNA binding or the potential to interact with other transcription factors (23).

To resolve the conflict in the published data on the role of GATA-1 phosphorylation we examined whether the binding activity of GATA-1, isolated from erythroleukaemia cells, was sensitive to dephosphorylation *in vitro*. Furthermore, we investigated whether the sensitivity of GATA-1 to phosphatase changed when MEL cells were induced to differentiate by DMSO and K562 by hemin. Both cell types are also induced with sodium butyrate (NaB) (18,24), which increases transcription of globin genes (25). Furthermore, we compared the effects of Trichostatin A (TSA) with those of NaB. Like NaB, TSA is an inhibitor of mammalian histone deacetylase, but it is effective at very low concentrations and is currently regarded as being far more specific than NaB (26). In addition it has been reported to be a MEL cell inducer (27,28). We also report the result of exposing both cell types to the reducing agent *N*-acetyl-L-cysteine (NAC). The effect of NAC on erythroleukaemic cells was investigated because we had previously observed that GATA-1 binding is redox sensitive, being much enhanced by reducing conditions (unpublished observations). The binding to nucleic acid of several transcription factors has been reported to be reduced or abolished when certain cysteine residues are oxidized or alkylated (29). Zinc finger proteins come into this category and the DNA binding by two zinc finger proteins, Sp1 and Egr-1, has been reported to be redox sensitive (29–31). Furthermore, some time ago it was reported that cysteine and other sulphydryl reducing agents could serve as inducers of haemoglobin synthesis in MEL cells (32).

As an additional test of the relationship between the phosphorylation state of GATA-1 and its DNA-binding affinity, we investigated whether exposure of MEL and K562 cells to a phosphatase inhibitor, okadaic acid (OA), would result in both enhanced binding to the probe and greater sensitivity to dephosphorylation. OA, a potent tumour promoter, specifically inhibits the serine/threonine phosphatases 1, 2A (33) and 3 (34) *in vivo*, resulting in the accumulation of phosphoproteins within the cell (35). OA has been reported to stimulate AP1 binding to a TRE (36), to activate NF-κB (37,38) and to enhance retinoic acid receptor binding (39), as a consequence of increased phosphorylation.

MATERIALS AND METHODS

Cell culture

MEL cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. K562 cells were maintained in RPMI medium with 10% fetal calf serum. Both cell lines were incubated in a 5% CO_2 atmosphere. Induction of MEL cells was initiated by incubation with either 1.4% w/v DMSO or 2 mM NaB and the cells were harvested after 3 days. K562 cells were induced with 0.05 mM bovine hemin or 2 mM NaB for 3 days prior to harvesting. Additionally, K562 cells were treated with 1 μM TSA (Wako Biochemicals) for 3 days.

RNA analysis

After fractionation of the cells into nuclear and cytoplasmic fractions the cytoplasmic fraction was diluted with 5 vol of buffer (200 mm NaCl, 20 mM Tris–HCl pH 7.2, 5 mM EDTA, 0.5% w/v SDS) and the RNA was extracted by two sequential extractions with phenol:chloroform (1:1 v/v) followed by an extraction with chloroform. The RNA was precipitated with 2.5 vol of ethanol. The amount of RNA in each preparation was quantitated spectrophotometrically and checked by agarose gel electrophoresis. The RNA probe used to determine α-globin levels was prepared from the vector pSp6Hα132 and the ribonuclease protection assay (RPA) was carried out as previously described (40), with bands quantitated on a phosphorimager.

Preparation of nuclear extract from MEL and K562 cells

Nuclear extracts were prepared from MEL and K562 cells as described (41) except that phenylmethylsulphonyl fluoride was replaced in all buffers by 1 mM [4-(2-aminoethyl)benzenesulfonyl-fluoride]·HCl (AEBSF; Calbiochem).

Electrophoretic mobility shift assay (EMSA)

The method used to assay the DNA binding activity of GATA-1 in extract preparations has been described (3). The protein concentration in nuclear extracts was determined using the BCA protein assay reagent (Pierce) and EMSA was carried out using aliquots containing equal amounts of protein. The double-stranded GATA-1-binding oligonucleotide used as a probe was αG2 (3,4). The heteropolymer poly(dI–dC)·poly(dI–dC) was used as the non-specific competitor at a concentration of 50 (with total nuclear extracts) or 1 μg/ml (with affinity-purified protein). EMSA and RPA gels were quantitated using a model GS-525 Molecular Imager (Bio-Rad).

