Ferric, not ferrous, heme activates RNA-binding protein DGCR8 for primary microRNA processing

Ian Barr^a, Aaron T. Smith^b, Yanqiu Chen^a, Rachel Senturia^a, Judith N. Burstyn^b, and Feng Guo^{a,1}

^aDepartment of Biological Chemistry, David Geffen School of Medicine, Molecular Biology Institute, University of California, Los Angeles, CA 90095; and

^bDepartment of Chemistry, University of Wisconsin, Madison, WI 53706

Edited by Harry B. Gray, California Institute of Technology, Pasadena, CA, and approved December 15, 2011 (received for review September 6, 2011)

The RNA-binding protein DiGeorge Critical Region 8 (DGCR8) and its partner nuclease Drosha are essential for processing of microRNA (miRNA) primary transcripts (pri-miRNAs) in animals. Previous work showed that DGCR8 forms a highly stable and active complex with ferric [Fe(III)] heme using two endogenous cysteines as axial ligands. Here we report that reduction of the heme iron to the ferrous [Fe(II)] state in DGCR8 abolishes the pri-miRNA processing activity. The reduction causes a dramatic increase in the rate of heme dissociation from DGCR8, rendering the complex labile. Electronic absorption, magnetic circular dichroism, and resonance Raman spectroscopies indicate that reduction of the heme iron is accompanied by loss of the cysteines as axial ligands. ApoDGCR8 dimers, generated through reduction and removal of the heme, show low levels of activity in pri-miRNA processing *in vitro*. Importantly, ferric, but not ferrous, heme restores the activity of apoDGCR8 to the level of the native ferric complex. This study demonstrates binding specificity of DGCR8 for ferric heme, provides direct biochemical evidence for ferric heme serving as an activator for miRNA maturation, and suggests that an intracellular environment increasing the availability of ferric heme may enhance the efficiency of pri-miRNA processing.

DiGeorge syndrome | Microprocessor | Pasha | redox | ligand switching

MicroRNAs (miRNAs) are a class of non-protein-coding RNAs about 22 nt in length (1, 2). They are involved in nearly every aspect of development and cell physiology and contribute to diseases such as cancer and DiGeorge syndrome (3–6). miRNAs are produced from long primary transcripts (pri-miRNA) that may be introns in messenger RNAs, or independent noncoding transcripts. The miRNA maturation pathway includes sequential cleavages in the nucleus and cytoplasm (7–9). DGCR8 is a RNA-binding protein that is essential for maturation of all canonical miRNAs (10–15). DGCR8 and its partner, the RNase III enzyme Drosha (16), specifically recognize and cleave pri-miRNAs in the nucleus to produce an intermediate called precursor miRNAs (pre-miRNAs). DGCR8 and Drosha copurify with each other from cell extracts and are collectively called the Microprocessor complex (10–12). Unlike Dicer, another RNase III enzyme that cleaves pre-miRNAs in the cytoplasm to generate miRNA duplexes, Drosha does not cleave pri-miRNA substrates in the absence of its RNA-binding partner DGCR8. DGCR8 makes a major contribution to the recognition of pri-miRNAs through highly cooperative binding and formation of higher-order structures (17–20).

Based on a yellow color that was associated with a recombinant human DGCR8 construct called NC1 (Fig. 1A), we found that DGCR8 binds heme (18). Each dimeric NC1 binds one heme molecule and heme-bound NC1 dimers are much more active in pri-miRNA processing than the heme-free monomers. This observation led us to suggest that heme is involved in regulation of miRNA maturation. To understand the potential function of heme in miRNA biogenesis, we have characterized the DGCR8–heme interaction using biochemical and structural methods. DGCR8 binds heme using a heme-binding domain (HBD) located in the central region of the 773-residue polypeptide chain,

N-terminal to two double-stranded RNA-binding domains (dsRBDs) (Fig. 1A). The HBD of DGCR8 contains an N-terminal dimerization subdomain that is primarily composed of a WW motif. Our crystal structure of the dimerization domain demonstrates that the WW motif and its C-terminal neighboring region form an extensive dimerization interface even in the absence of heme, and this domain seems to directly contribute a surface for heme binding (21). Recently, we found that the heme bound to native NC1 is ferric, that two cysteine (Cys) side chains bind to the heme iron in a ligation configuration that has not been observed in any other heme protein, and that the NC1–ferric heme complex is highly stable (22).

Most canonical heme proteins stably bind both the ferrous and ferric forms of heme. Here we characterize the DGCR8–ferrous heme complexes, and we find that reduction of the ferric heme iron in DGCR8 greatly increases the rate of heme dissociation. Spectroscopic data show that the dual cysteine ligands of the Fe(III) heme–DGCR8 complex are lost upon reduction of the heme iron. Taking advantage of the fast dissociation of Fe(II) heme from DGCR8, we generate the apoNC1 via reduction of the heme iron and find that ferric, but not ferrous, heme activates miRNA processing activity of DGCR8 *in vitro*. The biological implications of these findings are discussed.

