Zinc fingers as protein recognition motifs: Structural basis for the GATA-1/Friend of GATA interaction

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Edited by Stuart H. Orkin, Harvard Medical School, Boston, MA, and approved December 8, 2004 (received for review October 11, 2004)

GATA-1 and friend of GATA (FOG) are zinc-finger transcription factors that physically interact to play essential roles in erythroid and megakaryocytic development. Several naturally occurring mutations in the GATA-1 gene that alter the FOG-binding domain have been reported. The mutations are associated with familial anemias and thrombocytopenias of differing severity. To elucidate the molecular basis for the GATA-1/FOG interaction, we have determined the three-dimensional structure of a complex comprising the interaction domains of these proteins. The structure reveals how zinc fingers can act as protein recognition motifs. Details of the architecture of the contact domains and their physical properties provide a molecular explanation for how the GATA-1 mutations contribute to distinct but related genetic diseases.

transcription | factor | gene expression

The erythroid transcription factor GATA-1 has become a paradigm for understanding the control of gene expression by sequence-specific DNA-binding proteins. Numerous studies (reviewed in refs. 1 and 2) have revealed that the function of GATA-1 depends on its ability to bind both DNA and other proteins such as the DNA-binding proteins PU.1, Fli-1 Sp1, and erythroid Krüppel-like factor, as well as coregulators such as LIM-only 2 protein, cyclic AMP response element-binding protein-binding protein, and the zinc-finger protein friend of GATA (FOG). It is thought that different partnerships involving GATA-1 occur at different DNA elements, in different cell types, and at distinct stages of development, and that these regulatory networks contribute to the ability of GATA-1 to direct the complex patterns of gene expression required for the differentiation of blood cells.

GATA-family proteins contain two highly conserved treble-clef zinc fingers (ZnFs) domains (3): the N-terminal (NF) and the C-terminal (CF) fingers. These domains mediate both DNA and protein interactions, but despite high sequence similarity to each other (>50% identity), have quite distinct functions. The CF appears to be the main determinant of DNA-binding and recognizes sequences of the form T/AGATAA/G, whereas the NF can participate in binding to tandem T/AGATAA/G sites (ref. 4 and references therein) and can independently bind GATC motifs (5). The protein-binding specificities of the two ZnFs also differ; most notably, the NF alone is able to recruit FOG, an $\approx 1,000$ -aa protein (6) that contains five classic Cys–His–His (C_2H_2) and four related Cys–Cys–His–Cys (C_2HC) ZnFs (Fig. 14).

FOG is essential for the normal development of both erythrocytes and megakaryocytes (7) and the interaction of FOG with GATA-1 is indispensable for these events (8). Of the nine ZnFs in FOG (Fig. 14), four are not found in the tandem arrays that are generally associated with DNA-binding activity. Intriguingly, each of these four isolated domains can independently bind NF, consistent with the emerging view that single fingers can mediate protein–protein interactions (9, 10).

Here, we describe the solution structure of the protein–protein complex formed by the interaction domains of GATA-1 and FOG. The structure reveals for the first time how ZnF domains

can act as protein recognition motifs. We also show that NF can bind simultaneously both to FOG and to DNA, demonstrating that even domains of only ≈ 40 aa can harbor two completely distinct recognition surfaces. Finally, we define the molecular causes for several human blood disorders that arise from mutations in GATA-1.

Materials and Methods

Sample Preparation. The GATA-1 NF and dFOG finger 1 were overexpressed and purified as described (11). NF mutants were purified either in the same fashion or by size exclusion chromatography. Complexes were prepared by mixing stoichiometric quantities of each domain dissolved in 20 mM sodium acetate (pH 5.5). In each complex, one of the two components was uniformly ^{15}N , ^{13}C -labeled, whereas the other was unlabeled. Oligonucleotides (GCAACTGATCTGGACT and its complement) used for isothermal titration calorimetry were purchased from Sigma in the trityl-on form, simultaneously deprotected and purified by reverse-phase HPLC, annealed, and then purified on a Superdex 75 column.

