GATA-1 Self-association Controls Erythroid Development in Vivo*

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Ritsuko Shimizu‡, Cecelia D. Trainor‡, Keizo Nishikawa§, Makoto Kobayashi‡, Kinuko Ohneda‡, and Masayuki Yamamoto†

From the ‡Graduate School of Comprehensive Human Sciences and Center for Tsukuba Advanced Research Alliance, University of Tsukuba, Tennoudai 1-1-1, Tsukuba 305-8577, and Exploratory Research for Advanced Technology Environmental Response Project, Japan Science and Technology Agency, University of Tsukuba, Tsukuba 305-8577, Japan, and the §Laboratory of Molecular Biology, NIDDK, National Institutes of Health, Bethesda, Maryland 20892

GATA-1 is the key transcription factor for the development of the erythroid, megakaryocytic, eosinophilic, and mast cell lineages. GATA-1 possesses the ability to self-associate, and this characteristic has been suggested to be important for GATA-1 function. To elucidate the roles self-associated GATA-1 plays during hematopoietic cell development in vivo, in this study we prepared GATA-1 mutants in which three lysine residues potentially contributing to the self-association (Lys-245, Lys-246, and Lys-312) are substituted in combination with alanines. Of the mutants, 3KA harboring alanine substitutions in all three lysines showed reduced self-association activity without considerable interference in the modification of GATA-1 by acetylation. We generated transgenic mouse lines that express these GATA-1 mutants utilizing the Gata1 hematopoietic regulatory domain, and crossed the mice to Gata1 knockdown (GATA-1.05) mutant mice. Although NKA (K245A and K246A) and CKA (K312A) mutants almost fully rescued the GATA-1.05 mice from anemia and embryonic lethality, the 3KA mutant only partially rescued the GATA-1.05 mutant mice. Even with the higher than endogenous level expression, GATA-1.05/Y::3KA embryos were prone to die at various stages in mid-to-late gestation. Live birth and an anemic phenotype were restored in some embryos depending on the expression level of the 3KA transgene. The expression of the transferrin receptor and heme biosynthesis enzymes was impaired in the yolk sac and liver of the 3KA-rescued embryos. Immature erythroid cells with insufficient expression of the transferrin receptor accumulated in the livers of 3KA-rescued embryos. These results provide the first convincing line of evidence that the self-association of GATA-1 is important for proper mammalian erythroid development in vivo.

Significant progress has been made in the analysis of the transcriptional machinery operating during hematopoietic development. Elaborate regulation of hematopoiesis is suggested to be a consequence of highly orchestrated interactions of multiple transcription factors and cofactors. Modifications of transcription factors also appear to be important for the establishment of specificity and/or affinity of the interactions. The impact of the interactions and modifications of transcription factors on hematopoietic development in vivo is an important topic to be addressed.

GATA-1 is the key regulator of transcription in erythroid, megakaryocytic, eosinophilic, and mast cells (1–3). Essential roles for GATA-1 in erythroid and megakaryocytic cell development have been shown through in vitro differentiation analyses (4, 5). GATA-1-deficient embryonic stem cells fail to mature but undergo apoptosis (6, 7). Neither complete knock-out of GATA-1 nor knockdown mutation to 5% of the wild-type GATA-1 level (designated as the GATA-1.05 allele) could sustain primitive erythropoiesis in mouse embryos, whereas milder knockdown resulted in phenotypically normal mice (8–10), indicating that there is a threshold for GATA-1 to sustain proper hematopoietic cell differentiation (reviewed in Ref. 11).

We recently found that erythroid progenitors accumulate in the hematopoietic organs of heterozygous Gata1 knockdown mice (GATA-1.05/X); these cells are produced by the inhibition of apoptosis, in conjunction with stimulation of proliferation and inhibition of erythroid differentiation. The latter two mechanisms are provoked by the reduced expression of GATA-1 to 5% of wild-type level, whereas the former mechanism (i.e. inhibition of apoptosis) is an inherent function of GATA-1 that is still operating despite the severely reduced GATA-1 level. These erythroid progenitors eventually proceed to overt leukemic cells (12, 13).

GATA-1 contains two highly conserved zinc fingers, both of which are in the Cys-X2-Cys-X17-Cys-X2-Cys configuration. The C-terminal finger (CF)2 is essential for high affinity binding to the consensus GATA binding sequence, whereas the N-ter-

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1 To whom correspondence should be addressed. Tel.: 81-298-53-6158; Fax: 81-298-53-7318; E-mail: masi@tara.tsukuba.ac.jp.

2 The abbreviations used are: CF, C-terminal finger; NF, N-terminal finger; G1HRD, Gata1 gene hematopoietic regulatory domain; EKL, erythroid Kruppel-like factor; CBP, CREB-binding protein; FOG-1, friend of GATA-1; MBP, maltose-binding protein; p/CAF, p300-associated factor; RT, reverse transcription; Trfc, transferrin receptor 1; Als-e, erythroid 5-aminolevulinate synthase; Pbgd, porphobilinogen deaminase; Alad, 5-aminolevulinate dehydratase; GFP, green fluorescence protein; FSC, forward scatter; SSC, side scatter; E, embryonic day; GST, glutathione S-transferase; MEL, mouse erythroleukemia.
minal finger (NF) stabilizes DNA binding to palindromic GATA sites and recognizes a distinct GATC core sequence (14–17). In addition to DNA binding, these zinc fingers are important for interaction with other transcription factors, such as Sp1, erythroid Krüppel-like factor (EKLF), CREB-binding protein (CBP), FOG-1 (Friend of GATA-1), and PU.1 (7, 18–20). Similarly, GATA-1 associates with itself (or forms homodimers) using its zinc fingers (10, 21). These interactions enable GATA-1 to work as a powerful transcriptional activator (22).