Results

Fe(II) DGCR8 is Inactive in Pri-miRNA Processing. To test the importance of the heme iron redox state on the activity of DGCR8, we reduced the native Fe(III) heme-bound NC1 dimer and examined its activity in reconstituted pri-miRNA processing assays (18). Incubation of Fe(III) NC1 with excess dithionite resulted in slow reduction of the heme iron to Fe(II), which approached completion at room temperature in 30–60 min (Fig. 1B and C). At pH 8.0, the Fe(II) NC1 complex displayed a Soret peak at 425 nm and β -, α -bands at 530 and 557 nm, respectively; the Soret peak at 450 nm and the broad α/β -bands at 556 nm of the Fe(III) NC1 disappeared (Fig. 1B). At pH 8.0, the Fe(II) heme–NC1 complex tended to precipitate at concentrations higher than 2 μ M. We performed the reduction at pH 6.0 and found that Fe(II) NC1 remained soluble and had an absorption maximum at 390 nm, which was partially obscured by the dithionite absorption (Fig. 1C). The lack of distinct features in the electronic absorption spectrum of Fe(II) NC1 at pH 6.0 raised the question as to whether the Fe(II) heme is free in solution. We disfavor this possibility because the spectrum of Fe(II) NC1 is distinct from those displayed by Fe(II) heme alone or in the presence of DGCR8^{276–353}, a dimerization domain-only variant that cannot

Author contributions: I.B., A.T.S., J.N.B., and F.G. designed research; I.B., A.T.S., Y.C., and R.S. performed research; I.B., A.T.S., J.N.B., and F.G. analyzed data; and I.B., A.T.S., J.N.B., and F.G. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

¹To whom correspondence should be addressed. E-mail: fguo@mbi.ucla.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1114514109/-DCSupplemental.

Fig. 1. Reduction of the Fe(III) heme in human NC1 diminishes pri-miRNA processing. (A) Domain structure of human DGCR8. The dsRBDs and C-terminal tail (CTT) are required for cooperative association with pri-miRNAs and for triggering cleavage by Drosha. The human NC1, NC9, and frog DGCR8 HBD-His₆ proteins used in this study are represented by brackets. (B) Electronic absorption spectra of NC1 (7.1 μM) recorded before (dashed line) and after (solid line) incubation with solid dithionite at 25°C under Ar(g) for 60 min. (C) Electronic absorption spectra of NC1 (10 μM) reduced at pH 6.0 (50 mM MES) using 2 mM dithionite at room temperature under anaerobic conditions for 70 min. (D) Reconstituted pri-miR-30a processing assays were performed at 37°C for 45 min using recombinant His₆-Drosha^{390–1374} and various forms of DGCR8 as indicated. The Fe(III) and Fe(II) NC1 dimers were present at 25 nM and the NC9 monomer concentration was 100 nM. The relationship between the low molecular weight marker (LMWM) and the Decade RNA ladder was inferred from a comparison on a similar gel (Fig. S3).

bind heme (Fig. S1). Furthermore, the Fe(II) NC1 species at pH 6.0 and 8.0 are interconvertible (Fig. S2).

The Fe(II) NC1 complex was tested in reconstituted pri-miRNA processing assays with recombinant human Drosha^{390–1374}. The pri-miRNA processing reactions were performed anaerobically to avoid reoxidation of Fe(II) heme. Under these conditions, Fe(III) NC1 is highly active, whereas Fe(II) NC1 is inactive (Fig. 1D, lanes 3 and 4). Dithionite (1 mM) was present in the pri-miRNA processing reaction with Fe(II) NC1. To rule out the possibility that dithionite inactivated pri-miRNA processing, we added dithionite to pri-miRNA processing assays reconstituted using NC9, a DGCR8 construct that does not contain heme

due to the lack of the HBD but is active in pri-miR-30a (pri-miRNA of miR-30a) processing (Fig. 1A). The results showed clearly that dithionite does not interfere with the pri-miRNA processing activity of Drosha and NC9 (Fig. 1D, lanes 5 and 6). Thus, these results indicate that, unlike the Fe(III) heme-DGCR8 complex, the Fe(II) DGCR8 complex is not active in pri-miRNA processing.

Reduction of the DGCR8–Heme Complex Greatly Increases the Rate of Heme Dissociation. To understand the mechanism of the heme-reduction-mediated inactivation, we analyzed the Fe(II) NC1 using size exclusion chromatography (SEC). The Fe(II) NC1 protein eluted at the same volume as that of the Fe(III) NC1 (Fig. 2A), indicating that the dimeric state was unchanged upon reduction of the heme iron. However, little absorption at 390 nm was observed in the elution peak of Fe(II) NC1, suggesting that most Fe(II) heme had dissociated from DGCR8. The possibility of reoxidized heme remaining bound to NC1 was ruled out by the lack of absorption at 450 nm in the chromatogram.