In vitro phosphatase treatment of nuclear extracts

For dephosphorylation experiments λ phosphatase (λ-PPase), a Mn^{2+}-dependent protein phosphatase with activity towards serine, threonine and tyrosine residues, was used. Aliquots of extract were incubated at 30°C for 30 min in LS buffer (41,42) containing 200 μg/ml bovine serum albumin, 0.2 mM $MnCl_2$ and 400 000 U/ml of λ-PPase (New England Biolabs). When trial incubations were made it was found by omission that 2 mM $MnCl_2$, the recommended concentration for λ-PPase, destabilized GATA-1 binding and the concentration was reduced to 0.2 mM (New England Biolabs, personal communication).

For the experiment using potato acid phosphatase (PAP) (EC 3.1.3.2) (grade 1, Boehringer), the enzyme was resuspended at a 20-fold dilution into 20 mM HEPES pH 7.9, 2 mM $MgCl_2$ and aliquots were added to either total nuclear or affinity-purified MEL cell extract (for units/assay see Fig. 7 legend). Incubation was for 30 min at 20°C and EMSA was then carried out as described above.

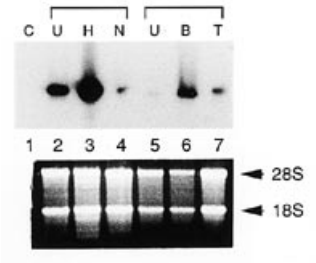

Figure 1. (**Upper**) RPA comparing α-globin gene mRNA levels in uninduced and induced K562 cells. Lane 1, tRNA control; lane 2, uninduced; lane 3, hemin-induced; lane 4, NAC-treated; lane 5, uninduced; lane 6, NaB-induced; lane 7, TSA-induced. Lanes 2–4 and 5–7 contain RNAs from different aliquots of K562 cells. Variability between batches of cells explains the different signal strengths in lanes 2 and 5. (**Lower**) Quantitation of RNA used in RPA by ethidium bromide stained agarose gel electrophoresis of RNA samples. Lane designations are as for upper panel. 28S and 18S rRNA bands are marked.

Immunoblotting

The method used has been described previously (41). Either a rat monoclonal GATA-1 antibody (N1) was used at a dilution of 1:2000 to detect mouse GATA-1 or a rabbit anti-peptide GATA-1 antibody (C61) was used for human GATA-1. The secondary antibodies, either anti-rat Ig diluted 1:1000 (Dako) or anti-rabbit Ig diluted 1:2000 (Amersham), were incubated with the blots for 2 h and the final washing was as previously described (41).

Polyclonal antibody

The sequence of the peptide used to raise the polyclonal GATA-1 antibody is listed: C61 SPVFQVYPLL..NSMEGIP(C). This antibody has been described previously (43). The C-terminal cysteine residue was added for linking purposes.

RESULTS

Induction of K562 and MEL cells

We compared the effects of hemin and NaB, known inducers of K562 cell erythroid differentiation, with those of TSA (25) and NAC. α-Globin mRNA levels were monitored using the RPA (Fig. 1, upper). RNA was extracted from uninduced and induced cells and quantitated by A_{260} absorbance. Loading was checked by agarose gel electrophoresis (Fig. 1, lower). Relative to the uninduced cells (lane 2), hemin induction resulted in a 4-fold increase in the level of α-globin mRNA (lane 3), whereas NAC treatment resulted in a 5-fold reduction (lane 4). In an extract from a separate aliquot of K562 cells, NaB (lane 6) caused an 8-fold enhancement relative to the uninduced control (lane 5). However, TSA (lane 7) produced little increase in α-mRNA above the uninduced sample, when RNA loading was taken into account.

As previously reported for other sulphydryl reducing agents (32), we found that NAC induced erythroid differentiation of MEL cells. However, K562 cells ceased division in 3 days after three divisions and the cells underwent non-apoptotic cell death after ~4–5 days of treatment (data not shown).

In conclusion, TSA is not as effective as hemin or NaB in inducing globin mRNA synthesis in K562 cells and, whereas NAC induces MEL cells, it eventually kills K562 cells.

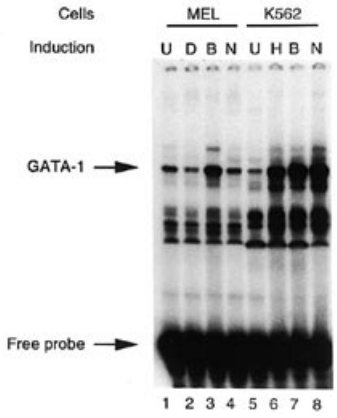

Figure 2. EMSA of equal amounts of MEL and K562 cell nuclear extracts, comparing the level of GATA-1 binding in uninduced and chemically induced cells. U, uninduced; D, DMSO-induced; H, hemin-induced; B, NaB-induced; N, NAC-treated. The probe was [^{32}P]αG2 (20 fmol/assay).