We have designed and conducted a transgenic complementation rescue analysis utilizing the Gata1 gene hematopoietic regulatory domain (GIHRD) that recapitulates Gata1 expression in vivo (23–25). GATA-1 expression under the regulatory influence of GIHRD can fully rescue the GATA-1.05 knockdown mouse from anemia and embryonic lethality (26). This method was also used for in vivo functional analysis of various GATA-1 domains, and it has been demonstrated that NF is dispensable for primitive erythropoiesis, whereas both CF and NF are strictly required for proper definitive erythropoiesis (27).

The transgenic complementation rescue approach has been extended to analyze various motifs within GATA-1. For instance, the GATA-1 and FOG-1 interaction was found critical for megakaryocyte development in vivo through the analysis of mice harboring Val-205 mutant (28). An intriguing observation in the analysis was that, in contrast to the case for the entire NF-deletion mutant, high level accumulation of the Val-205 mutant sustains hematopoietic progenitor differentiation to the erythroid lineage despite impaired ability to interact with FOG-1 (28). Rescued mice with Val-205 mutant transgene develop to adult stage.

Whereas disruption of the self-association ability of GATA-1 was found to reduce markedly the positive autoregulation mechanism for the Gata1 gene in zebrafish embryos (29, 30), the physiological significance of the self-association mechanism remains to be assessed. Therefore, to gain insights into the contribution of GATA-1 self-association to the regulation of erythropoiesis in vivo, especially in mammalian systems, in this study we attempted another transgenic complementation rescue analysis exploiting GATA-1 mutants that lack the self-association ability. We modified two lysine residues (Lys-245 and Lys-246) located at the C-terminal side of NF, along with one (Lys-312) located at the C-terminal side of CF. The combination of these three lysine mutations (3KA) reduces self-association ability significantly but affects the acetylation of GATA-1 only mildly. The 3KA mutant expressed under the control of GIHRD hardly rescues GATA-1 knockdown mutant mice from embryonic lethality. Although 3KA mutant embryos survive the primitive hematopoietic stage, they succumb to anemia during definitive hematopoiesis, and die at various late embryonic stages. The expression of the transferrin receptor and heme biosynthesis enzymes is markedly reduced, indicating that self-association is indispensable for GATA-1 to direct proper erythroid development through regulating the expression of specific target genes.

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**EXPERIMENTAL PROCEDURES**

**Immunoblot Analysis**—Whole cell extracts were prepared from bone marrow. Bone marrow cells in 300-μl SDS sample buffer were sonicated and subsequently heat-denatured. Samples were separated by SDS-10% PAGE, and transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore). The membrane was probed with anti-mouse GATA-1 antibody (N6, Santa Cruz Biotechnology, Santa Cruz, CA (31)) and horseradish peroxidase-conjugated sheep anti-rat IgG (ICN Biomedicals) as primary and secondary antibodies, respectively.

**Expression and Purification of Wild-type GATA-1 and Mutant Proteins**—Maltose-binding protein (MBP)-GATA-1, -NKA, -CKA, -3KA, and MBP were expressed in *Escherichia coli* strain BL21 codon plus RIL (Stratagene). The cells were grown overnight at 37 °C in LB medium containing 50 μg/ml ampicillin and 50 nm zinc acetate and induced with isopropyl-1-thio-β-d-galactopyranoside for 1 h at 37 °C. Cells were lysed in phosphate-buffer saline by sonication, and the proteins were purified by affinity chromatography over an amylose resin column (New England Biolabs). His-tagged human p300 protein was purified from Sf9 cells infected with recombinant baculo-virus, which is a kind gift from Drs. Ito and Kraus (32, 33).

**In Vitro Acetyltransferase Assays**—Acetyltransferase assays were performed as previously described with a minor modification (34). Briefly, proteins were incubated with 100 ng of p300 or p/CAF in the presence of [14C]acetyl-CoA for 1 h at 30 °C in acetyltransferase buffer (50 mM Tris-HCl (pH 8.0), 10% glycerol, 0.5-mM dithiothreitol, 1-mM p-aminobenzenesulfonyl fluoride, 0.1 mM EDTA, and 10 mM sodium butyrate). Reaction mixtures were separated by SDS-10% PAGE and analyzed with a phosphorimaging device (BAS 1600, Fujifilm).

