CREB-Binding Protein Acetylates Hematopoietic Transcription Factor GATA-1 at Functionally Important Sites

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The transcription factor GATA-1 is a key regulator of erythroid-cell differentiation and survival. We have previously shown that the transcriptional cofactor CREB-binding protein (CBP) binds to the zinc finger domain of GATA-1, markedly stimulates the transcriptional activity of GATA-1, and is required for erythroid differentiation. Here we report that CBP, but not p/CAF, acetylates GATA-1 at two highly conserved lysine-rich motifs present at the C-terminal tails of both zinc fingers. Using [3H]acetate labelling experiments and anti-acetyl lysine immunoprecipitations, we show that GATA-1 is acetylated in vivo at the same sites acetylated by CBP in vitro. In addition, we show that CBP stimulates GATA-1 acetylation in vivo in an EIA-sensitive manner, thus establishing a correlation between acetylation and transcriptional activity of GATA-1. Acetylation in vitro did not alter the ability of GATA-1 to bind DNA, and mutations in either motif did not affect DNA binding of GATA-1. CBP associates with GATA-1 in erythroid环境. For example, the N-terminal zinc finger of EKLF is necessary for binding to CBP and diminish the response to CBP in transient expression. Here we report that CBP, but not p/CAF, acetylates GATA-1 at two highly conserved lysine-rich motifs present at the C-terminal tails of both zinc fingers. Using [3H]acetate labelling experiments and anti-acetyl lysine immunoprecipitations, we show that GATA-1 is acetylated in vivo at the same sites acetylated by CBP in vitro. In addition, we show that CBP stimulates GATA-1 acetylation in vivo in an EIA-sensitive manner, thus establishing a correlation between acetylation and transcriptional activity of GATA-1. Acetylation in vitro did not alter the ability of GATA-1 to bind DNA, and mutations in either motif did not affect DNA binding of GATA-1. CBP associates with GATA-1 in vivo in the absence of EIA.

Our previous studies indicated that a widely expressed transcriptional cofactor, CREB-binding protein (CBP), is a potent coactivator of GATA-1 (4). CBP associates with GATA-1 in vitro and in vivo and markedly stimulates its activity. Expression of the adenovirus oncoprotein E1A, which interferes with the action of CBP, blocks the effects of CBP on GATA-1 activity, inhibits erythroid differentiation, and reduces the expression of several GATA-1-dependent genes (4).

Multiple mechanisms have been invoked to account for the activity of CBP and its close relative p300. First, CBP interacts with TFIIB (23), TATA-binding protein (1, 12, 37, 40), and RNA polymerase II (10, 21, 27) and thus serve as a bridging molecule between DNA-bound transcription factors and the basal transcription machinery. Second, CBP interacts with numerous transcription factors via dedicated domains and could thus provide a scaffold for the assembly of a high-molecular-weight “enhanceosome” complex (for a review, see reference 38). Such multifunctional interactions may account for the observed synergy between transcription factors that use CBP and p300 as cofactors. Third, CBP and p300 possess intrinsic and associated histone acetyltransferase activity (3, 9, 28, 39, 46). Acetylated histones are associated with an open chromatin configuration, which in turn might facilitate the accessibility of DNA to other transcription factors (for a review, see references 31 and 35). Finally, CBP and p300 were recently shown to acetylate nonhistone proteins such as the tumor suppressor protein p53 (16), the erythroid Kruppel-like factor (EKL) (48), and the basal transcription factors, TFIIIE and TFIIIF (19). In the case of p53, acetylation of the regulatory domain led to a dramatic increase in DNA binding (16) in vitro, whereas the functional consequences of EKL, TFIIIE, and TFIIIF acetylation remain to be explored.