Contrasts in the effects of induction on GATA-1 binding in K562 and MEL cells

Changes in the level of GATA-1 binding after chemical treatment of MEL and K562 cells were monitored by EMSA (Fig. 2) using the oligonucleotide αG2 (Materials and Methods). Nuclear extracts were prepared from uninduced and induced cells and aliquots of extract containing equal amounts of protein were assayed. Relative to uninduced MEL cells (lane 1), GATA-1 binding decreased slightly following DMSO induction (lane 2), increased with NaB induction (lane 3) and remained unchanged in NAC-treated cells (lane 4). In contrast, with K562 cells, hemin, NaB and NAC treatment all resulted in considerably higher levels of GATA-1 binding activity (compare control lane 5 with lanes 6–8). The main band was confirmed to be GATA-1 by supershifting with either monoclonal (MEL) or polyclonal (K562) antibodies (data not shown). Although the identities of the other bands are unknown, the weak band of lower mobility than GATA-1 is possibly a hetero- or homodimeric complex (44,45), whereas the band with a mobility just greater than GATA-1 is possibly the 40 kDa alternative GATA-1 translation product (46).

GATA-1 protein levels in nuclear extracts from uninduced and induced MEL and K562 cells were compared by immunoblotting to determine whether the observed changes in GATA-1 binding resulted from alterations in the level of GATA-1 protein or in the affinity of the factor for DNA. The levels of GATA-1 in MEL cells were assayed in equal amounts of protein using the GATA-1 monoclonal antibody N6 (Fig. 3A). A single band of ~50 kDa was detected. Relative to uninduced MEL cells (lane 1) less GATA-1 was found in the DMSO-induced (lane 2) and NAC-treated cells (lane 4), while more factor was present in NaB-treated cells (lane 3), parallelling the EMSA result (Fig. 2). An immunoblot was also carried out to determine the levels of GATA-1 in equal amounts of nuclear protein from K562 cells using a polyclonal antibody (Fig. 3B). GATA-1, the most prominant band (arrowed), is ~54 kDa. Comparing the level of GATA-1 protein from uninduced (lane 1) with hemin- (lane 2), NaB- (lane 3) and NAC-treated cells (lane 4) shows that in contrast to EMSA all have lower levels of GATA-1 relative to the control.

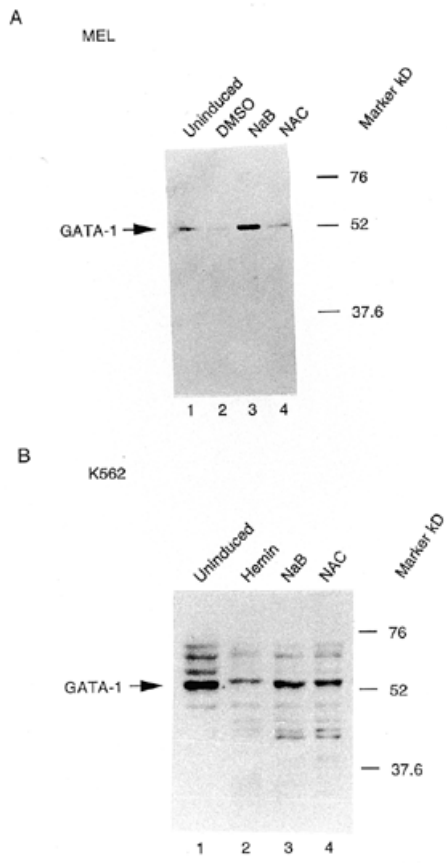

Figure 3. (**A**) Immunoblot showing GATA-1 protein levels in MEL nuclear extracts. Lane 1, uninduced; lane 2, DMSO-induced; lane 3, NaB-induced; lane 4, NAC-induced. The positions and sizes (kDa) of the prestained protein markers are shown. The primary antibody used was the GATA-1 monoclonal antibody N1. The position of GATA-1 is designated by an arrow. (**B**) Immunoblot showing GATA-1 protein levels in K562 nuclear extracts. Lane 1, uninduced; lane 2, hemin-induced; lane 3, NaB-induced; lane 4, NAC-treated. The positions and sizes (kDa) of the prestained protein markers are shown. The primary antibody used was the GATA-1 polyclonal antibody C61. The position of GATA-1 is designated by an arrow.