**GST Pulldown Assay**—MBP and glutathione S-transferase (GST) fusion GATA-1 proteins were produced in *E. coli* BL21(DE3)pLysS competent cells (Novagen) as described (30). GST fusion proteins containing the GATA-1 finger domain (200–322 amino acids) were purified with glutathione-Sepharose beads (Amersham Biosciences) and mixed with graded amount of MBP fusion protein (0, 2, 5, and 10 μg). Proteins were incubated in binding buffer (40 mM HEPES, 50 mM KCl, 5 mM MgCl₂, 0.2 mM EDTA, 1 mM dithiothreitol, and 0.5% Nonident P-40) at 4 °C for 1 h. Glutathione-Sepharose beads were subsequently washed three times with the binding buffer, and bound proteins were eluted in SDS sample buffer and separated in SDS-7% polyacrylamide gel electrophoresis. 100 ng of MBP fusion protein was used as an input control. Immunoblot analysis was performed using anti-MBP antibody (Santa Cruz Biotechnology). Results were quantified by the use of an LAS-3000 phosphorimaging device (Fujifilm).

**RT-PCR**—Total RNA from the spleen or bone marrow of transgenic founder mice was prepared by using the RNA-sol extraction system (Tel-Test). The cDNAs were synthesized with superscript reverse transcriptase (Invitrogen). For analyzing the expression of GATA-1 mutants, the amount of reverse-transcribed cDNA was adjusted by dilution to give an amount equivalent to that of the endogenous GATA-1 cDNA amplicon. The expression of total Gata1 mRNA was detected by real-time
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quantitative RT-PCR analysis with an ABI Prism 7700 sequence detection system. Primers used were 5′-CAGAACCAGC-CTCTCATTCC and 5′-TAGTGGTGGTGGTGGTGGT. The TaqMan fluorescence probe used was 5′-CCCAGGAGG-GAATTGTGCAGCAGA. For semi-quantitative RT-PCR analysis of mRNAs, the amount of cDNA was diluted to give an amount equivalent to that of the Hprt ampiclon. Sequences of the primers used were 5′-TGGATCTAGAGTGCTACTG and 5′-GGTAGTCTGGCCAGACTTTCCTG for transferrin receptor 1 (Trfc). The other primers used in this study were as described previously (27, 28). Each primer pair was designed to span at least one intron to distinguish amplons originating from cDNA and genomic DNA.

Statistical Analysis—Statistical analyses were performed with a Student’s t test. Probability values of p < 0.05 were considered as statistically significant.

RESULTS

A GATA-1 Mutation That Impairs Self-association but Not Acetylation Modification—To gain further insights into the contribution of GATA-1 self-association to the regulation of erythropoiesis in a mammalian in vivo system, in this study we established transgenic lines of mice that harbor a GATA-1 mutant specifically lacking the self-association ability. In addition to contributing to the interaction surface between GATA-1 molecules, the lysine residues in the NF and CF tail regions of mouse GATA-1 (mGATA-1) are also acetylated by CBP/p300 (35–37). Therefore, to distinguish the effects of self-association from those caused by acetylation, it was necessary to generate a GATA-1 mutant molecule that cannot self-associate but can be acetylated. To this end, we modified two lysine residues of the KKR motif (Lys-245 and Lys-246) located at the N-terminal side of CF (CKA), as shown in Fig. 1A.

We established three independent GATA-1 mutants that contain these lysine to alanine substitutions alone and in combination, NKA, CKA, and 3KA (NKA+CKA), as shown in Fig. 1A. To examine the susceptibility of these mutant GATA-1 molecules to acetylation, we performed an in vitro acetylation assay. As shown in Fig. 1B, an MBP fusion protein containing 3KA mutant GATA-1 was acetylated by p300 to ~60% of the wild-type level. This result shows very good agreement with the report of a GATA-1 mutant acetylation attained by CBP (37); the substitution of Lys-312 to alanine was reported not to influence the acetylation of GATA-1 by CBP, perhaps because of the compensation by adjacent lysine residues.

We next examined whether or not the self-association ability of GATA-1 was impaired by the introduction of NKA, CKA, or 3KA mutation by a GST pulldown assay. GST-tagged GATA-1 finger domain (corresponding to amino acids 200–322) harboring the NKA, CKA, or 3KA mutation was incubated with graded amounts of wild-type or mutant GATA-1 proteins fused to MBP, and resulting complexes were captured with glutathione-Sepharose beads, followed by immunoblotting with anti-MBP antibody (Fig. 1C). In this analysis the MBP-GATA-1 protein was detected as a doublet (a major band plus a minor band with slower mobility) in both wild-type and mutant proteins. As shown in Fig. 1D, the band intensity shown in C was quantified by phosphorimaging analysis. The band intensity was normalized with that from input protein loaded on the same gel. Results are shown as average ± S.D. of three independent experiments. Asterisks, p < 0.05 relative to values from wild-type GATA-1.