In this report, we further define the mechanisms of transcriptional activation of GATA-1 by CBP. We found that CBP acetylates GATA-1 at two highly conserved lysine-rich motifs near the zinc finger domains. In contrast to a recent report which appeared while this paper was under review (7), we found that acetylation of GATA-1 did not affect its binding to DNA and that substitutions or deletions in these motifs did not affect DNA binding of mammalian-cell-expressed GATA-1. We also found that mutations in the C-terminal acetylation motif, but not in the N-terminal motif, reduce the binding to CBP and diminish the response to CBP in transient-transfection assays. We further demonstrate that CBP stimulates GATA-1 acetylation in vivo and that coexpression of E1A abolishes GATA-1 acetylation.

Certain functions of GATA-1 critically depend on an erythroid environment. For example, the N-terminal zinc finger of GATA-1 is required for function in erythroid cells but is dispensable for transactivation of some GATA-1-dependent reporter constructs in transiently transfected NIH 3T3 cells (45).
Conversely, a potent activation domain at the N terminus of GATA-1 is required for transactivation in the NIH 3T3 cell assay while this domain is dispensable for function in erythroid and megakaryocytic cells (6, 25, 42, 45). Therefore, we examined the function of the acetylation sites in an assay that measures GATA-1 activity by its ability to trigger terminal differentiation when introduced into the GATA-1-deficient erythroid-cell line G1E. This assay revealed that mutations in either motif reduce GATA-1 function without affecting its ability to bind DNA. These results indicate that the acetylated motifs are of biological importance independent of a role in DNA binding. They further suggest that acetylation of GATA-1 might provide a novel mechanism of controlling GATA-1 activity.

MATERIALS AND METHODS

Plasmids. Vectors expressing CBP (pCMV5-CBP) (a gift from M. Rosenfeld), GATA-1 (pXM-G1), E1A (pEFla-E1A), and GATA-1 reporter construct M1G2H have been described previously (20, 25). To construct CBP HAT− (a histone acetyltransferase [HAT]-deficient version of CBP), Leu1690 and Cys1691 were changed to Lys1690 and Leu1691 by overlapping PCR (26). This retroviral construct expressing GATA-1, MGF-GATA-1, and various glutathione-S-transferase (GST)–GATA-1 fusion constructs have been described previously (11, 45). GST-CBP-HAT containing the HAT domain of murine CBP (amino acids [aa] 1196 to 1718) (3) was generated by cloning a PCR-amplified fragment into pGEX4T. GST-pCAF-HAT (a gift from M. Montminy) contains the HAT domain of human pCAF (46) (aa 352 to 382). To introduce Lys-into-Ala mutations into the acetylation motifs of GATA-1, overlapping PCR was used. Sequencing analysis confirmed the absence of PCR errors. The N-terminal acetylation motif (LIRPKRKMIVSOKRA; aa 241 to 254) was mutated to LLRAARMSVKRA (N-mut). The C-terminal motif (KASGKKKGGSKNLG; aa 308 to 323) was mutated to KASGARGAGAAARGSNLAG (C-mut). In the construct termed NC-mut, both motifs were mutated. The mutant GATA-1 constructs were inserted into pXM, pGEX2T, MFG, and pBluescript.