NMR Spectroscopy. NMR data were acquired on a Bruker 600-MHz spectrometer. All experiments were carried out at 280 K at concentrations of 400–600 μM . Spectra were analyzed by using XEASY (12). Backbone and side chain resonance assignments were obtained by using standard triple resonance experiments (13), namely HNCA, HN(CO)CA, HNCACB, CBCA(CO)NH, HCCH-TOCSY, HCCH-COSY, and HNCO. Chemical shift indices were calculated as described (14). Torsion angle restraints were obtained from HNHA experiments (15) and TALOS (16). Intermolecular distance constraints were obtained by using 2D NOESY and 2D ^{12}C -filtered/ ^{13}C -edited and ^{15}N -filtered/ ^{13}C -edited NOESY experiments (mixing time, 100 ms) (17); all constraints were set to upper distance limits of 6 Å.

Structure Determination and Analysis. Preliminary structure calculations were performed in DYANA (18) and later in ARIA (19, 20). Zinc coordination was incorporated in the ARIA calculations (21). HADDOCK (22) was used to dock mNF and dFOG-F1, using the previously determined structures of both domains (11, 23) and 35 intermolecular NOEs derived from analysis of NOE data. Ambiguous restraints (15) based on chemical shift perturbation studies were also added, but did not significantly improve the quality of the structures. A total of 500 rigid body docking trials were carried out by using the standard HADDOCK protocol, with the 100 lowest energy solutions used for subsequent semiflexible

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: FOG, friend of GATA; ZnF, zinc finger; NF, N-terminal ZnF; CF, C-terminal ZnF.

Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 1Y0J).

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Therefore, we determined the solution structure of the complex formed between mNF and dFOG-F1 by using restraints derived from multidimensional NMR spectroscopy. Because the relatively weak association constant ($\approx 1 \times 10^5 \text{ M}^{-1}$) gave rise to exchange kinetics that broadened many resonances in the spec-

both carry valine in this position, and the additional van der Waals interactions may give rise to a stronger interaction for these fingers. Similarly, the contact residue S218_{dFOG} is substituted with arginine in several GATA-binding FOG ZnFs. As shown in Fig. 3B, an arginine side chain would be well positioned to make electrostatic interactions with both E200_{NF} and E203_{NF}. Finally, the two residues that are absolutely conserved in all GATA-binding FOG ZnFs, namely I215_{dFOG} and Y230_{dFOG}, appear on the periphery of the interface (Fig. 2 Left). Whereas Y230_{dFOG} makes direct contact with a number of residues in NF, I215_{dFOG} does not appear to directly contact NF. However, mutation of this residue to alanine abrogates the GATA/FOG interaction. It is possible that I215_{dFOG} assists in establishing the nature of the GATA-binding surface of FOG by excluding solvent and modifying the electrostatics of residues at the interface. This residue also packs against Y230_{dFOG}, and might aid in positioning the Y230_{dFOG} side chain.

Binding Properties of the GATA-1 NF. Although the interaction between GATA-1 and FOG-1 is essential for normal erythropoiesis, the molecular mechanisms underlying this requirement are not well understood. Furthermore, FOG-1 can act as either a corepressor (31) or a coactivator (6) in different assays, and we do not currently understand the basis for this observation. The corepressor activity of FOG could arise from FOG blocking the binding of GATA-1 to DNA, or FOG could serve as a scaffold for the recruitment of additional gene silencing proteins. In contrast, the finding that FOG can work as a coactivator is most consistent with a mechanism where it associates with GATA-1 bound at a gene control element. However, the coassociation of GATA-1 and FOG on a single DNA element has not to our knowledge been demonstrated directly. We used magnetic beads coupled to DNA containing a single GATA element to determine whether murine GATA-1 can contact both DNA and FOG simultaneously (Fig. 4A). As shown in lane 5, GATA-1 is retained by the wild-type promoter sequence, but binding to this site is abrogated by mutation of the A/TGATAA/G motif (lane 6). When FOG is added to the reaction mixture (lane 3), it is also retained on the promoter, but only in the presence of GATA-1 (lane 4). Thus, FOG can be recruited to DNA by GATA-1.