FIGURE 1. The GATA-1–3KA mutant reduces the self-association ability of GATA-1 without considerable interference of acetylation. A, illustration of the substitution mutants of GATA-1 employed for the transgenic complementation rescue assay. NKA, Lys to Ala substitutions in Lys-245 and Lys-246; CKA, Lys to Ala substitution in Lys-312; 3KA, both motifs mutated. B, in vitro acetylation assay of GATA-1 using mouse p300. Wild-type and 3KA mutant GATA-1 fused with maltose-binding protein (MBP) were used as substrates for p300. Upper panel, 14C-labeled proteins were visualized with autoradiography. Lower panel, relative amounts of tested fusion proteins are shown by Coomassie Brilliant Blue staining. Migration positions of the GATA-1 proteins are indicated by arrows, and autoacetylated p300 is indicated by an asterisk. C, self-association of GATA-1 proteins was tested in vitro in a pull-down assay. GST-fused GATA-1 finger protein (encoding residues 200–322) harboring NKA, CKA, or 3KA mutation was incubated with graded amounts (0, 2, 5, and 10 µg) of corresponding GATA-1 proteins fused to MBP. The proteins were captured by glutathione-Sepharose beads and analyzed by immunoblot to detect MBP fusion proteins. 0.1 µg of MBP fusion protein was loaded onto the same gel as input control. Equal volumes of the GST fusion protein–Sepharose bead fractions were used for the pulldown assay, and protein amounts were comparable (bottom panel). D, the band intensity shown in C was quantified by phosphorimaging analysis. The band intensity was normalized with that from input protein loaded on the same gel. Results are shown as average ± S.D. of three independent experiments. Asterisks, p < 0.05 relative to values from wild-type GATA-1.
four different protein lanes when visualized by Coomassie Brilliant Blue staining (Fig. 1C, bottom panel). These results thus indicate that both the N-terminal (245 and 246) and C-terminal (312) lysine residues are involved in the self-association of GATA-1 protein in vitro, and these residues act cooperatively.

The C-terminal Lys-312 residue seems to contribute more critically to the GATA-1 self-association than the N-terminal lysine residues do.

Expression of GATA-1 Mutant in Vivo under the Regulation of GATA-1 Hematopoietic Regulatory Domain—We cloned NKA, CKA, and 3KA mutants of GATA-1 individually into a vector harboring the G1HRD, which directs expression of the transgenes in both primitive and definitive erythroid lineages (23, 26). These transgenic constructs were then microinjected into fertilized eggs using the standard procedure (38), and several transgenic mouse lines were established for each GATA-1 construct. The expression level of the transgenes was determined by semi-quantitative RT-PCR as described previously (27). Because all transgenic constructs contain 110-bp extra sequence insertion into 5′-untranslated region, a single PCR reaction enabled us to determine the expression level of all constructs as well as the difference of expression level between transgene-derived and endogenous GATA-1 mRNA.

As shown in Fig. 2A, we obtained three to five transgenic mouse lines per construct. These mouse lines were classified in accordance with the expression level of transgene-derived GATA-1 mRNA determined by the semi-quantitative RT-PCR using the cDNA templates from the spleens of transgenic females. Each transgenic line was categorized into High (transgene-derived mRNA level was severalfold higher than endogenous GATA-1 mRNA level; H), Medium (comparable to endogenous GATA-1 mRNA level; M), and Low expressers (less abundant than the endogenous GATA-1 mRNA; L). We next examined the expression GATA-1 mRNA derived from both Gata1 transgene and endogenous Gata1 gene by real-time quantitative RT-PCR. Showing very good coincidence with the results of conventional semi-quantitative RT-PCR analyses (Fig. 2A), we found that the expression of 3KA-H1 mRNA was much higher than that of 3KA-M in this quantitative RT-PCR analysis.
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(Fig. 2B). We found no hematological abnormality in these transgenic lines of mice (data not shown).

To verify whether the accumulation of mutant GATA-1 protein inside the transgenic animals was comparable with that expected from the transgene-derived mRNA level, we prepared total protein lysates from the 3KA transgenic male mice bone marrow and performed immunoblot analysis using N6 anti-GATA-1 antibody (31). A 2.5-, 4-, and 4-fold increase of the expression level of immunoreactive proteins was detected in bone marrow cell lysates prepared from 3KA-M, 3KA-H2, and 3KA-H1 transgenic mice, respectively, compared with that of the wild-type mouse (Fig. 2C). In the immunoblotting experiment, we found a low molecular weight protein band, with expression levels that correlate well with full-length GATA-1. This phenomenon has been observed reproducibly in the immunoblots of GATA-1 in hematopoietic tissues, but not for the GATA-1 from cultured cells, suggesting that the band may correspond to a limited proteolysis product of GATA-1 (35). These findings demonstrate that the GATA-1 mutant proteins do accumulate in the hematopoietic organs of transgenic mice, showing very good coincidence with the results of semi-quantitative and quantitative RT-PCR.

GATA-1 harbors two nuclear localization signals, the RPKKR (243–247 amino acids) and KGKKK (312–316 amino acids) motifs, and the alanine-substituted lysines were located in either of the two GATA-1 nuclear localization signals. Therefore, to examine nuclear localization of the mutant GATA-1 proteins, the subcellular localization of the transgene-derived mutant GATA-1 proteins was examined by immunohistochemistry using N6 antibody and the yolk sacs of E9.5 embryos. Whereas GATA-1 could not be detected in the GATA-1.05/Y embryonic yolk sacs without the transgene (Fig. 2E), N6 antibody detected 3KA mutant GATA-1 in the nucleus of erythroid cells in the yolk sacs of GATA-1.05/Y embryo bearing 3KA transgene (Fig. 2F) as well as wild-type embryos (Fig. 2D). This is in very good agreement with the previous report that a GATA-1 mutant with multiple lysine-to-alanine substitutions, including Lys-245, Lys-246, and Lys-312, did accumulate in the nucleus (39). Taken together, these results indicate that the mutant GATA-1 proteins derived from the transgenes were expressed in the nucleus of hematopoietic cells in vivo at levels compatible with the amounts of their mRNAs.