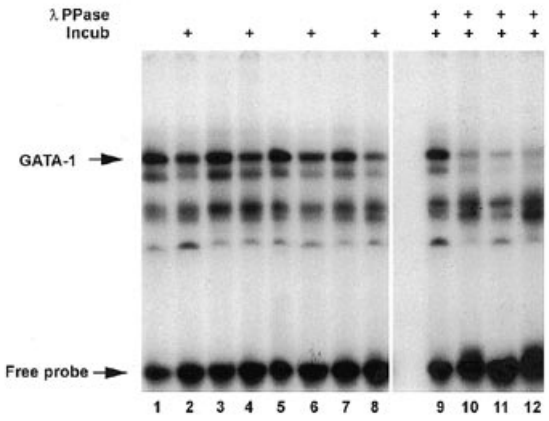

Figure 4. EMSA of K562 nuclear extracts showing the effect on GATA-1 binding of pretreatment with λ-PPase prior to assay. The lanes containing unincubated extract, extract incubated with buffer only and extract incubated with buffer and λ-PPase are designated (details in Materials and Methods). Lanes 1, 2 and 9, uninduced; lanes 3, 4 and 10, hemin-induced; lanes 5, 6 and 11, NaB-induced; lanes 7, 8 and 12, NAC-treated. The position of full-length GATA-1 is shown. The probe used was αG2 (20 fmol/assay).

The combined results of the EMSA and immunoblot data of chemically treated MEL cells show that the changes in the level of GATA-1 binding are largely accounted for by changes in the amount of GATA-1 protein and not by significant changes in the affinity of GATA-1 for the probe. In contrast, the changes in the level of GATA-1 binding in treated K562 cell extracts are not reflecting changes in protein level and therefore indicate an increased affinity for the αG2 probe.

The binding affinity of GATA-1 in induced K562 cells is increased by phosphorylation

To investigate whether the increase in binding of GATA-1 after chemical induction of K562 cells was the result of a change in level of phosphorylation, aliquots of uninduced and induced nuclear extracts (normalized to have equivalent binding activities) were pretreated with a protein phosphatase, with activities directed towards serines, threonines and tyrosines, prior to EMSA. We have made use of two such phosphatases, PAP and λ-PPase, which give equivalent results when compared. Comparison of unincubated with incubated cell extracts shows that incubation in the phosphatase buffer alone caused a small decrease in the level of GATA-1 binding (Fig. 4, compare lanes 1, 3, 5 and 7 with 2, 4, 6 and 8). However, after incubation of the uninduced extract with phosphatase, the affinity of GATA-1 for the probe was not decreased further (compare lanes 2 and 9). In contrast, extracts from hemin-, NaB- and NAC-treated cells all had decreased GATA-1 binding after extract incubation with phosphatase (compare lanes 4, 6 and 8 with 10–12).

For comparison with NaB, we also examined whether phosphorylation of GATA-1 was altered in K562 cells after induction by TSA, a histone deacetylase inhibitor of greater specificity than NaB (26). As already noted, with uninduced nuclear extract little change in GATA-1 binding was seen on phosphatase treatment (Fig. 5, compare lanes 2 and 7) whereas in extract from NaB-induced cells GATA-1 binding was significantly decreased by phosphatase treatment (compare lanes 4 and 8). Similarly, GATA-1 binding in extract from TSA-treated cells was also reduced by phosphatase treatment (compare lanes 6 and 9). As a control, the levels of Sp1 binding, both prior to and subsequent to phosphatase treatment, were examined, as it has been reported that dephosphorylation of Sp1 promotes increased binding (47,48). In agreement with this, dephosphorylation of uninduced, NaB- and TSA-treated cell extracts resulted in increased binding of Sp1 to its recognition sequence (Fig. 5, compare lanes 10–12 with 13–15).

In summary, this experiment shows that, despite its greater specificity for histone deacetylases, TSA still gives rise to GATA-1 with increased sensitivity to phosphatase, indicating an enhanced level of phosphorylation.

