Expression, purification, and characterization of human hemoglobins Gower-1 (ζ2ε2), Gower-2 (α2ε2), and Portland-2 (ζ2β2) assembled in complex transgenic–knockout mice

Zhenning He and J. Eric Russell

Embryonic ζ- and ε-globin subunits assemble with each other and with adult α- and β-globin subunits into hemoglobin heterotetramers in both primitive and definitive erythrocytes. The properties of these hemoglobins—Hbs Gower-1 (ζε2), Gower-2 (αε2), and Portland-2 (ζβ2)—have been incompletely described as they are difficult to obtain in quantity from either primary human tissue or conventional expression systems. The generation of complex transgenic–knockout mice that express these hemoglobins at levels between 24% and 70% is described, as are efficient methods for their purification from mouse hemolysates. Key physiological characteristics—including P50, Hill coefficient, Bohr effect, and affinity for 2,3-BPG—were established for each of the 3 human hemoglobins. The stability of each hemoglobin in the face of mechanical, thermal, and chemical stresses was also determined. Analyses indicate that the ζ-for-α exchange distinguishing Hb Portland-2 and Hb A alters hemoglobin O2-transport capacity by increasing its P50 and decreasing its Bohr effect. By comparison, the ε-for-β exchange distinguishing Hb Gower-2 and Hb A has little impact on these same functional parameters. Hb Gower-1, assembled entirely from embryonic subunits, displays an elevated P50 level, a reduced Bohr effect, and increased 2,3-BPG binding compared to Hb A. The data support the hypothesis that Hb Gower-2, assembled from reactivated ε globin in individuals with defined hemoglobinopathies and thalassemias, would serve as a physiologically acceptable substitute for deficient or dysfunctional Hb A. In addition, the unexpected properties of Hb Gower-1 call into question a common hypothesis for its primary role in embryonic development. (Blood. 2001;97: 1099-1105)

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assembled from αααα- and he-globin subunits (ααααββ) displayed O2-binding properties similar to those of control Hb ααααββββ. This result strengthened the hypothesis that physiologically important characteristics of fully human Hb Gower-2 (αβββ) might be similar to those of Hb A (αβββ).10 In contrast, substantial differences in the properties of Hbs hααααββ and hααααββ suggested that the physio-

logical characteristics of fully human Hb Portland-2 (ββββ) and Hb A might differ in several important respects.10 In addition to providing an estimate of the biochemical and physiological properties of the semi-embryonic hemoglobins, these studies also indicated the potential value of human embryonic globin subunits as substitutes for adult globin subunits in individuals with defined thalassemias or hemoglobinopathies.

The current study extends our previous work by assessing key biochemical and physiological properties of fully human semi-embryonic and embryonic hemoglobins purified from complex transgenic–knockout mice. We describe a strategy for generating mice expressing high levels of human Hbs Gower-1 (ααααββ), Gower-2 (αβββ), and Portland-2 (ββββ), as well as specific methods for their rapid and efficient purification. The key biochemical characteristics of each hemoglobin are subsequently determined, including their O2 affinities, subunit cooperativities, and changes in O2 affinity in response to allosteric modifiers and variations in ambient pH. We also assess the stability of each of these hemoglobins in response to defined mechanical, chemical, and thermal stresses. Based on the data, we speculate on the evolution-ary basis for hemoglobin switching and the potential value of these poorly understood hemoglobins to patients with congenital α–β-globin chain defects.

Materials and methods

Transgen and knockout mice

The generation and characterization of transgenic mice expressing high levels of human αααα, ββββ, γγγγ, and εεεε globins have previously been described.18-21 Mice with heterozygous knockout of their endogenous αααα-globin genes (genotype maxαααα) or ββββ-globin genes (genotype mββββ) were generously provided by Y. W. Kan and Judy Chang (University of California, San Francisco)22 and O. Smithies (University of North Carolina, Chapel Hill),23 respectively. All mouse husbandry and experimentation were performed using protocols approved by the IACUC of the University of Pennsylvania.

Hemoglobin purification

Whole blood was collected from decapitated mice in 200 μL phosphate-buffered saline (PBS)–heparin (20 U/mL) or PBS–EDTA (27 mM), and the hemoglobins were promptly converted to the carbonmonoxy form by bubbling the sample with CO. Erythrocytes were subsequently washed with PBS–heparin (20 U/mL) or PBS–EDTA (27 mM), and the Hemoglobins were precipitated by desktop centrifugation, and the A540 of the supernatant was determined.

Electrophoretic analysis

The identity and purity of each hemoglobin preparation was verified by denaturing Triton–acid–urea44,45 and nondenaturing cellulose acetate elec-
trophoresis17 using methods recommended by the manufacturer (Helena Laborato-

ries, Beaumont, TX).