Self-association of GATA-1 Is Necessary for Erythropoiesis—To examine the importance of the self-association of GATA-1 protein for its function, we performed complementation rescue analysis of GATA-1 knockdown mice from embryonic lethality (26, 27). Because the GATA-1 gene is located on the X chromosome, hemizygous male (GATA-1.05/Y) mice cannot live beyond 12.5 embryonic days (E12.5), whereas heterozygous female (GATA-1.05/X) mice are viable and fertile, albeit they have various degrees of anemia during the neonatal period due to random X chromosome inactivation (9). We have shown that transgene-derived wild-type GATA-1 accumulated under the regulation of G1HRD can overcome the GATA-1.05/Y embryonic lethality (26), so the rationale of this analysis is that GATA-1.05/Y mice bearing a mutant transgene would die in utero, if the function of GATA-1 mutant were defective.

We crossed transgenic male mice harboring the GATA-1-NKA, -CKA, or -3KA mutant with GATA-1.05/X female mice and analyzed the genotype of pups by PCR. Results of this rescue experiment are summarized in Fig. 3, and numbers in the figure show pups analyzed in individual studies. Although no live pups were obtained from GATA-1.05/Y mice lacking a transgene (data not shown), GATA-1.05/Y mice bearing the NKA transgene were rescued from embryonic lethality with the number statistically expected, when the expression level of the NKA transgene was compatible with that of the endogenous GATA-1 (Fig. 3, gray bars). Similarly, the CKA mutant could fully rescue the GATA-1.05/Y mice from embryonic lethality, even with low level expression of the transgene (Fig. 3, white bars). We did not notice any hematological abnormalities upon examination of the NKA and CKA transgene-rescued mice (data not shown).

In stark contrast, transgenic lines of mice expressing the GATA-1–3KA mutant showed significant variation in the rescue depending on their transgene expression level (Fig. 3, black bars). When the 3KA mutant was expressed at medium level, no rescued pups were observed, but when GATA-1–3KA mutant was expressed at higher levels (3KA-H1 and -H2), rescued mice were obtained, albeit at a much lower frequency (~14 and 7%, respectively) than Mendelian expectations. Intriguingly, GATA-1.05/Y mice expressing the 3KA mutant (GATA-1.05/Y:3KA-H1) that escaped from the embryonic lethality showed moderate anemia but no thrombocytopenia (Table 1). This finding suggests that the GATA-1–3KA mutation specifically affects erythropoiesis, and this mutant protein does not rescue the GATA-1.05/Y mice efficiently from embryonic lethality, even if expressed at high level. In summary, this series of transgenic complementation rescue assays demonstra-
strates that the three lysine residues manipulated in this study are vital for definitive erythropoiesis. The normal phenotype of GATA-1.05 mice rescued with NKA or CKA transgenes suggests that there is no specific requirement for any of the three individual lysine residues and that lysines in the N-terminal and C-terminal fingers might be able to compensate for each other.

Embryonic Analysis Reveals That GATA-1 Self-association Is Vital for Both Primitive and Definitive Erythropoiesis—Because GATA-1.05/Y mice bearing the GATA-1–3KA mutant transgene died in utero, we next examined whether the requirement for GATA-1 self-association is different between primitive and definitive erythropoiesis. To examine this possibility, we crossed GATA-1.05/X mice with male 3KA-M transgenic mice and analyzed the genotype of embryos at the various stages.

At E10.5, GATA-1.05/Y embryos were severely pale (Fig. 4D) compared with wild-type embryos (Fig. 4A). Similarly, GATA-1.05/Y embryos bearing the 3KA mutant transgene (GATA-1.05/Y::3KA-M; Fig. 4, B and C) were usually distinguishable easily from both GATA-1.05/Y and wild-type littermates, because a decreased level of circulating red blood cells was observed. This result suggests that the self-association of GATA-1 is important for primitive hematopoiesis, but in the few embryos rescued by the 3KA mutant, primitive erythropoiesis was somehow sustained at a sufficient level to keep the embryos alive.

At E13.5, GATA-1.05/Y::3KA-M embryos that escaped premature death during the aforementioned primitive hematopoietic crisis showed severe anemia (Fig. 4G) compared with both wild-type (Fig. 4E) and GATA-1.05/X heterozygous (Fig. 4F) littersmates, and no live GATA-1.05/Y::3KA-M embryos were observed beyond E14.5 (data not shown). This finding indicates that the self-association capability of GATA-1 is crucial to sustain definitive erythroid cell development, because the mutant embryos could not develop without the contribution of the self-associated GATA-1.

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TABLE 1
Hematopoietic indices of rescued mice

| Mouse type     | Mouse number | Age  | Hemoglobin | Hematocrit | Red blood cell count | White blood cell count | Platelet count |
|----------------|--------------|------|------------|------------|----------------------|------------------------|---------------|
| 3KAR-H1        | 3            | 418 ± 70 | 11.4 ± 0.4* | 39.7 ± 2.2* | 774 ± 78*           | 131 ± 39*             | 83.9 ± 14.1   |
| Wild type      | 9            | 407 ± 28 | 12.9 ± 0.5 | 44.0 ± 1.9 | 887 ± 40            | 66 ± 26               | 97.4 ± 34.1   |
| GIR           | 2            | 482     | 13.9       | 45.6       | 998                  | 101                    | 76.1          |

* p < 0.05.