Acetylation site mapping. Peptides corresponding to residues 236 to 255 and 304 to 323 of murine GATA-1 were synthesized with an Applied Biosystems 430A synthesizer. The purity was estimated to be 85% based on high-pressure liquid chromatography (HPLC) analysis. Twenty micrograms of peptide was incubated with 1 nM of [3H]acetyl-CoA per ml (16 Ci/mmol; ICN) and 50 nM Trichostatin (Sigma) and incubated for 90 min. The cells were lysed in 350 mM NaCl-50 mM Tris-HCl (pH 7.5)-0.5% Igepal–1 mM EDTA–0.5 mM DTT–10 mM sodium butyrate–1 μg of aprotinin per ml–1 μg of leupeptin per ml–0.2 mM PMSF. Following centrifugation at 10,000 × g for 5 min, the supernatants were diluted to 150 mM NaCl with H2O containing 1 mM EDTA, 0.5 mM DTT, 10 mM sodium butyrate, 1 μg of aprotinin per ml, 1 μg of leupeptin per ml, and 0.2 mM PMSF and immunoprecipitated with monoclonal GATA-1 antibodies (Nc; Santa Cruz) or irrelevant isotype-matched antibodies. The antibodies were captured by adding 30 μl of a 50% slurry of protein G-Sepharose. Immunoprecipitated samples were washed five times in 150 mM NaCl-50 mM Tris-HCl (pH 7.5)-0.5% Igepal–1 mM EDTA–0.5 mM DTT–10 mM sodium butyrate–1 μg of aprotinin per ml–1 μg of leupeptin per ml–0.2 mM PMSF, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, fixed, enhanced with Autorad (National Diagnostics), and autoradiographed. Immunoprecipitations with anti-acetyl lysine (anti-AK) antibodies (18) were performed under the same conditions as described above. Anti-AK antibodies were a gift from Colyn Crane-Robinson.

Retroviral infections. BOSC23 cells (a gift from W. Pear) (32) were used to generate retrovirus containing GATA-1 cDNAs. BOSC23 cells grown in T25 flasks to 80% confluence were transiently transfected with 8 μg of DNA per flask by the calcium phosphate precipitation method in the presence of 25 μM chloroquine. Fresh medium was added 10 h following transfection. At 24 h later, G1E cells were transferred to fresh medium and grown for an additional 48 h before being subjected to benzidine staining of hemoglobin (45).
Of note, in both motifs the pattern of acetylation is different from that observed in p53 (16), indicating that the amino acid context surrounding the acetylation sites directs substrate specificity.

Acetylation of the peptide spanning the C-motif occurred with higher efficiency than that of the peptide spanning the N-motif, as judged by the absolute number of incorporated acetate residues (Fig. 3, compare the top and bottom panels). Given that both motifs are acetylated with comparable efficiency in the context of full length GATA-1 (Fig. 2), this suggests that other portions of GATA-1 contribute to substrate recognition by CBP-HAT. Residue 252, which is conserved among different members of the GATA family, remained intact in the NC-mut construct and might account for its residual acetylation (Fig. 2).

Given the importance of the amino acid context in directing acetylation toward specific lysine residues, we asked whether a substitution at lysine 312, which resides in the C-motif, leads to reduced acetylation in the context of full-length N-mut (N-mut 312A). Surprisingly, we found that N-mut 312A is acetylated to a similar degree to N-mut (data not shown), indicating that lysine residues adjacent to lysine 312 can serve as alternative substrates and that the specificity of CBP-HAT toward certain residues is not absolute. Therefore, to ensure complete loss of acetylation for our functional studies, we used GATA-1 mutants which contain multiple lysine-to-alanine substitutions in each motif, as shown in Fig. 2.

In vivo acetylation of GATA-1. To determine whether acetylation of GATA-1 occurs in vivo, we pulse-labeled MEL cells with [3H]acetate for 90 min and then subjected them to cell lysis and immunoprecipitation with monoclonal GATA-1 antibodies. The autoradiograph shown in Fig. 4A demonstrates that GATA-1 antibodies, but not isotype-matched control antibodies, precipitate [3H]acetate-labelled GATA-1, indicating that acetylation of GATA-1 occurs in intact erythroid cells. Similar results were obtained with [3H]acetate-labelled COS cells transfected with GATA-1 (data not shown). A large number of cellular proteins are acetylated at their N terminus, which could account at least in part for the observed [3H]acetate incorporation. To ensure that acetylation of GATA-1 occurs in vivo at the same sites acetylated by CBP in vitro, we used an antibody, anti-AK, which reacts with acetyl-lysine residues but not with unacetylated lysine residues (18). Lysates from transfected COS cells were immunoprecipitated with an-
ti-AK and then subjected to Western blotting with anti-GATA-1 antibodies. Anti-AK precipitated significant amounts of wild-type GATA-1 (Fig. 4B, top), while precipitation of N-mut and C-mut was reduced and only very little NC-mut was precipitated (Fig. 4B, top). Control Western blots demonstrate the presence of comparable amounts of transfected GATA-1 constructs in the lysates used for immunoprecipitation (Fig. 4B, bottom). These results indicate that GATA-1 is acetylated in vivo at the same sites acetylated by CBP in vitro. In addition, both lysine-rich motifs appear to contribute equally to total acetylation, again similar to what is observed with in vitro-acetylated GATA-1. As in the in vitro acetylation experiments, residual acetylation observed with NC-mut might be due to acetylation of lysine 252.