The binding activity of GATA-1 from uninduced and induced MEL cells is similarly affected by phosphatase treatment

An EMSA (using equal amounts of protein in each assay) showing the effect of phosphatase treatment of MEL extracts on GATA-1 binding is shown in Figure 6. It can be seen that, as with

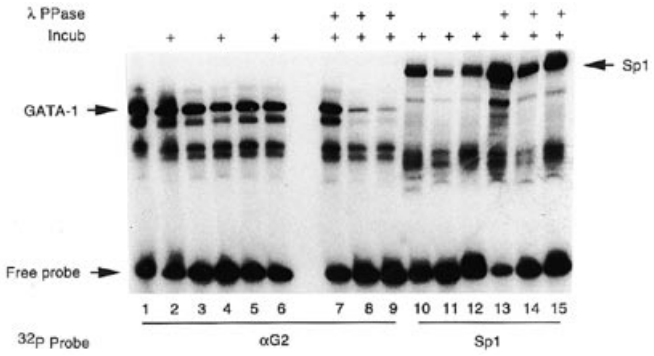

Figure 5. EMSA of K562 nuclear extracts showing a comparison of the effect on GATA-1 and Sp1 of pretreating extracts with λ-PPase prior to assay. Lanes 1, 2, 7, 10 and 13, uninduced; lanes 3, 4, 8, 11 and 14, NaB-induced; lanes 5, 6, 9, 12 and 15, TSA-induced. The probe used was either αG2 (lanes 1–9) or Sp1 (lanes 10–15), both at 20 fmol/assay. Other details are as for Figure 4.

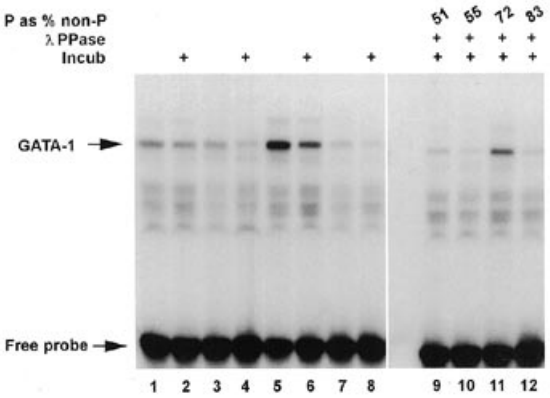

Figure 6. EMSA of MEL nuclear extracts showing the effect on GATA-1 binding of pretreatment with λ-PPase prior to assay. Lanes 1, 2 and 9, uninduced; lanes 3, 4 and 10, DMSO-induced; lanes 5, 6 and 11, NaB-induced; lanes 7, 8 and 12, NAC-induced. Other details are as for Figure 4.

GATA-1 from K562 cells, incubation of extract with the phosphatase buffer alone weakened GATA-1 binding (compare lanes 1, 3, 5 and 7 with 2, 4, 6 and 8, respectively). Furthermore, when GATA-1 binding from λ-PPase-treated extract is compared with non-phosphatased incubated extracts it can be seen that GATA-1 binding in both induced and uninduced extracts was weakened by phosphatase treatment (compare lanes 2, 4, 6 and 8 with 9–12, respectively). Quantitation of the data in this experiment shows that binding of GATA-1 from the uninduced and DMSO-induced cells was reduced by comparable amounts, ~2-fold, whereas GATA-1 from NaB- or NAC-treated cells was less affected by the λ-PPase (see Fig. 5 annotation). The magnitude of the reduction is greater in other experiments (see Fig. 7 for example) and most likely reflects non-saturating levels of the enzyme in this experiment.

Overall the results so far show that GATA-1 binding is reduced by dephosphorylation. Clearly chemical induction of K562 cells resulted in a distinctly different response to that seen with MEL cells, in that no change in GATA-1 binding was seen after phosphatase treatment of uninduced K562 cell extract, whereas

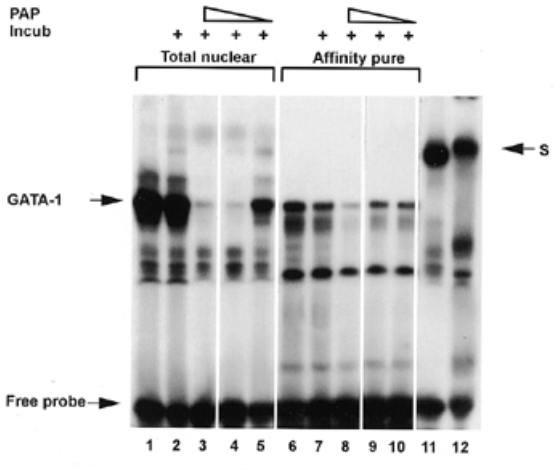

Figure 7. EMSA of MEL total nuclear extract (lanes 1–5 and 11) and affinity-purified MEL GATA-1 (lanes 6–10 and 12), showing the effect on GATA-1 binding of pretreating the extract with PAP. Lanes 2 and 7 are controls with buffer alone and the number of units of PAP used per assay were: lanes 3 and 8, 0.036; lanes 4 and 9, 0.012; lanes 5 and 10, 0.006. The probe used was [^{32}P]αG2. Supershifts of GATA-1 with monoclonal antibody N6 are shown in lanes 11 and 12; S denotes the supershifted band. The difference in mobility of this band between total nuclear extract and affinity-purified GATA-1 may reflect conformational changes exposed by the antibody and consequent upon chromatography in detergent and high salt.

extracts from all chemically treated cells showed a large decrease in GATA-1 binding. The similarity of uninduced and induced MEL cells also indicates that the reported phosphorylation of Ser310 within the finger region, which occurs on DMSO induction (21), causes little change in the binding affinity of GATA-1.