To further corroborate the instability of the Fe(II) heme-DGCR8 complex, we studied the reduction of frog DGCR8 HBD-His₆ (frog HBD) (Fig. 2B). We previously showed that the frog HBD binds Fe(III) heme in the absence of the dsRBDs and displays spectroscopic properties very similar to those of the human NC1 (22). Upon 1-h incubation with 2 mM dithionite at pH 8.0 and 37°C, the frog HBD remained soluble, and the 366- and 450-nm split Soret peaks were replaced by a single sharp peak at 424 nm (Fig. 2B). The broad absorption envelope at approximately 557 nm, characteristic of the α/β region of an Fe(III) heme, was replaced by two new, distinct peaks at 529 and 558 nm, characteristic of β- and α-bands of an Fe(II) heme (Fig. 2B). The similar absorption peaks of Fe(II) frog HBD and Fe(II) NC1 at pH 8.0 (Figs. 1B and 2B) suggest that the environments of the heme are alike in these proteins. At pH 6.0, Fe(II) frog HBD precipitated.

We previously showed that Fe(III) NC1 was highly stable, with no heme transfer to apomyoglobin over 4 d (22). Similarly, the Fe(III) frog HBD complex did not transfer heme when incubated with apomyoglobin for 5 d (Fig. 2C). Apomyoglobin has an extremely high affinity for heme ($K_d = 3 \times 10^{-15}$ M), making it an effective heme scavenger (23). In sharp contrast, when the Fe(II) frog HBD was incubated with a molar excess of apomyoglobin at room temperature, nearly complete transfer of Fe(II) heme from the frog HBD to myoglobin was observed within the experimental time of 1–2 min, as indicated by the shift of Soret peak from 424 to 431 nm (Fig. 2B). The transfer of heme from frog HBD to myoglobin was so rapid that we could not measure the rate using a stopped-flow apparatus. Therefore, we conclude that reduction

Fig. 2. Reduction of the heme iron in DGCR8 greatly decreases the stability of the heme-protein complex. (A) Size exclusion chromatograms of Fe(III) and Fe(II) NC1 at pH 6.0 (50 mM MES). Fe(II) NC1 was prepared as described in Fig. 1C. (B) Electronic absorption spectra of frog HBD (8 μM) at pH 8.0 [50 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPS)]. The Fe(III) form of the protein (dotted line) was reduced to the Fe(II) form (solid line) through addition of solid dithionite for 60 min. Subsequently, 66 μL of 80 μM apomyoglobin was added to 200 μL of the Fe(II) HBD solution (dashed line, normalized to compensate for dilution). (C) Fe(III) frog HBD (7 μM) was incubated with a sixfold excess of apomyoglobin at room temperature. The absorbance at 450 nm [Fe(III) DGCR8] and 409 nm (metmyoglobin) are plotted.

of the heme iron causes a dramatic increase of the rate of heme dissociation from DGCR8.

Reduction of the DGCR8-Heme Complex is Accompanied by Ligand Switching. Reduction of the DGCR8 heme results in the loss of the two cysteine ligands that were bound to the Fe(III) heme. When dithionite is added to Fe(III) frog HBD at pH 8.0 and 37°C, the 451-nm peak slowly loses intensity while a sharper Soret peak at 424 nm grows in. The Soret band shift from 450 to 424 nm suggests that no cysteine(thiolate) ligand is bound to the Fe(II) DGCR8 heme, because six-coordinate low-spin thiolate-ligated Fe(II) hemes exhibit red-shifted Soret peaks (440–460 nm) (24), whereas five-coordinate high-spin thiolate-ligated Fe(II) hemes exhibit Soret peaks in the 406- to 412-nm region (25). The Fe(II) frog HBD 424-nm band has a relatively low molar absorptivity ($\epsilon_{424} \sim 55 \text{ mM}^{-1} \text{ cm}^{-1}$) and the α/β region of the spectrum, although consistent with that of a low-spin Fe(II) heme, is broad. These features suggest that multiple spin and/or coordination states may be present (26). Further evidence for the formation of intermediates and/or multiple products is provided by the fact that the reduction process is not isosbestic. Fe(II) NC1 exhibits a different Soret maximum at pH 6.0 from those of Fe(II) NC1 and Fe(II) HBD at pH 8.0, indicating that the heme spin and coordination state changes with pH.

Magnetic circular dichroism (MCD) spectroscopy further supports the conclusion that the Fe(II) frog HBD has lost the bis-Cys ligation present in the Fe(III) complex and exists as a mixture of spin and coordination states at pH 8.0. The α/β region of the Fe(II) frog HBD MCD spectrum is dominated by a derivative-shaped, temperature-independent A term with a cross-over position of 558 nm (α -band), consistent with the presence of a six-coordinate, low-spin Fe(II) heme (Fig. 3A). However, the Soret region of the Fe(II) frog HBD MCD spectrum is dominated by an inverted C term with trough-cross-over-peak positions of 422–431–442 nm, consistent with the presence of a five-coordinate, high-spin Fe(II) heme. The magnetic saturation behavior of the most intense peak of the C term, 442 nm (Fig. 3A, *Inset*), taken at 2.5, 4.0, 8.0, 15, and 25 K has a characteristic shape and nonoverlapping nature that further confirm the presence of high-spin, $S = 2$, Fe(II) heme. At 50 K, the MCD spectrum of Fe(II) frog HBD shows greater intensity in the Soret C term than the A term in the α/β region, implying that high-spin Fe(II)

heme is the major species at pH 8.0 (27). The electronic absorption and MCD peak positions and intensities of Fe(II) DGCR8 do not match those of thiolate-bound Fe(II) human cystathionine β -synthase or thiol-bound Fe(II) myoglobin H93G, suggesting that Cys is not bound to the Fe(II) heme of DGCR8 (28, 29).