We further sought to determine whether the NF can simultaneously contact DNA and FOG. Therefore, we repeated the pull-down by using a GATC DNA element from the ALAS2 promoter, which has been shown to bind the GATA-1 NF (5), and purified recombinant domains from GATA-1 and FOG. In the absence of CF, NF was indeed able to simultaneously bind DNA and FOG finger 6 (Fig. 4B). Notably, the NF/DNA complex selectively bound the full-length FOG construct in preference to truncations that were more abundant in the input, which is in accord with the structure of GATA/FOG complex (Fig. 4C, lilac). These data support the view that FOG associates with DNA-bound GATA-1 rather than affecting its DNA-binding activity. The repression observed on some promoters is thus most likely to be due to the recruitment of additional cofactors such as chromatin modifying complexes.

Analysis of GATA-1-Related Human Blood Disorders. At least five distinct *GATA-1* mutations that are associated with familial anemias and/or thrombocytopenias have been reported. These mutations all lie in the NF domain and result in single amino acid substitutions: V205M (33), G208S (34), R216Q (35), D218G (36), and D218Y (37). These substitutions give rise to distinct but related genetic disorders. For instance, the V205M and D218Y mutations both give rise to severe anemia and thrombocytopenia, whereas the G208S and D218G mutations give rise to thrombocytopenia but have less significant effects on erythropoiesis. The simplest explanation for these observations is that

Fig. 4. Simultaneous binding of GATA-1 to DNA and FOG-1. (A) Pull-down showing that full-length GATA-1 can simultaneously bind to the murine α -globin promoter and recruit full-length FOG-1. MEL cell nuclear extract (lane 1), supplemented with COS nuclear extract from untransfected [COS-(mock); lanes 5 and 6] or transfected [COS(FOG); lanes 3 and 4] cells, was incubated with DNA containing either a wild-type (WT) or a mutant (mut) GATA site coupled to magnetic beads. Complexes were blotted with antibodies against GATA-1 and FOG. (B) Pull-down showing that murine GATA-1 NF can simultaneously bind a GATC site and FOG-1 finger 6. Magnetic beads coupled to a GATC-containing oligonucleotide were incubated with purified MBP-NF and GST-FOG-F6 and run on denaturing gels. Gels were blotted with anti-MBP and anti-GST. A number of breakdown products of GST-FOG-F6 are visible in the input. (C) DNA- and protein-binding surfaces of the GATA/FOG complex. The DNA-binding surface of GATA-1 NF is inferred from the NMR structure of the GATA-1 CF bound to DNA (38). The complex is shown in the same orientation as in Fig. 1D.

the mutations alter the structure and functions of the NF in different ways.

To assess the consequences of each of these mutations, we expressed and purified the mutant NFs and assessed their biophysical properties. Far-UV CD spectra (Fig. 5A) reveal that all mutants contain substantial levels of secondary structure, but display a blue-shifted minimum (≈ 5 nm). One-dimensional ^1H NMR spectra of these mutants (Fig. 5B) show that only R216Q has a well defined tertiary fold, as judged by the presence of well separated resonances in the range of 9 to 9.5 ppm. The combination of CD and NMR data indicates that the other mutants probably exist in solution as molten-globule-like states that contain substantial amounts of secondary structure but lack a well defined tertiary structure. These ensembles most likely undergo conformational fluctuations on a millisecond to microsecond time scale. Thus, the mutations V205M, G208S, D218G, and D218Y all destabilize the structure of NF, which may contribute to the mechanism by which they impair GATA-1 activity *in vivo*.