The binding of affinity-purified GATA-1 from uninduced MEL cells is decreased after phosphatase treatment

To determine if the decrease in binding affinity of GATA-1 following dephosphorylation was a direct effect on the factor, GATA-1 was purified from uninduced MEL cell nuclear extract by affinity chromatography on a column containing the strong binding site oligonucleotide, αG2. Aliquots from nuclear extract and from a column-bound fraction were pretreated with three concentrations of phosphatase prior to EMSA (Fig. 7). Incubation of either total nuclear extract or the purified fraction in buffer alone slightly reduced GATA-1 binding (compare lanes 1 and 2, 6 and 7), whereas the binding was markedly reduced for unpurified and purified samples on incubation with phosphatase (compare lanes 2 with 3–5, and 7 with 8–10). The column-purified factor was confirmed to be GATA-1 by comparing a supershift of GATA-1 in unpurified nuclear extract (lane 11) with the purified factor (lane 12) using a GATA-1 monoclonal antibody.

This experiment using phosphatase treatment of affinity-purified GATA-1 demonstrates that the decrease in binding caused by dephosphorylation is a direct effect on GATA-1.

GATA-1 from K562 but not MEL cells exposed to okadaic acid exhibits enhanced binding

The data presented above show that, with the exception of uninduced K562 cells, GATA-1 binding from all other K562 and

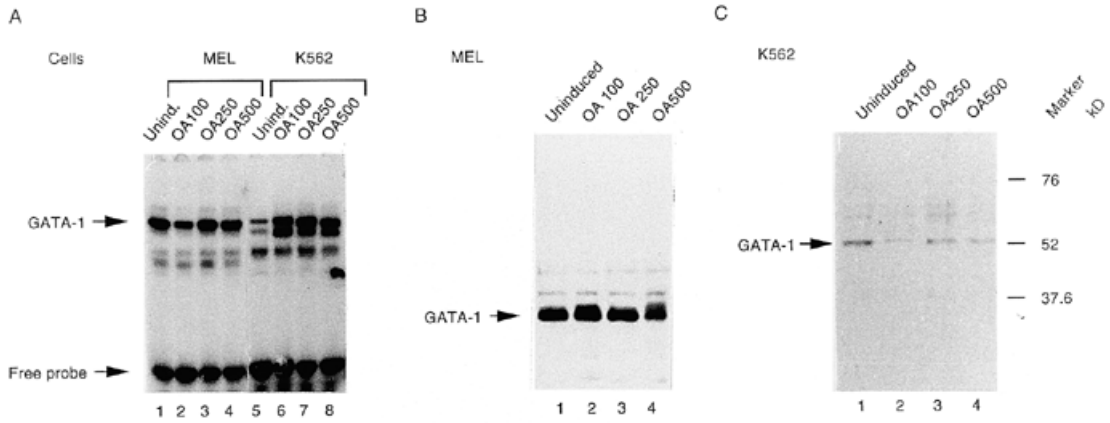

Figure 8. (**A**) EMSA showing a comparison of GATA-1 binding activity in untreated and OA-treated MEL (lanes 1–4) and K562 (lanes 5–8) cell nuclear extracts. (**B**) Immunoblot showing a comparison of GATA-1 protein levels in untreated and OA-treated MEL cells. (**C**) Immunoblot showing GATA-1 protein levels in untreated or OA-treated K562 nuclear extracts. OA concentrations are in nM. The probe used was [^{32}P]αG2 (20 fmol/assay). The positions and sizes (kDa) of the prestained protein markers are shown. The primary antibody used was the GATA-1 polyclonal antibody C61. The position of GATA-1 is designated by an arrow.