To determine whether CBP can stimulate GATA-1 acetylation in vivo, we cotransfected NIH 3T3 cells with GATA-1 and CBP and then subjected them to anti-AK immunoprecipitation and anti-GATA-1 Western blotting. The results demonstrate that coexpression of CBP leads to a strong increase in acetylation of wild-type GATA-1 but not of NC-mut (Fig. 4C). Thus, CBP induces GATA-1 acetylation in vivo at the same site as it does in vitro. Of note, coexpression of E1A, but not of mutant E1A defective for CBP binding (E1A Δ2–36), abolished in vivo acetylation of GATA-1 (Fig. 4C). Control Western blots showed that neither CBP nor E1A expression significantly altered GATA-1 protein levels (data not shown). This establishes a strong correlation between acetylation of GATA-1 and its transcriptional activity (4).

The failure to detect acetylated GATA-1 in NIH 3T3 cells in the absence of cotransfected CBP is due to the presence of substantially lower basal levels of acetylated GATA-1 protein than in COS cells (data not shown).

**Acetylation does not affect DNA binding of GATA-1.** In contrast to p53, where acetylation occurs outside the DNA-binding domain (16), the acetylation motifs in GATA-1 are directly
The C-terminal acetylation motif of GATA-1 is required for binding to and stimulation by CBP in transient-transfection assays. To test whether acetylation by CBP affects transcriptional activity of GATA-1, we cotransfected GATA-1 acetylation mutants together with CBP and a synthetic GATA site-containing reporter plasmid into NIH 3T3 cells. CBP expression increased wild-type GATA-1 and N-mut activity about eightfold, in agreement with our previous studies (Fig. 5A) (4). In contrast, C-mut and NC-mut were severely impaired in their response to CBP. Since CBP interacts with the zinc finger region of GATA-1 (4), we considered the possibility that mutations in the C-terminal motif affect interaction with CBP. To address this question, we performed in vitro protein-binding assays with bacterially expressed GST-CBP containing the GATA-1-binding domain (aa 1805 to 1891) and in vitro-translated GATA-1 constructs. The results show that C-mut and NC-mut bind GST-CBP less avidly than wild-type GATA-1 and N-mut do (Fig. 5B). This establishes a correlation between the ability of CBP to bind to GATA-1 and to enhance its activity, indicating that a direct interaction between these molecules is required for transcriptional stimulation.

Since mutations in the C-motif do not distinguish between the effects of binding to and acetylation by CBP, we directly tested the requirement of the acetyltransferase domain of CBP for GATA-1 activation. We introduced a double point mutation into the acetyltransferase domain of CBP (CBP-HAT), which completely abolishes in vitro acetylation of free histones (22) and GST-GATA-1 (results not shown). In transient-transfection assays, CBP-HAT activated GATA-1 to the same extent as wild-type CBP did (Fig. 5C). Similar results were obtained with a CBP construct bearing a deletion in the acetyltransferase domain (Δ1458–1479) (reference 26 and data not shown). Thus, in transient-transfection assays of nonerythroid cells with an artificial reporter construct, GATA-1 activation by CBP does not require intrinsic acetyltransferase activity of CBP. This suggests that acetylation might be mediated by other
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Acetyltransferases associated with CBP or that GATA-1 activation might involve an acetylation-independent mechanism (see Discussion).