Resonance Raman spectroscopy reinforces the conclusion that the heme in Fe(II) frog HBD exists as a mixture of spin and coordination states. The most intense oxidation state marker band, ν_4 , displays a dramatic downshift in energy from 1,372 to 1,358 cm^{-1} , consistent with the reduction of Fe(III) to Fe(II) heme (30, 31) (Fig. 3C). The most prominent spin and coordination state marker band, ν_3 , shifts from 1,501 cm^{-1} upon heme reduction and becomes split between 1,470 cm^{-1} (high-spin, five-coordinate) and 1,489 cm^{-1} (low-spin, six-coordinate), implicating a mixture of spin and coordination states for the Fe(II) heme in frog HBD (31, 32). At higher energy, the oxidation and coordination state marker bands ν_2 and ν_{10} are identified at 1,579 and 1,615 cm^{-1} , respectively, similar to those of other Fe(II) heme proteins that have mixed coordination and ligation states (30–32). Thus, from the electronic absorption, MCD, and resonance Raman data we conclude that, when the DGCR8 heme is reduced, the two cysteine ligands are lost, and one or two new ligands bind to the heme. The exact nature of the interaction between Fe(II) heme and DGCR8 (i.e. the identity of the new ligands and the specificity of the Fe(II) heme–protein interaction) will be reported elsewhere.

Association of ApoNC1-P351A with Fe(II) Heme. Neither the wild-type human NC1 nor the frog HBD can be expressed in dimeric heme-free (apo) forms. However, the NC1-P351A mutant has reduced affinity for heme and can be expressed in *Escherichia coli* as a heme-free dimer. The purified apoNC1-P351A dimer is very soluble at pH 8.0 and binds Fe(III) heme in vitro to reconstitute the complex with its characteristic absorption peaks at 365, 447, and 556 nm (22). Here we incubated heme-free NC1-P351A dimer with various molar equivalents of Fe(II) heme at pH 8.0 under anaerobic conditions; the electronic absorption spectra displayed peaks at 424, 529, and 557 nm, respectively, which are very close to those of reduced NC1 (Fig. 1B and Fig. S4A). This result suggests that an Fe(II) heme–NC1-P351A complex has been reconstituted. SEC analysis showed the reconstituted Fe(II)

Fig. 3. The Fe(II) heme in frog HBD is a mixture of five-coordinate, high-spin and six-coordinate, low-spin species without a cysteine thiolate ligand. (A) The electronic absorption spectrum (*Upper*) of Fe(II) frog HBD (11.7 μM) in 50 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPS) (pH 8.0), 400 mM NaCl, and 1 mM sodium dithionite at 37°C. MCD spectra (*Lower*) of the Fe(II) frog HBD (29.4 μM) in 20 mM EPPS (pH 8.0), 160 mM NaCl, 60% (vol/vol) glycerol, and 7 mM sodium dithionite at 4, 8, 15, 25, and 50 K. (*Inset*) The magnetic saturation behavior of the MCD C-term intensity at 442 nm taken at 2.5, 4.0, 8.0, 15, and 25 K. (B and C) Resonance Raman spectra are shown for Fe(III) (dotted line) and Fe(II) (solid line) frog HBD for low energy (B) and high energy (C) regions. Fe(III) HBD (153 μM) and Fe(II) HBD (136 μM) samples were in 45 mM EPPS (pH 8.0), 360 mM NaCl, and 10% (vol/vol) glycerol; Fe(II) HBD also contained 7 mM sodium dithionite. For the Fe(III) protein, spectra were acquired by excitation with a 457.9-nm line with 20 mW of power at the sample; for the Fe(II) protein, spectra were acquired by excitation with a 413.1-nm line with 14 mW of power at the sample.

NC1-P351A complex is labile (Fig. S4B), similar to the Fe(II) wild-type NC1.

ApoNC1 Produced Through Reduction Binds Both Fe(III) and Fe(II) Heme in Vitro. To directly test the effect of heme binding on the activity of DGCR8 in vitro, it is desirable to generate heme-free wild-type protein. To accomplish this, we took advantage of the labile nature of the Fe(II) NC1 complex. The heme-bound NC1 dimer was reduced at pH 6.0 using dithionite, and the Fe(II) heme was removed through incubation with apomyoglobin followed by SEC (Fig. 4A). The A_{280}/A_{450} ratio of the NC1 protein increased from 2.8 to 27 through this procedure, indicating that approximately 90% of heme was removed. The small amount of heme that remained bound to NC1 contained Fe(III). Incubation of the apoNC1 protein with equimolar Fe(III) heme restored the 366-, 450-, and 556-nm peaks (Fig. 4A), indicating that the Fe(III) heme–NC1 complex was successfully reconstituted. The 366- and 450-nm peaks of the reconstituted Fe(III) NC1 complex are of nearly equal intensities. In contrast, the 366-nm peak of the native NC1 complex is always less intense than the 450-nm peak. This observation indicates that the reconstituted complex may have subtle differences relative to the native complex. Incubation of Fe(II) heme with apoNC1 at pH 6.0 produced a complex with an absorption peak at approximately 390 nm (Fig. 4A), similar to that of the reduced NC1 (Fig. 1C).