MEL cell extracts was reduced after incubation with phosphatase. As a further test of the conclusion that the increased GATA-1 binding seen on induction of K562 cells is a consequence of an increase in phosphorylation, both K562 and MEL cells were treated with three concentrations of OA, an inhibitor of serine/threonine phosphatases. An EMSA using equal amounts of protein in each assay was carried out to monitor the effects on GATA-1 binding (Fig. 8A). Relative to the control (lane 1), GATA-1 binding from MEL cells was slightly decreased at the lowest concentration of OA (lane 2) but was unchanged at the higher concentrations (lanes 3 and 4). In contrast, with K562 cells all three concentrations of OA produced a significant increase in binding of GATA-1 to the probe (compare control lane 5 and lanes 6–8). Furthermore, the mobility of GATA-1, and to a lesser extent the higher mobility band, was progressively increased in extracts from cells exposed to increasing concentrations of OA. Such an effect of hyperphosphorylation has been reported previously for the transcription factors Fra-1 and Fra-2 (49). Factor binding to the probe after OA treatment was confirmed to be GATA-1 for both MEL and K562 by supershifting with GATA-1 antibodies (data not shown)

To determine the level of GATA-1 protein in control and OA-treated cells, aliquots of each extract were immunoblotted. In an attempt to separate the different phospho forms of GATA-1 from untreated and OA-treated MEL cells a low density crosslink gel was used (50). Relative to the control (Fig. 8B, lane 1), MEL cells treated with the three concentrations of OA showed no significant change in the level of GATA-1 protein (Fig. 8B, lanes 2–4). It is probable that this gel system is not adequate for separating the different phospho forms of GATA-1, because fractionation was only becoming evident at the highest concentration of OA (lane 4). The analysis of GATA-1 protein levels in control and OA-treated K562 cells is shown in Figure 8C. Relative to the control (lane 1), cells treated with OA (lanes 2–4) contained lower levels of GATA-1.

In summary, treatment of MEL and K562 cells with OA had contrasting effects: while GATA-1 binding was increased for K562 cells there was little change for MEL. The increase in binding in K562 cells could not be explained by increasing protein and is therefore consistent with an increase in phosphorylation of GATA-1. These results therefore confirm both the difference in the state of phosphorylation of GATA-1 in K562 and MEL cells and that phosphorylation enhances DNA binding.

DISCUSSION

In this paper we have shown that GATA-1 is less phosphorylated in uninduced K562 cells than in MEL cells and that the response of the two cell lines to chemical inducers is different. With uninduced K562 cell extract, GATA-1 binding was insensitive to incubation with phosphatase, while both DNA binding and sensitivity to phosphatase was increased substantially by chemical inducers. The GATA-1 protein levels, however, were not substantially changed. In uninduced MEL cells, GATA-1 is in the phosphorylated form already and, with the exception of butyrate induction, showed little change in either GATA-1 binding activity or protein levels in response to induction. The contrasting phosphorylation states of GATA-1 in K562 and MEL cells was confirmed by treatment of the cells with the phosphatase inhibitor, OA: GATA-1 binding was increased in K562 but not in MEL cells.

The role of GATA-1 phosphorylation

It is difficult to reconcile our DNA binding data, indicating that the affinity of GATA-1 is increased by phosphorylation, with that of Crossley and Orkin, who could find little effect on the DNA binding activity of GATA-1 synthesized in COS cells when the phosphorylated serines were mutated to alanines (21). The important differences between the assays used in each case are yet to be identified. However, the result of Taxman *et al.* (22), showing that the binding of baculovirus-expressed GATA-1 is sensitive to treatment by alkaline phosphatase, is in agreement with our findings.

We used only the αG2 probe to monitor changes in GATA-1 binding and it is conceivable that the affinity of GATA-1 for other sites could be changed to a greater or lesser extent than was seen with this particular site. A particularly notable and well-studied example

of this phenomenon is the factor p53, with binding to different motifs being regulated in opposite ways by phosphorylation (51). In addition, GATA-1 has been shown to undergo homotypic (44,52) and heterotypic interactions, with transcription factors such as Sp1 and EKLF (45,53), FOG1 (54) and CDP (55). A direct interaction, in erythroid cells, has been demonstrated between the LIM protein LMO2, the LIM-binding protein Ldb1/NL1, E2A, the basic helix–loop–helix protein TAL1/SCL and GATA-1 allowing binding to a unique site (56–58). Thus it is possible that changing the phosphorylation state of GATA-1 affects *in vivo* interactions with other factors and thereby its DNA sequence specificity or transcriptional activation potential.

It has been suggested that one possible role for GATA-1 phosphorylation during the differentiation of primary chick erythroid cells is the translocation of GATA-1 from the cytoplasm to the nucleus (59). The nuclear form of GATA-1 was hyperphosphorylated, leading the authors to suggest that phosphorylation of GATA-1 could be the cause of the nuclear translocation. Consistent with this, GATA-1 is already abundant and phosphorylated in proerythroblast and MEL nuclei (21,60; this study). However, in the case of K562 cells, phosphorylation is induced without a change in the level of nuclear GATA-1 protein. It is unlikely therefore that the role of phosphorylation in these cells is to effect nuclear translocation.