The following additional considerations have to be made when assaying GATA-1 and CBP functions. First, the chromatin structure of stably integrated genes regulated by GATA-1 and CBP is likely to be important, especially when evaluating the role of histone and factor acetylation. Second, regulation of GATA-1 and CBP activity might require the architecture of natural GATA-1-dependent gene promoters. Third, it is established that some GATA-1 functions are critically dependent on an erythroid environment (see below). To address these issues, we used the GATA-1-deficient erythroid cell line G1E, which can be induced to terminally differentiate upon introduction of GATA-1.

Both N and C acetylation motifs are required to trigger erythroid differentiation. GATA-1-deficient G1E cells proliferate as immature erythroblasts and undergo terminal differentiation and hemoglobinization upon infection with GATA-1-expressing retrovirus (45). Previous structure-function studies of GATA-1 yielded important differences in domain requirements depending on whether these studies were performed with NIH 3T3 cells or in G1E cells (25, 45) (see the introduction). To assess the role of GATA-1 acetylation in an erythroid context with chlorinated GATA-1 target genes, we determined the ability of various GATA-1 mutants to induce G1E cell differentiation. Retroviruses harboring wild-type and mutant GATA-1 constructs were used to infect G1E cells, and the extent of erythroid differentiation was monitored by benzidine staining for hemoglobin. Infection with wild-type GATA-1-carrying virus induced hemoglobinization in about 7% of cells, which approximates the proportion of infected cells, similar to what has been reported previously (45). The number of benzidine-positive cells obtained with GATA-1 was used as a standard (100%) against which to compare the other GATA-1 constructs. A substantially reduced number of benzidine-positive cells was seen when either N-mut or C-mut was expressed (10% and 22% of wild-type levels, respectively) (Fig. 7A), while NC-mut was completely inactive, with no detectable benzidine-positive cells. Consistent with these findings, a GATA-1 mutant which lacks the entire C terminus (Δ308, residues 308 to 413 deleted), including the C-terminal acetylation motif, showed an activity comparable to that of C-mut (Fig. 7A). In contrast, a similar deletion construct (Δ331, residues 331 to 413 deleted) which leaves the C motif intact was almost as active as wild-type GATA-1. A non-DNA-binding mutant of GATA-1, which bears a mutation in the C-terminal zinc finger (C261P) (25), showed no activity in this assay (Fig. 7A). To confirm that expression levels and DNA binding of all GATA-1 constructs were comparable in erythroid cells, nuclear extracts were prepared from the infected G1E cells and analyzed by gel mobility shift assays with a GATA-1-binding site as probe. To distinguish GATA-1 from endogenous GATA-2, which migrates at a similar position, monoclonal anti-GATA-1 antibodies were used to generate a GATA-1-specific supershift. The amounts of GATA-1 supershifted with GATA-1 antibodies were comparable in all samples (Fig. 7B). Together, these experiments establish the requirement of both acetylation motifs for GATA-1 activity in erythroid cells. These results contrast with those obtained in NIH 3T3 cells, where mutations at the N-motif had no detectable effect, and underscore the importance of the cellular environment for the analysis of transcription factor activity.