Fe(III), not Fe(II), Heme Activates DGCR8 for Pri-miRNA Processing. We tested the activity of the reconstituted Fe(III) and Fe(II) heme NC1 complexes using in vitro pri-miRNA processing assays (Fig. 4B–D). Three pri-miRNA fragments, pri-miR-30a, pri-miR-21, and pri-miR-380, were used in these assays. The native NC1 complex was much more active than apoNC1 (lanes 3 and 4, 10 and 11, 17 and 18). The reconstituted Fe(III) NC1 complex was as active as native NC1 (lanes 5, 12, and 19), whereas the reconstituted Fe(II) NC1 complex was as inactive as the apoNC1 (lanes 6,

13, and 20). To prevent oxidation of Fe(II) heme, these assays were performed under anaerobic (N_2 gas) conditions. As controls, we also performed pri-miRNA processing assays using native NC1 under aerobic conditions (lanes 7 and 14) and observed activity indistinguishable from that in anaerobic conditions (lanes 3 and 10). This result suggests that the oxygen in the air does not affect the activity of Fe(III) NC1. Overall, these experiments clearly demonstrate that heme-free DGCR8 has very low pri-miRNA processing activity, and that Fe(III) heme activates DGCR8, whereas Fe(II) heme does not.

ApoNC1 Binds Pri-miRNAs with Reduced Cooperativity. To understand why the apoNC1 dimer is defective in pri-miRNA processing, we used a filter-binding assay to examine its interaction with pri-miR-30a. The binding data were best fit using a cooperative dimer model, in which two apoNC1 dimers bind cooperatively to a pri-miR-30a RNA, with a $\sqrt{K_d}$ of 6.0 ± 1.6 nM (Fig. 4E) and a Hill coefficient (n) of 1.6 ± 0.2 (Fig. 4F). Our control experiments and previous work showed that Fe(III) heme-bound NC1 binds pri-miR-30a with similar affinities, but with a higher Hill coefficient (*ca.* 3) (18). Thus, heme removal from DGCR8 likely inhibits pri-miRNA processing via reducing the binding cooperativity and hampering the formation of productive higher-order structures. This observation is consistent with our recent report demonstrating that DGCR8 recognizes pri-miRNAs through highly cooperative binding and formation of higher-order (trimer of dimers) structures (17).

Discussion

This study advances our understanding of the function of heme in miRNA maturation by demonstrating that Fe(III) heme binds to the apoNC1 dimer to activate its pri-miRNA processing activity, and that DGCR8 has an unusual specificity for Fe(III) heme over Fe(II) heme. In our initial discovery of the DGCR8–heme interaction, we observed that heme-bound NC1 dimer is much more

Fig. 4. Fe(III), not Fe(II), heme reconstitutes DGCR8 for pri-miRNA processing in vitro. (A) Electronic absorption spectra of apoNC1 (dotted line), prepared as described in *Materials and Methods*, and after incubation with equimolar Fe(III) (solid line) or Fe(II) (dashed line) heme. The protein solutions contained 50 mM MES (pH 6.0), 400 mM NaCl, and 1 mM DTT. (B–D) Uniformly 32 P-labeled pri-miR-30a (B), pri-miR-21 (C), and pri-miR-380 (D) were incubated at 37 °C for 45 min, with either Drosha^{390–1374} alone (lanes 2, 9, and 16) or Drosha^{390–1374} together with various forms of NC1 as indicated (lanes 3–7, 10–14, and 17–20). All reactions were performed in anaerobic conditions, except the ones analyzed in lanes 7 and 14. (E) Filter-binding assays demonstrate that apoNC1 associates with pri-miR-30a with lower cooperativity than that of the Fe(III) heme-bound NC1 (18). The data were best fit using a cooperative dimer model. (F) Hill plot of data from E around the binding transition. The result shown is one of three experiments. K is defined as $10^{(x-\text{intercept})}$. Low molecular weight marker, LMW.

active than the heme-free monomer in reconstituted pri-miRNA processing reactions (18). However, we could neither add heme to the heme-free monomer to activate NC1 nor remove heme from the heme-bound dimer to inactivate it. Later, we realized that the existence of the NC1 “monomer” is at least partially attributable to a NC1 heterodimer in which one subunit is cleaved during bacterial overexpression and purification (22). Characterization of the Fe(II) DGCR8 complexes led to the successful removal of heme from NC1 via reduction to produce the apoNC1 dimer, which allowed us to directly link heme binding with pri-miRNA processing. Importantly, using the apo-NC1 dimer, we were able to show definitively that only Fe(III) heme, and not Fe(II) heme, activates NC1 for pri-miRNA processing.