We also determined whether the mutants retained binding functionality. Isothermal titration calorimetry was used to quantify the ability of each mutant to bind to either DNA or dFOG-F1 (Fig. 5C and Table 1). No measurements were made with D218Y, because of difficulties in obtaining pure, stable protein. V205M, G208S, and D218G were all able to bind to a 16-bp oligonucleotide containing a GATC site with similar affinities to wild-type NF. R216Q showed no measurable binding to DNA. We then tested the ability of the domains to interact with dFOG-F1. R216Q and D218G bound to dFOG-F1 with affinities that were indistinguishable from that of wild-type GATA-1 NF, whereas V205M and G208S did not bind dFOG-F1.

Fig. 5. GATA-1 NF mutations associated with familial blood disorders. (A) Far-UV CD spectra of NF mutants, showing that each forms substantial secondary structure, compared to the wild-type protein in the absence of Zn(II)

Table 1. DNA- and FOG-binding affinities of naturally occurring GATA-1 mutants

Protein	k_a	
	NF:DNA, M ⁻¹	NF:FOG, M ⁻¹
mNF	$(1.9 \pm 0.1) \times 10^6$	$(4.5 \pm 1.8) \times 10^4$
V205M	$(2.0 \pm 1.6) \times 10^7$	No binding
G208S	$(9.1 \pm 5.2) \times 10^6$	No binding
R216Q	No binding	$(9.4 \pm 0.3) \times 10^4$
D218G*	$>10^7$	$(1.9 \pm 0.3) \times 10^4$

Association constants were measured by isothermal titration calorimetry. Numbers are presented with standard deviations obtained from fitting the data to a simple 1:1 model.

*The sharpness of the binding curve obtained in the D218G/DNA titration prevented accurate determination of the binding affinity, and the affinity is therefore presented as a lower limit.

Discussion

ZnFs as Protein-Binding Domains. ZnF proteins comprise 3% of human genes. Although ZnFs are best recognized as sequence-specific DNA-binding motifs, it has become clear that they also can function as RNA- and protein-recognition motifs. This study provides a demonstration of the molecular mechanisms through which pairs of proteins can interact via their ZnF domains.

Notably, all of the FOG ZnFs that contact GATA-1 are not part of tandem arrays of ZnFs. Thousands of such “isolated” ZnFs exist, and it is likely that many serve as protein recognition motifs. The surface used by FOG ZnFs to recognize GATA-1 overlaps with the surface normally used by classical ZnFs to bind to DNA, indicating that the classical ZnF has acted throughout evolution as a versatile structural scaffold, onto which different binding functions have been “grafted.” In line with this idea, we recently demonstrated that the third classical ZnF from FOG mediates a specific interaction with the coiled-coil protein TACC3 (9). Indeed, given that a single classical ZnF is capable of mediating protein–protein interactions, and that an array of such domains is necessary for high affinity DNA binding, it is likely that the latter function arose later as a consequence of gene duplication events.

GATA-1-Related Blood Disorders. The structure of the GATA/FOG complex, together with our biophysical data, provides insights into the molecular basis for hematological disorders that arise from inherited mutations in GATA-1. Fig. 5*D* shows the location of each of the mutations on NF. It is immediately apparent that the FOG- and DNA-binding surfaces of NF are nonoverlapping, consistent with our finding that NF can participate in both interactions simultaneously.

R216 clearly lies on the DNA-binding surface of NF; the corresponding residue forms an ionic interaction with the phosphate backbone in the structure of the CF/DNA complex (38). Mutation to glutamine will remove this interaction. Our isothermal titration calorimetry data reveal that the R216Q mutant does not bind DNA. Despite this, CD and NMR data indicate that this mutant is well folded, and is also still able to bind

(black). (B) One-dimensional ^1H NMR spectra of the proteins in A. V205M, G208S, D218G, and D218Y do not form compact tertiary structures in the same way as the wild-type or R216Q. (C) Isothermal titration calorimetry data showing the titration of dFOG-F1 into V205M (*right*) and the titration of V205M into a 16-bp oligonucleotide (*left*). (D) Locations of the NF mutations (yellow) in the GATA/FOG complex. The complex is shown in the same orientation as in Fig. 1. The DNA-binding surface of NF is shown in purple, whereas the FOG-binding face is shown in gray. R216 lies on the NF/DNA interface, V205 and G208 lie on the FOG contact surface, and D218 lies between these two opposing surfaces.

dFOG-F1 with wild-type affinity. This latter observation is not surprising, given the relative locations of the FOG- and DNA-binding sites. Thus, the only mechanism that we have detected through which R216Q causes disease is the abrogation of DNA-binding by the NF. The NF is thought to play a relatively minor role in DNA-binding *in vivo*, and the relatively mild phenotype of individuals carrying the R216Q mutation is consistent with this view.