DISCUSSION

It has long been known that acetylated histones are associated with transcriptionally active chromatin (2, 33). A strong correlation between histone acetylation and DNase I sensitivity has been demonstrated at the chicken β globin gene (17). Moreover, a number of transcription factors have recently been added to the list of acetyltransferase substrates (16, 19, 48). We have previously shown that the HAT CBP physically interacts with GATA-1 and stimulates its activity in an EIA-sensitive manner (4). Interference with CBP function by forced expression of E1A led to a block in erythroid differentiation and failure to induce GATA-1-dependent genes, including the α- and β-globin genes. Deletion of the N-terminal 23 aa of E1A, which abrogates binding to CBP/p300 but not to Rb family proteins, led to loss of the ability of E1A to block GATA-1 activity and erythroid differentiation (4). Of note, this E1A mutant retained the critical amino acids required for binding to p/CAF, which can also directly bind to E1A (34), suggesting that the effects of E1A are largely due to specific inhibition of CBP/p300 function. Since GATA-1-binding sites in the locus control regions of the globin genes contribute to
maintaining DNase I hypersensitivity, this raised the possibility that GATA-1 acts by recruiting CBP to the locus control regions, thereby locally increasing histone acetylation. The results of the present study suggest that the interaction between CBP and GATA-1 entails an additional function, namely, acetylation of GATA-1 itself.

We demonstrated that CBP acetylates murine GATA-1 in vitro at two conserved lysine-rich motifs near the two zinc fingers. Using the CBP-related protein p300, Boyes et al. (7) mapped the major in vitro acetylation sites to the analogous motifs in chicken GATA-1, demonstrating the high degree of conservation between chicken and mouse GATA-1 in CBP- and p300-mediated acetylation.

Using anti-AK antibodies and in vivo labeling experiments we found that GATA-1 is acetylated in vivo, consistent with the findings of Boyes et al. (7). Using site-specific GATA-1 mutants, we further showed that acetylation in intact cells occurred at the same sites acetylated by CBP in vitro.

We also demonstrate that CBP can stimulate GATA-1 acetylation in vivo at the relevant sites and that expression of E1A abolishes acetylation completely. This demonstrates that CBP can strongly stimulate the acetylation of a transcription factor in vivo and that this activity is inhibited by E1A. These data further demonstrate that GATA-1 activity correlates well with its acetylation status (4).

While it is possible that other acetyltransferases such as SRC-1 and ACTR or unknown acetyltransferases acetylate GATA-1, several observations suggest that CBP is a major GATA-1 acetyltransferase. First, we have previously shown that CBP and GATA-1 associate in vivo; second, pCAF does not acetylate GATA-1; third, there is remarkable specificity among different acetyltransferases regarding substrate and residue specificity (24, 36, 48).

To examine the mechanism by which CBP regulates GATA-1 activity, we showed that in vitro acetylation did not detectably alter DNA binding of either full-length GATA-1 or...
the zinc finger region alone. Substitutions of lysine residues with arginine or alanine residues, which maintain and neutralize the positive charge, respectively, did not affect DNA-binding and dissociation rates of GATA-1 constructs expressed in mammalian cells, further supporting the lack of involvement of the acetylated motifs in DNA binding. These findings are consistent with the observation that GATA-1 mutants bearing a deletion of the entire C terminus including the C-motif (3408) bind DNA normally (25, 42). Furthermore, the solution nuclear magnetic resonance spectroscopy structure of the C-terminal zinc finger of chicken GATA-1 bound to DNA demonstrated that the site corresponding to the C-terminal acetylation motif of murine GATA-1 does not make direct contact with DNA (29). Our findings contrast with those of Boyes et al., who reported a significant change in DNA binding upon in vitro acetylation (7). These discrepancies might be the result of differences between chicken and mouse GATA-1 and between CBP and p300. However, in agreement with our findings, mutations in the chicken GATA-1 acetylation sites do not alter DNA binding (7). Clearly, the strong acetylation-induced increase in DNA binding of a peptide spanning the zinc finger region of chicken GATA-1 observed by Boyes et al. in vitro is not reflected in the moderate stimulation of chicken GATA-1 activity by p300 in transient-activation assays (7). It is possible that the in vitro conditions do not reflect the actual changes in GATA-1 function triggered upon acetylation.