DGCR8 is a heme protein with high specificity for binding Fe(III) heme. Previous studies showed that the association rates (k_{on}) for heme binding to globin variants, BSA, and several other heme proteins are similar to each other ($1-10 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$) and are independent of protein structure (33, 34). Furthermore, the k_{on} values of Fe(III) heme for apomyoglobin and BSA, as measured using fluorescence quenching, are almost the same as those of Fe(II) heme (33). Thus, the affinities of these proteins for heme, which vary up to 10^6 -fold, are primarily determined by the rates of heme dissociation (k_{off}). The estimated k_{off} for the DGCR8–heme complex increased from $<2 \times 10^{-6} \text{ s}^{-1}$ for the Fe(III) complex ($t_{1/2} \gg 5 \text{ d}$ assuming that the K_d is higher than that of myoglobin, $k_{off} = \ln 2/t_{1/2}$) to $>1 \times 10^2 \text{ s}^{-1}$ (too fast to be measured using the stopped-flow method). The $>10^7$ -fold difference in k_{off} of DGCR8 for Fe(III) and Fe(II) heme suggests that the thermodynamic stability of Fe(III) complex is much greater than that of the Fe(II) complex.

The specificity of DGCR8 for Fe(III) heme is likely contributed by the use of two cysteine side chains as axial ligands. The affinity of a typical heme protein for heme is contributed by the axial ligands that bind to the iron, a hydrophobic pocket that surrounds the porphyrin ring, and amino acid residues that make specific interactions with the heme periphery, including salt bridges to the heme propionates. In myoglobin, mutation of the proximal histidine ligand to glycine reduces the affinity for heme by a factor of 10^4 , which provides an estimate of the contribution for heme binding by this axial ligand (33). Mutation of cysteine-352 in both NC1 subunits completely abolishes heme binding during bacterial expression, demonstrating the importance of dual cysteine axial ligation in the binding of Fe(III) heme to DGCR8 (18). Further evidence for the importance of dual cysteine ligation is provided by the $>10^7$ -fold increase of k_{off} for heme when the DGCR8 heme is reduced and both cysteine ligands are lost. That Fe(II) heme remains bound to DGCR8, albeit more weakly, is presumably attributable to other DGCR8–heme interactions and to the new ligand or ligands that replace the cysteines.

Reducing the heme abrogates function, presumably due to structural changes in the DGCR8 protein arising from ligand switching at the heme. Previously reported data suggest that at least one of the two Fe(III) heme-bound cysteine ligands, and possibly both, is deprotonated (22). When the heme is reduced from Fe(III) to Fe(II), unfavorable electrostatic interaction between the more electron rich Fe(II) and the anionic thiolate ligands drives them both off the heme. Interestingly, we see no evidence for DGCR8-bound heme with only a single cysteine ligand, either in the Fe(III) or Fe(II) forms. During the slow reduction of Fe(III) to Fe(II) frog HBD, no five-coordinate thiolate-ligated intermediate was observed. Similarly, when Fe(III) human DGCR8 HBD was titrated with up to 2 molar equivalents of methylmercury, a linear decrease of the absorption peak at 450 nm was observed with no intermediate observed that could be attributable to single-cysteine-ligated species (22).

The activity of DGCR8 is dependent on the redox state of its heme cofactor in vitro, suggesting that heme may be a redox-sensitive regulator of cellular RNA processing. One working

model regarding the biological function of the heme–DGCR8 interaction is that heme may serve as a ligand of DGCR8 to activate pri-miRNA processing (the heme sensing model). The weak pri-miRNA processing activity of the heme-free NC1 dimer and the activation by Fe(III) heme strongly support this model. The lack of activation of DGCR8 by Fe(II) heme further suggests that pri-miRNA processing might be regulated specifically by the availability of Fe(III) heme (the ferric heme sensing model). Heme is produced in the Fe(II) form, and the last step of the heme biosynthesis pathway, incorporation of Fe(II) into protoporphyrin IX by ferrochelatase, occurs on the mitochondrial inner membrane in animal cells (35). Little is known about how heme is transported from the site of Fe(II) insertion to where it is incorporated into host proteins, or how the oxidation state of heme may be altered during trafficking (36). Our data suggest that an intracellular environment favoring conversion of Fe(II) heme to Fe(III) heme may increase the efficiency of pri-miRNA processing.

The results reported herein suggest an opportunity for controlling pri-miRNA processing in therapeutics. DGCR8 is among the 35–60 genes at the 22q11.2 locus that are heterozygously deleted in DiGeorge syndrome (37, 38). Haploinsufficiency of DGCR8 in mouse models results in reduced processing of a subset of miRNAs in the brain and in deficits in synaptic plasticity in the prefrontal cortex that are associated with symptoms of the syndrome (4–6). The unique heme-binding properties revealed in our studies suggest that it may be possible to alter pri-miRNA processing efficiency using heme derivatives without affecting other heme proteins. This approach may be used for correcting miRNA processing defects in DiGeorge syndrome and other diseases without genetic manipulation.