To examine whether overexpression of the GATA-1–3KA mutant can compensate for the observed defects in the mutant mice, we intercrossed males of 3KA-H1 transgenic line with GATA-1.05/Y::3KA-M embryos that escaped pre-implantation death due to impaired erythropoiesis (Table 2 and data not shown). Finally, GATA-1.05/Y::3KA-H1 pups were born in a ratio of $<15\%$ as expected (Fig. 3). In summary, these results demonstrate that the ability of GATA-1 to self-associate is required to sustain both primitive and definitive erythropoiesis.

The Self-association of GATA-1 Is Required to Regulate the Transcriptional Activity of Some of Its Target Genes—To analyze the cause of hematological defect in GATA-1.05/Y::3KA-M embryos, we examined the expression of GATA-1 and its target genes. We found that the transgenic expression of GATA-1–3KA mutant mRNA in E9.5 yolk sac varied significantly in the 3KA-M transgene-rescued embryos, even among the embryos from the same litter (data not shown; see Fig. 4, B and C). Results of semi-quantitative RT-PCR analysis for one mildly

FIGURE 4. GATA-1.05/Y::3KA-M embryos at two different embryonic stages. A–D, analysis of E10.5 embryos. Although wild-type embryo has abundant red blood cells in its heart and aorta (A), GATA-1.05/Y embryo at E10.5 is pale, and red blood cells are not observed (B). GATA-1.05/Y embryos bearing the 3KA transgene have various degrees of anemia; in one embryo red blood cells are clearly observed (C), whereas the other embryo is as pale as GATA-1.05/Y embryo (D). E–G, Analysis of GATA-1.05/Y::3KA-M embryos at E13.5. GATA-1.05/X heterozygous female embryo (F) shows anemia caused by the random X chromosome inactivation compared with wild-type littermate (E). GATA-1.05/Y::3KA-M embryo shows severe anemia (G).


TABLE 2
Embryo analysis of GATA-1.05/Y::3KA-H1

| Embryo age | Total number of pups | GATA-1.05/Y | GATA-1.05/Y::3KA-H1 |
|------------|----------------------|-------------|---------------------|
|            | Number of live pups | Number of dead pups | Number of live pups | Number of dead pups |
| E9.5       | 42                   | 5           | 0                   | 1                   |
| E10.5      | 17                   | 2           | 0                   | 2                   |
| E11.5      | 46                   | 2           | 1                   | 5                   |
| E12.5      | 78                   | 0           | 7                   | 4                   |
| E13.5      | 86                   | 0           | 4                   | 11                  |
| E15.5      | 78                   | 0           | 1                   | 3                   |

FIGURE 5. Expression of GATA-1 target genes in the rescued embryos.

A, mRNA abundances of GATA-1 target genes were determined in E9.5 yolk sacs from wild-type (lanes 1–3 and 4–6) and two GATA-1.05/Y::3KA-M (3KAR-M) (lanes 7–9) and four GATA-1.05/Y::3KA-H1 (3KAR-H1) (lanes 1–6) embryos by semi-quantitative RT-PCR. The products were analyzed after 21, 24, and 27 cycles for Trfc; 27, 30, and 33 cycles for Gata1, Alas-e, and Hprt; 30, 33, and 36 cycles for Gata2, Pbgd, e-globin, Eklf, Nfe2p45, and Fog1. B, expression of the GATA-1 target genes was examined in E12.5 fetal livers from three wild-type (lanes 1–2, lanes 3–4, and lanes 5–6) and four GATA-1.05/Y::3KA-H1 (3KAR-H1) (lanes 7–8, lanes 9–10, lanes 11–12, and lanes 13–14) embryos. The products were analyzed after 21 and 24 cycles for Trfc; 27 and 30 cycles for Gata1, Alas-e, Alad, Pbgd, β-globin, Eklf, Nfe2p45, and Hprt; and 30 and 33 cycles for Gata2.

anemic and one severely anemic GATA-1.05/Y::3KA-M embryo are shown in Fig. 5A. In the E9.5 yolk sacs of mildly anemic embryos (Fig. 5A, columns 7–9), wild-type levels of GATA-1 mRNA (columns 1–6) were present. On the other hand, the expression of GATA-1 mRNA was decreased relative to wild type, in the rescued embryos that were suffering from severe anemia (columns 10–12).

We also examined the expression of several erythroid genes, such as erythroid 5-aminolevulinate synthase (Alas-e), porphobilinogen deaminase (Pbgd), and Eklf. Whereas expression of these genes was severely reduced in the yolk sac of GATA-1.05/Y embryos (27), the expression was restored in the rescued embryos, in proportion to mutant GATA-1 levels (Fig. 5A). The expression of Fog1 was comparable in the yolk sac of anemic embryos with that of the wild type. In addition, other erythroid genes that have been implicated to be under the regulatory influence of GATA-1, including Nfe2p45, e-globin, and Gata2, were also influenced by the GATA-1–3KA expression level. Importantly, the expression of Trfc (transferrin receptor) was reduced in the rescued embryos regardless of the expression level of the GATA-1 mutant.