The mutation V205M lies at the center of the FOG-binding surface of NF. As shown in Fig. 2, the V205 side chain fits snugly into a cavity on the surface of the FOG finger and is completely buried in the complex. Methionine cannot occupy the same cavity, and the fact that we observed no FOG binding with this mutant confirms the importance of V205 in the GATA/FOG interaction. This mutation causes severe anemia and thrombocytopaenia, suggesting that abrogation of the GATA-1/FOG interaction profoundly impairs both erythropoiesis and thrombopoiesis. This conclusion is in accord with the phenotype observed for FOG-knockout mice (7).

Interestingly, this mutant retains full DNA-binding ability, despite the fact that its ¹H NMR spectrum indicates reduced levels of tertiary structure. This observation is in agreement with the findings of Crispino *et al.* (8), which showed that the mutation V205G (which removes the hydrophobic valine side chain) disrupted the interaction of NF with FOG but not with DNA. We have recently made a similar observation for the binding of the zinc-finger transcriptional regulator basic Krüppel-like factor (39) to DNA: partially folded BKLK mutants retained full DNA-binding capacity. These data demonstrate that mild destabilization of a protein fold, even to the extent of disrupting the tertiary structure, does not necessarily compromise function. Changes in the enthalpic and entropic contributions to binding often dampen effects on the free energy change, a phenomenon referred to as enthalpy–entropy compensation. This situation presumably minimizes the impact of random mutations on an organism.

The G208S mutant, like V205M, is partially folded, unable to bind to dFOG-F1 *in vitro*, but retains DNA-binding ability. In the NF structure, G208 takes up a positive ϕ angle ($\approx 100^\circ$), so it is not surprising that mutation of this residue disrupts the structure of NF to some degree. The disruption occurs on the periphery of the FOG-binding surface. Interestingly, the phenotype for patients carrying this mutation is less severe than that of V205M (thrombocytopenia only), and previous gene reporter assays have indicated that the effect of the G208S mutation is less pronounced. In fact, we also showed recently that G208S appears to weakly interact with FOG-F6 (34).

D218G is perhaps the most intriguing mutation. Although the DNA-binding affinity could not be accurately defined because of the properties of the binding isotherm, it was clear that the mutation does not significantly compromise binding to either DNA or dFOG-F1. D218 is located in the second β -hairpin of NF and lies on a surface midway between the DNA- and FOG-binding faces of NF (Fig. 5D). This residue does not contact FOG and is not expected to contact DNA, so how then does mutation of D218 give rise to thrombocytopaenia and mild dyserythropoiesis? It has been noted that binding of D218G to some FOG fingers (e.g., mammalian FOG-finger 1) is reduced, and this may cause problems. Alternatively, this residue may be important for the recognition of other partners of GATA-1, such as CBP, Fli-1 or PU.1.

Our data show how ZnFs can act as protein recognition modules, and demonstrate how even domains as small as 30–40 aa can present two (or perhaps more) completely separable functional surfaces. These findings also shed light on the molecular origins of GATA-1-related blood disorders.

This work was supported by Australian Research Council and National Health and Medical Research Council (NHMRC) grants (to J.P.M. and M.C.). J.P.M. is a NHMRC Senior Research Fellow. J.M.M. is a Charles and Sylvia Viertel Medical Research Fellow. C.K.L., R.J.Y.S., A.H.Y.K., and F.E.L. are recipients of Australian Postgraduate Awards. L.A.C. is a NHMRC C. J. Martin Fellow.

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