Materials and Methods

Expression, Purification, Reduction, Heme Removal, and Reconstitution of DGCR8 Proteins. The human NC1 (heme-bound wild-type and heme-free P351A mutant), DGCR8^{276–353}, and frog HBD-His₆ proteins were expressed in *E. coli* and purified as previously described (18, 21, 22). The purified proteins were in 20 mM Tris (pH 8.0), 400 mM NaCl, and 1 mM DTT, and in some spectroscopic studies were exchanged into desired pH buffers, as indicated in the figures and figure legends, using centrifugal concentrators. Reduction of heme-bound NC1 and frog HBD was performed in an anaerobic chamber filled with N₂(g), unless stated otherwise. To produce apoNC1, freshly purified Fe(III) heme-bound NC1 was reduced at pH 6.0 with 2 mM sodium dithionite at room temperature in an anaerobically sealed cuvette until the 450-nm absorbance peak disappeared (ca. 1 h). Excess apomyoglobin was added to scavenge Fe(II) heme dissociated from NC1, and the proteins were separated using SEC at pH 6.0, as described below. For reconstitution experiments, hemein chloride was dissolved in 1.4 M NaOH at 100 mM, incubated at room temperature for approximately 30 min, and then diluted in water to give a 100-μM stock solution. An Fe(II) heme stock solution (100 μM) was prepared by addition of 5 mM dithionite to a hemein solution. Sodium dithionite (1 mM) was included in the apoNC1 and apoNC1-P351A protein solutions during reconstitution with Fe(II) heme.

Electronic Absorption Spectroscopy. Electronic absorption spectra were recorded using either a DU800 spectrophotometer (Beckman-Coulter; bandwidth $\leq 1.8 \text{ nm}$) (Figs. 1, 2, and 4, and Figs. S1, S2, and S4A) or a double-beam Varian Cary 4 Bio spectrophotometer with a temperature controller (Agilent Technologies) with its spectral bandwidth set to 0.5 nm (Fig. 3). The samples containing Fe(II) heme were analyzed in sealed cuvettes under N₂ or Ar gas.

Size Exclusion Chromatography. The SEC analysis was performed at room temperature using a Superdex-200 10/300 GL column (GE Healthcare). The running buffer contained 400 mM NaCl, 1 mM DTT, and either 50 mM MES (pH 6.0) (Fig. 2A and in production of apoNC1) or 20 mM Tris (pH 8.0) (Fig. S4B), and was always degassed.

Expression and Purification of His₆-Drosha^{390–1374}. The His₆-Drosha^{390–1374} protein was overexpressed in Sf9 insect cells using the baculovirus system and was purified using Ni affinity chromatography. Details may be found in *SI Text*.

In Vitro Pri-miRNA Binding and Processing Assays. The assays were carried out at pH 8.0 as described previously (18), with adaptation for anaerobic conditions where indicated. Details may be found in [SI Text](#).

MCD Spectroscopy. MCD spectra were recorded and analyzed as described previously (22). The Fe(II) frog HBD (ca. 29 μ M) was prepared from the Fe(III) protein at 37 °C via addition of 7 mM sodium dithionite. Samples were transferred via gas-tight syringes into cells purged with Ar(g), flash-frozen, and stored in N₂(l).

Resonance Raman Spectroscopy. Resonance Raman spectra were obtained with an excitation wavelength of either 413.1 nm from a Coherent I-302C Kr⁺ laser or 457.9 nm from a Coherent I-305 Ar⁺ laser in a backscattering 135° sample geometry. An Acton Research triple monochromator was

used with a grating of 2,400 grooves/mm. Low incident laser powers of ≤ 20 mW were focused with a cylindrical lens onto the sample. A Princeton Instruments Spex 1877 triple spectrograph outfitted with a cooled, intensified diode array detector was operated under computer control. Samples were placed in a quartz Dewar filled with ice water to reduce local heating. Peak positions were calibrated relative to a Na₂SO₄ standard peak at 983 cm⁻¹.

ACKNOWLEDGMENTS. We thank J. Valentine and K. Barnese for use of the anaerobic chamber, J. Feigon and T.C. Brunold for use of spectrophotometers, S. Weitz for sharing reagents, and M. Phillips in the University of California, Los Angeles–Department of Energy Biochemistry Instrumentation Facility for technical support. This project is supported by National Institutes of Health Grants GM080563 (to F.G.) and HL065217 (to J.N.B.).