We observed little change in GATA-1 protein or DNA-binding activity in DMSO-induced MEL cells, in agreement with previous studies (61–64). It is clear that the terminal stage of erythroid differentiation of MEL cells does not require an increase in GATA-1 DNA-binding activity. In contrast, on induction of K562 cells, GATA-1 binding activity increased, in the absence of any increase in protein levels, as a result of phosphorylation, suggesting that an increase in GATA-1 binding activity is required for the terminal erythroid differentiation of these cells. Since K562 cells retain bipotentiality (17,18), our observations may indicate that phosphorylation and increased binding activity of GATA-1 coincide with erythroid commitment.

Erythroid induction by NaB and TSA

Previously the induction of haemoglobin in K562 cells by TSA was shown to be <2-fold, whereas butyrate produced a >3-fold increase (25). In addition, it was reported that butyrate caused a 6-fold increase in γ-globin mRNA levels, after induction for 72 h (65). In the studies reported here butyrate stimulated an 8-fold increase in α-globin mRNA, whereas TSA produced only a 2-fold increase. Hence our results are in broad agreement with previous work and confirm that butyrate is a better inducer of K562 cells than TSA.

Both of these compounds inhibit histone deacetylase (26,66,67) and the mode of action of TSA may reside entirely in this function, as it has not been found to affect enzyme activities such as phosphatases or kinases (68). It is likely, therefore, that the mechanism by which TSA enhances GATA-1 phosphorylation in K562 cells involves changes in gene expression which eventually result in altered signal transduction.

Butyrate, however, is known to affect many cellular pathways (69). Recently it was shown to activate the promoter of the WAF1/Cip1 gene in a human colon cancer cell line through Sp1 binding sites, but no change was observed in the binding affinity of Sp1 for these sites (70). However, it has been reported that dephosphorylation of Sp1 increases DNA binding (47,48) and that butyrate can stimulate serine/threonine phosphatase activity (71). In our experiments with K562 cells, the binding activity of Sp1 was decreased after NaB treatment. Thus, for these cells, the butyrate may be activating kinase activity.

Rivero and Adunyah reported that NaB rapidly induced tyrosine phosphorylation of several proteins including MAP kinase (ERK-1) which is, as a consequence, up-regulated (72). ERK-1 can phosphorylate GATA-1 and GATA-2 *in vitro* and the ERK-1 pathway is thought to be involved *in vivo* in GATA-2 phosphorylation (50; T.Enver, personal communication). Recently it has been shown that NaB modulates the activity of serine/threonine kinases which have also been proposed to have a role in erythroid induction (73,74). Thus it appears that NaB can act to change signal transduction, which could well lead to the increase seen in the level of GATA-1 phoshorylation in K562 cells.

The increase in GATA-1 protein seen in MEL cells on NaB induction could result from either an increase in expression of the GATA-1 gene or a change in the turnover rate of the factor. Interestingly, overexpression of GATA-1 blocks differentiation of DMSO-induced MEL cells, probably by regulating cyclin E-dependent kinase activity (60). However, in the NaB-treated cells the increased level of GATA-1 did not inhibit the up-regulation of globin gene transcription or the block on cell division caused by NaB.

NAC and antioxidants

NAC treatment of K562 and MEL cells produced only minor changes in GATA-1 protein levels; however, DNA-binding activity was increased in K562 cells. NAC has been widely reported to affect signal transduction pathways but it appears that there are considerable differences in the responses of different cell types to NAC treatment. For example it was reported recently that the c-jun N-terminal kinase (JNK) is a target for activation by antioxidants in T cells (75); in contrast the activation of JNK and stress-activated kinases by alkylating agents was reported to be blocked in several cell lines by NAC and other antioxidants (76). In addition to GATA-1 (this study), NAC treatment has been shown to induce DNA binding and transactivation by AP-1 (77), but to block NF-κB activity (78,79). In the case of GATA-1, we have shown that the NAC-induced increase in DNA binding reflects increased phosphorylation.

In conclusion, our data show that a variety of chemical agents can increase the phosphorylation state of GATA-1 in K562 cells, concomitant with the cessation of cell division and either terminal erythroid differentiation or, in the case of NAC, eventual cell death. In contrast, the phosphorylation state of GATA-1 in MEL cells is already maximal for DNA binding. Phosphorylation of GATA-1 may therefore play a role in finally restricting cell fate to the erythroid lineage.

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