The expression of GATA-1 target genes was also analyzed in the liver of E12.5 GATA-1.05/Y::3KA-H1 embryos. In agreement with the analysis shown in Fig. 2A, approximately eight times more GATA-1 mRNA was expressed in GATA-1.05/Y::3KA-H1 embryos than in wild-type embryos, and consistent results were obtained, in all E12.5 embryos examined (Fig. 5B). Importantly, although the expression level of GATA-1–3KA mutant was much more abundant in rescued embryos than wild-type GATA-1 in wild-type embryos, expression of Alas-e, Eklf, and Trfc was severely impaired in the liver of GATA-1.05/Y::3KA-H1 embryos (Fig. 5B). Thus, self-association of GATA-1 is vital for the expression of these genes in both primitive and definitive hematopoiesis.

Self-association of GATA-1 Is Required for Maximum Expression of Transferrin Receptors on the Surface of Erythrocytes—The transferrin receptor (TRFC) is important for cellular iron uptake and indispensable for the development of erythrocytes (40). GATA-1 is expressed in erythroid and megakaryocytic precursors, and CD71 (TRFC) expression is coincident with high level expression of GATA-1 (41). Thus, the TRFC gene may be under the influence of GATA-1. To further assess the link between TRFC expression and GATA-1 self-association, we carried out flow cytometric analysis utilizing GH1HRD-GFP (green fluorescence protein) transgenic line of mice. Much stronger expression of GFP was observed in the liver of E13.5 GATA-1.05/Y::3KA-H1 embryos than in wild-type littermates. Because rescued embryos were easily distinguishable from the wild-type littermates due to obvious anemia (Fig. 6, A and B), GH1HRD may be activated during the stress erythropoiesis.

The flow cytometric analysis (Fig. 6E) showed that the intensity of GFP in the liver of GATA-1.05/Y::3KA-H1 embryos (red line histogram) was stronger than that of wild-type littermates (solid green histogram). Importantly, the number of GFP-positive cells in the GATA-1.05/Y::3KA-H1 embryos was comparable with those in wild-type embryos, indicating that erythroid progenitors expressing GATA-1
reside in GATA-1.05/Y::3KA-H1 embryos. Forward scatter (FSC) and side scatter (SSC) characteristics showed that GFP-expressing cells of wild-type fetal liver (Fig. 6C) were concentrated into low FSC and low SSC fraction, whereas those of GATA-1.05/Y::3KA-H1 fetal liver were shifted into a larger cell fraction (Fig. 6D).

We next examined expression of CD71 in the GFP-expressing cells. Intriguingly, the vast majority of GFP-expressing cells in the fetal liver were CD71-positive, but the peak intensity of CD71 was ~5-fold less in the rescued embryos than in the wild-type embryos (Fig. 6F). The GFP-positive cells in the GATA-1.05/X heterozygous females were split into the small and large fractions of FSC/SSC characteristics (data not shown) and into the CD71low and CD71high fractions (Fig. 6F, blue line histogram). Because of the random X chromosome inactivation, there are two types of erythroid progenitors in the GATA-1.05/X females. One is harboring the X chromosome with inactivated GATA-1.05 allele, whereas the other is harboring the X chromosome with inactivated wild-type Gata1 allele. Erythroid progenitors lacking GATA-1 stay in CD71low immature state and cannot differentiate into CD71high mature cells. This deficit cannot be restored by high level expression of GATA-1–3KA mutant.

DISCUSSION

Whereas self-association of GATA-1 influences its transactivation activity, it has been difficult to elucidate the physiological significance of the mechanism in vivo. This is mainly because of the difficulty in identifying regions of the protein that, when mutated, will eliminate only the self-association ability of GATA-1, while leaving other GATA-1 functions intact. A number of transcription factors and cofactors that influence the transactivation activity of GATA-1 interact with GATA-1 through the NF and/or CF domains (7, 18–20), and these regions also house the lysine residues that are substrates for acetylation of GATA-1 (35, 36). Thus, it is hard to dissect the contribution of the self-association mechanism from other functions of GATA-1 mediated through this region and required for hematopoiesis in vivo. We decided to utilize our established transgenic complementation rescue assay (26) to more precisely define the requirements for self-association. We generated three independent GATA-1 mutant molecules, in which three lysines potentially important for the self-association were substituted with alanines, and established several transgenic mouse lines for each mutant. Transgenic mouse lines that express these GATA-1 mutants at various levels were used to rescue the GATA-1.05 knockdown mutant of GATA-1. The results of this complementation rescue analysis unequivocally demonstrate that the ability to self-associate is critical for GATA-1 to regulate both primitive and definitive erythropoiesis.

Two KKR motifs located C-terminal to both NF (NF tail) and CF (CF tail) have been reported to be important for GATA-1 self-association (42). Disruption of either one of the KKR motifs caused a decrease of self-association following deterioration of the transactivation activity of GATA-1. It is interesting to note that these tail regions are also known as the substrate regions for acetylation. Acetylation increases the transactivation activity of GATA-1 mediated through this region and required for hematopoiesis in vivo. We decided to utilize our established transgenic complementation rescue assay (26) to more precisely define the requirements for self-association. We generated three independent GATA-1 mutant molecules, in which three lysines potentially important for the self-association were substituted with alanines, and established several transgenic mouse lines for each mutant. Transgenic mouse lines that express these GATA-1 mutants at various levels were used to rescue the GATA-1.05 knockdown mutant of GATA-1. The results of this complementation rescue analysis unequivocally demonstrate that the ability to self-associate is critical for GATA-1 to regulate both primitive and definitive erythropoiesis.