- Ambros V (2004) The functions of animal microRNAs. *Nature* 431:350–355.
- Bartel DP (2009) MicroRNAs: Target recognition and regulatory functions. *Cell* 136:215–233.
- Croce CM (2009) Causes and consequences of microRNA dysregulation in cancer. *Nat Rev Genet* 10:704–714.
- Stark KL, et al. (2008) Altered brain microRNA biogenesis contributes to phenotypic deficits in a 22q11-deletion mouse model. *Nat Genet* 40:751–760.
- Fenelon K, et al. (2011) Deficiency of Dgcr8, a gene disrupted by the 22q11.2 microdeletion, results in altered short-term plasticity in the prefrontal cortex. *Proc Natl Acad Sci USA* 108:4447–4452.
- Schofield CM, et al. (2011) Monoallelic deletion of the microRNA biogenesis gene Dgcr8 produces deficits in the development of excitatory synaptic transmission in the prefrontal cortex. *Neural Dev* 6:11.
- Kim VN, Han J, Siomi MC (2009) Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol* 10:126–139.
- Faller M, Guo F (2008) MicroRNA biogenesis: There's more than one way to skin a cat. *Biochim Biophys Acta* 1779:663–667.
- Sashital DG, Doudna JA (2010) Structural insights into RNA interference. *Curr Opin Struct Biol* 20:90–97.
- Gregory RI, et al. (2004) The Microprocessor complex mediates the genesis of microRNAs. *Nature* 432:235–240.
- Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ (2004) Processing of primary microRNAs by the Microprocessor complex. *Nature* 432:231–235.
- Han J, et al. (2004) The Drosha-DGCR8 complex in primary microRNA processing. *Genes Dev* 18:3016–3027.
- Landthaler M, Yalcin A, Tuschl T (2004) The human DiGeorge syndrome critical region gene 8 and its D. melanogaster homolog are required for miRNA biogenesis. *Curr Biol* 14:2162–2167.
- Wang Y, Medvid R, Melton C, Jaenisch R, Blueloch R (2007) DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. *Nat Genet* 39:380–385.
- Yi R, et al. (2009) DGCR8-dependent microRNA biogenesis is essential for skin development. *Proc Natl Acad Sci USA* 106:498–502.
- Lee Y, et al. (2003) The nuclear RNase III Drosha initiates microRNA processing. *Nature* 425:415–419.
- Faller M, et al. (2010) DGCR8 recognizes primary transcripts of microRNAs through highly cooperative binding and formation of higher-order structures. *RNA* 16:1570–1583.
- Faller M, Matsunaga M, Yin S, Loo JA, Guo F (2007) Heme is involved in microRNA processing. *Nat Struct Mol Biol* 14:23–29.
- Han J, et al. (2006) Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. *Cell* 125:887–901.
- Sohn SY, et al. (2007) Crystal structure of human DGCR8 core. *Nat Struct Mol Biol* 14:847–853.
- Senturia R, et al. (2010) Structure of the dimerization domain of DiGeorge Critical Region 8. *Protein Sci* 19:1354–1365.
- Barr I, et al. (2011) DiGeorge Critical Region 8 (DGCR8) is a double-cysteine-ligated heme protein. *J Biol Chem* 286:16716–16725.
- Hargrove MS, et al. (1994) Stability of myoglobin: A model for the folding of heme proteins. *Biochemistry* 33:11767–11775.
- Dawson JH, Andersson LA, Sono M (1983) The diverse spectroscopic properties of ferrous cytochrome P-450-CAM ligand complexes. *J Biol Chem* 258:13637–13645.
- Sono M, Stuehr DJ, Ikeda-Saito M, Dawson JH (1995) Identification of nitric oxide synthase as a thiolate-ligated heme protein using magnetic circular dichroism spectroscopy. Comparison with cytochrome P-450-CAM and chloroperoxidase. *J Biol Chem* 270:19943–19948.
- Adar F (1978) Electronic absorption spectra of hemes and hemoproteins. *The Porphyrins*, ed D Dolphin (Academic, New York), IIIA, pp 167–210.
- Alberta JA, Andersson LA, Dawson JH (1989) Spectroscopic characterization of secondary amine mono-oxygenase. Comparison to cytochrome P-450 and myoglobin. *J Biol Chem* 264:20467–20473.
- Pazicni S, et al. (2004) The redox behavior of the heme in cystathionine beta-synthase is sensitive to pH. *Biochemistry* 43:14684–14695.
- Perera R, et al. (2003) Neutral thiol as a proximal ligand to ferrous heme iron: implications for heme proteins that lose cysteine thiolate ligation on reduction. *Proc Natl Acad Sci USA* 100:3641–3646.
- Spiro TG, Strekas TC (1974) Resonance Raman spectra of heme proteins. Effects of oxidation and spin state. *J Am Chem Soc* 96:338–345.
- Hu S, Smith KM, Spiro TG (1996) Assignment of protoheme resonance Raman spectrum by heme labeling in myoglobin. *J Am Chem Soc* 118:12638–12646.
- Tomita T, Gonzalez G, Chang AL, Ikeda-Saito M, Gilles-Gonzalez MA (2002) A comparative resonance Raman analysis of heme-binding PAS domains: Heme iron coordination structures of the BfFixL, AxPDEA1, EcDos, and MtDos proteins. *Biochemistry* 41:4819–4826.
- Hargrove MS, Barrick D, Olson JS (1996) The association rate constant for heme binding to globin is independent of protein structure. *Biochemistry* 35:11293–11299.
- Igarashi J, et al. (2008) The roles of thiolate-heme proteins, other than the P450 cytochromes, in the regulation of heme-sensor proteins. *Acta Chim Slov* 55:67–74.
- Dailey HA (2002) Terminal steps of haem biosynthesis. *Biochem Soc Trans* 30:590–595.
- Schultz IJ, Chen C, Paw BH, Hamza I (2010) Iron and porphyrin trafficking in heme biogenesis. *J Biol Chem* 285:26753–26759.
- Karayorgou M, Simon TJ, Gogos JA (2010) 22q11.2 microdeletions: Linking DNA structural variation to brain dysfunction and schizophrenia. *Nat Rev Neurosci* 11:402–416.
- Shiohama A, Sasaki T, Noda S, Minoshima S, Shimizu N (2003) Molecular cloning and expression analysis of a novel gene DGCR8 located in the DiGeorge syndrome chromosomal region. *Biochem Biophys Res Commun* 304:184–190.