Sequence analysis of in vitro-acetylated peptides revealed that lysine 312 is the major acetylated residue in the C-motif while lysine 252 and lysine 246 are the predominant sites in the N-motif. Lysine 252, which is conserved among GATA-1 of different species, resides outside the canonical RXKK motif and might account for the residual acetylation observed in vitro with the NC-mut construct. We are in the process of testing the function of GATA-1 carrying a point mutation at this site. Nevertheless, even with lysine 252 intact, mutations at the N-motif lead to reduced acetylation and diminished biological function, indicating that the N-terminal acetylation motif is important.

While sequencing of acetylated synthetic peptides allows the identification of acetylation sites in the context of an intact amino acid sequence, i.e., without the potential artifacts which might result from the introduction of mutations, the results obtained by this method must be interpreted in the context of other experimental approaches. For example, we noted that acetylation of the peptide spanning the N-motif occurred with lower efficiency than did that of the peptide spanning the C-motif. However, when assayed in the context of full-length GATA-1, disruption of the C-motif reduced acetylation to only about half of that observed with wild-type GATA-1, indicating that the extents of acetylation at the N-motif and at the C-motif are comparable. Thus, sequences outside of the stretch of amino acids contained in the peptides might contribute to maximal acetylation efficiency, perhaps by increasing the binding affinity between GATA-1 and CBP.

In performing in vitro acetylation reactions, it is important to keep in mind that recombinant, purified acetyltransferases might display a substrate spectrum distinct from that observed in the presence of additional cofactors. For example, the recombinant purified yeast acetyltransferase Gcn5 acetylates only free histones but not histones packaged into nucleosomes (8). However, when assayed as a multicomponent complex, Gcn5 acetylates nucleosomal histones as well (for example, see reference 15). Therefore, cofactors bound to CBP or to GATA-1 might modulate the specificity and/or efficiency of the CBP acetyltransferase.

Transient-transfection experiments designed to test the functional role of acetylation showed that the C-motif but not the N-motif of GATA-1 is required for stimulation by CBP. Since the C-motif contributes to CBP binding, this suggests that physical association or subsequent acetylation or both are important for stimulating GATA-1 activity. Mutations in the C-motif do not interfere with acetylation at the N-motif in vitro (Fig. 2) or in vivo (Fig. 4), suggesting that the interaction between C-mut and CBP is sufficient for efficient catalysis. To address directly whether CBP-mediated acetylation is required for GATA-1 activation, we used two CBP mutants which lack HAT activity, one containing a point mutation and the other bearing a deletion in the HAT domain. Both constructs failed to acetylate GATA-1 and histones in vitro but activated GATA-1 with an efficiency comparable to that of wild-type CBP in transiently transfected NIH 3T3 cells. Together, these findings indicate that in transient-expression assays, acetylation of GATA-1 and histones by CBP is not required for GATA-1 stimulation. At least two explanations could account for this result. First, it is important to note that in transiently transfected nonerythroid cells with artificial reporter genes, CBP might stimulate GATA-1 activity by a mechanism different from that operational in erythroid cells with fully chromatinized GATA-1 target genes. The reporter gene used in the transient-transfection assays contains a single GATA site positioned closely to a TATA box. Therefore, in this setting, it is possible that CBP augments GATA-1 activity by serving as a link between GATA-1 and the basal transcription machinery. A functional domain analysis of CBP is being used to address this question. Second, it is possible that acetylation is mediated by p300/CBP-associated acetyltransferases, including ACTR (9) and SRC-1 (39, 47). Experiments are under way to test the role of the CBP-associated acetyltransferases during GATA-1 activation.

In performing similar transient-transfection experiments, Boyes et al. observed that a p300 mutant bearing a 50-aa deletion in the HAT domain failed to stimulate chicken GATA-1 activity (7). It is possible that such a deletion abrogated functions of p300 unrelated to its HAT activity.