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GATA-1 and GATA-3 (7, 35, 43), whereas CF tail contains a physical interaction site with CBP (7). Quite recently, it was reported that acetylated GATA-1 is a target of ubiquitination, leading to its own degradation (44).

Our in vitro acetylation experiment with p300 showed that GATA-1–3KA mutant retains ~60% the acetylation capacity of wild type. In contrast, self-association capacity of the 3KA mutant decreased to approximately ~5% to 330% of wild-type level. In addition, the affect of the 3KA mutation on transcription of GATA-1 target genes is not consistent with GATA-1 acetylation. The acetylation of GATA-1 should cause a global increase in transcription of genes that are normally positively regulated by GATA-1, by activating the protein and stabilizing its interaction with chromatin (7, 39). We have shown that the 3KA mutant leaves some positively regulated GATA-1 target genes unchanged and has both positive and negative effects on others. The in vivo rescue experiment further revealed that the phenotypic differences between GATA-1.05/Y::NKA and GATA-1.05/Y::3KA are not derived from the difference in the acetylation but should be derived from the difference in the dimerization. Thus, we conclude that GATA-1 must be capable of self-association to sustain erythropoiesis.

One important observation in this study is that the 3KA mutant could not restore the GATA-1 function in a transgenic complementation rescue assay, demonstrating that Lys-216, Lys-217, and Lys-312 residues are indispensable for GATA-1 function in vivo. Transgenic expression of NKA mutant (i.e. substitution of KKR in NF tail with AAR; see Fig. 1A) at the endogenous GATA-1 level almost fully rescued hematopoiesis in GATA-1.05/Y mice during embryonic and postnatal stages. Similarly, CKA mutant (i.e. K312A mutant) could fully rescue GATA-1.05/Y mutant from embryonic lethality. However, markedly fewer pups than expected were rescued by the low expresser NKA mutants in both L1 and L2 lines. Because we repeatedly observed that low level expression of wild-type GATA-1 efficiently rescues GATA-1.05/Y mutant mice (27), there may be some contribution of the KKR motif in the NF tail to GATA-1 function. We speculate that the CF tail region may substitute for loss of the NF tail KKR function in vivo and vice versa.

Another important observation in this study is that the rescue profile by the 3KA mutant is tightly linked to the expression level of the transgene. Indeed, the 3KA-M mutant could rescue primitive erythropoiesis to some extent but not definitive erythropoiesis of GATA-1.05/Y embryos. The 3KA-H mutant has a capacity to overcome the embryonic lethality during primitive hematopoiesis. However, 3KA-H-rescued mice are still prone to die during the definitive hematopoiesis stage. The few (15% of expected) GATA-1.05/Y adult mice obtained by rescue with the 3KA-H mutant were anemic, further highlighting the importance of GATA-1 self-association.

What are the biological roles of GATA-1 self-association? One plausible explanation is that some GATA-binding sites may require a specific structure controlled by intra- and/or intermolecular association of GATA-1. In fact, DNA binding via NF is important for the binding activity of GATA-1 to double GATA sites and the GATA-pal site (14, 16, 45). We surmise that various stereographic structures of GATA-1 may be formed in the transcription factor complexes, in which single or dimeric GATA-1 molecules would provide a mechanism for regulating the orchestrated expression of numerous GATA-1 target genes. Alternatively, an increase in the local concentration of GATA-1 by self-association may recruit transcriptional cofactors, such as CBP/p300 and SWI/SNF, more efficiently (46, 47). High density of GATA-1 in the promoter regions may contribute to establish and/or maintain an open chromatin configuration. Wild-type GATA-1 expressed under the GIHRD could fully rescue the GATA-1.05 mutant from embryonic lethality even if the expression level was below the endogenous level (27). We speculate that, because wild-type GATA-1 possesses intact self-association ability, it can increase the local concentration of GATA-1 enough to compensate for the reduced expression.

We previously reported that a six-lysine-to-alanine substitution mutation of zebrafish GATA-1 (6KA mutant), which lost the ability to self-associate without influencing the acetylation potential, regulated the transcription of the Gata1 gene through a double GATA motif in the zebrafish Gata1 gene (30). In contrast, such reduction of transgenic GATA-1 expression was not observed in this transgenic rescue study driven by GIHRD, even in the embryos rescued by the high accumulation of 3KA mutant. One plausible explanation for this difference is that some of the self-association activity still remains in the mouse 3KA mutant, whereas the ability is considerably more weakened in the zebrafish 6KA mutant. Therefore, transactivation activity of 3KA mutant for its own promoter may be compensated by the abundant accumulation of 3KA mutant protein. Although we could not establish a link between the autoregulatory mechanism for Gata1 expression and GATA-1 self-association in our system, the decrease of specific target gene transcription by the 3KA mutant suggests that GATA-1 self-association may play an important role in its own gene expression.

In summary, we provide the first line of evidence for the physiological significance of GATA-1 self-association during erythroid maturation. In addition, whereas many previous studies point to a critical requirement for GATA-1 to regulate many erythroid genes, this study has revealed the importance of GATA-1 as a regulator of the transferrin receptor.

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