While Boyes et al. did not test the function of the C-motif (the strongest acetylation site), mutations in the chicken GATA-1 N-motif resulted in a reduced response to p300, leading the authors to establish a correlation between chicken GATA-1 acetylation at the N-motif and transcriptional activation. Two open questions remain regarding this interpretation: first, the observed reduction in the p300 response is marginal (2.5-fold in wild-type GATA-1 compared to 1.4- and 1.9-fold in two acetylation mutants), especially in a transient-transfection assay. Second, the authors did not provide information about the acetylation status of these sites in vivo. In our experiments, mutations in the N-motif had no effect on DNA binding or transactivation by CBP in transient-transfection assays. Furthermore, binding of GATA-1 to a single GATA-1 site does not require the N-terminal zinc finger (25). Therefore, we believe that the mechanism by which the N-motif contributes to the GATA-1 function remains to be elucidated but is unlikely to involve regulation of DNA binding. In evaluating these findings, we point out that a biological function of the N-motif required the assay of GATA-1 constructs in erythroid cells with GATA-1-dependent genes present in their natural state (see below).

Since critical GATA-1 functions were uncovered only in maturing erythroid cells (6, 41, 45), we used the GATA-1-deficient cell line G1E to study the functional role of the acetylation sites. Mutations in either the N- or C-motif significantly reduced activity, while mutations in both motifs abrogated activity entirely. Thus, only in erythroid cells did we
uncover a requirement for the N-motif. Mutations in the N-motif did not interfere with binding to CBP or to Fog (data not shown), the recently identified cofactor which selectively binds to the N-terminal zinc finger of GATA-1 (41), excluding the possibility that mutations simply disrupt the interaction of GATA-1 with its known cofactors. Deletion of the C terminus of GATA-1 leaving the C-motif intact (Δ331) resulted only in a small loss of activity, whereas a slightly larger deletion which removes the C-motif (Δ308) led to a much more pronounced loss of function without affecting DNA binding. Together, these results demonstrate that mutations in the acetylation motifs impair biological activity without affecting DNA binding, which demonstrates that the DNA-binding and acetylation functions can be uncoupled.

Interestingly, the ability of GATA-1 to induce megakaryocytic conversion of the early myeloid cell line 416B also depends in part on an intact C-motif (42). In these assays, Δ308 was significantly less active than Δ331, which raises the possibility that CBP also synergizes with GATA-1 in the regulation of megakaryocytic gene expression. Together, these results indicate that the C-terminal acetylation site is of great biological importance and are consistent with a requirement for CBP for GATA-1 function in vivo.

Acetylation of lysine residues neutralizes their positive charge and changes the size of the residue. In general, such changes could conceivably entail alterations in protein function similar to those incurred upon phosphorylation. For example, acetylation could lead to changes in protein conformation. Such a mechanism has been proposed for p53, where acetylation in the regulatory domain leads to increased DNA-binding activity (16). Alternatively, acetylation could directly influence protein-DNA interactions, as has been suggested for binding of histone tails to DNA, or it could affect protein-protein interactions, as described for binding of histone tails to the yeast transcriptional repressor Tup1 (13).

The mechanism by which either acetylation motif in GATA-1 exerts its function in G1E cells remains to be determined. Gel shift analysis with nuclear extracts of the virally infected G1E cells demonstrated the presence of equal amounts of DNA-bound GATA-1 proteins, and immunofluorescence microscopy confirmed the nuclear localization of all constructs. This suggests that acetylation in either motif does not prevent nuclear localization, DNA binding, or protein stability. Several additional mechanisms by which acetylation regulates GATA-1 activity could be envisioned. It is conceivable that acetylation leads to a conformational change in GATA-1, resulting in increased activity through exposure of an activation domain. Alternatively, the acetylation motifs might represent docking sites for still unidentified coactivators or repressors, and acetylation might positively or negatively regulate their binding affinity. More extensive studies, including structural analyses, will be required to determine the mechanistic role of GATA-1 acetylation.

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