Oxygen equilibrium curves

Purified CO-hemoglobins were resuspended to a final concentration of approximately 7.5 μM in P50 buffer (50 mM Bis-Tris, pH 7.4, 100 mM NaCl, 5 mM EDTA) and converted to the oxy form by photolysis under 100% O2 using an ice water–cooled rotary condenser.26 Conversion to the oxyhemoglobin form was judged complete by an A540/A576 ratio of less than 0.95. Oxygen equilibrium curves (OECs) were subsequently determined on a HEMOX analyzer (TCS, Southampton, PA) at 20°C. Studies of 2,3-
bisphosphoglycerate binding (2,3-BPG; Sigma, St Louis, MO) were carried out in P50 buffer (pH 7.4), whereas Bohr effect studies were carried out in P50 buffer adjusted to defined pH values.

Stability determinations

Mechanical. Using a modified version of a previously described method,27 Hbs were diluted to approximately 13 μM with 10 mM potassium phosphate buffer (pH 8.0) and converted to the oxyhemoglobin form (see above). Aliquots (2 mL) were shaken for defined intervals at a setting of 2000 on a Maxi-Mix III type 65800 shaker (Thermolyne, Dubuque, IA), and denatured hemoglobins were precipitated by a 5-minute desktop spin. The soluble hemoglobin was determined as A542 spectrophotometry of the supernatant.

Chemical. Purified hemoglobins were diluted to approximately 0.1 mM in buffer (0.1 mM Tris, pH 7.4) and converted to the oxyhemoglobin form as described above. Aliquots diluted 10-fold in prewarmed Tris buffer containing 17% (vol/vol) isopropanol were incubated at 37°C for 5 minutes.28 Precipitated hemoglobins were clarified by desktop centrifugation, and the A540 of the supernatant was determined.

Thermal. Purified hemoglobins were diluted to approximately 50 μM in buffer (0.1 mM Tris, pH 7.4) and converted to the oxyhemoglobin form as described above. Test and control aliquots were incubated for 2 hours at 50°C and 4°C, respectively, and spun for 10 minutes on a desktop centrifuge.29 The supernatant was diluted 10-fold with developer solution [11.9 mM NaHCO3, 0.77 mM KCN, 0.61 mM K3Fe(CN)6], insoluble hemoglobins were precipitated by desktop centrifugation, and the A540 of the supernatant was determined.

Results

Generation of adult mice expressing high levels of human embryonic and semi-embryonic hemoglobins

The construction of transgenes and the generation of mice expressing hαααα, hαααα, hββββ, and hββββ globins in definitive erythrocytes has previously been described.16-20 The high-level, developmental-stage inappropriate expression of transgenic hαααα and hββββ globins was achieved by linking their encoding genes to transcriptional control elements from the hαααα and hββββ globin genes, respectively (Figure 1A).18 Full-length genes encoding hαααα and hββββ globins, containing their native transcriptional control elements, were anticipated to be expressed at high levels in adult erythrocytes and consequently were not modified.19,20 Each transgene was linked to a micro β-locus control region to insure its high-level, integration position-independent expression.18-20,30 Single lines expressing high levels of each transgenic globin were identified by phenotypical screening of hemolysates using denaturing globin electrophoresis.18,24 These lines were used in the experiments described in the current work.

A breeding strategy was designed to generate mice expressing high levels of human embryonic or semi-embryonic hemoglobins with minimal mouse globin background (Figure 1B). We had
previously noted that the expression of each of the 4 human globin transgenes increased substantially in mice carrying one or more knockout mutations of the related endogenous adult globin gene homolog, \( \textit{18,19} \). The level of h\( _{2} \) and h\( _{\alpha} \) induction was sufficient to rescue the viability of mice with homozygous-lethal deletions of their endogenous mo-globin genes, \( \textit{18,31,32} \) whereas the viability of mice with homozygous-lethal deletions of the m\( _{\beta} \)-globin genes could be rescued by the expression of either transgenic h\( _{\delta} \) or h\( _{\beta} \) globins. \( \textit{18,31,32} \) We reasoned that the assembly of hemoglobin heterotetramers from transgenic human \( \alpha \)-like and \( \beta \)-like globins would be similarly enhanced in mice carrying both mo- and m\( _{\beta} \)-globin knockout alleles.

**Design of methods to purify human hemoglobins from transgenic hemolysates**

A combination of genetic and biochemical strategies was used to facilitate the preparation of human Hbs from transgenic mice. We screened more than 125, 335, and 89 candidate pups expressing Hbs Gower-1, Gower-2, and Portland-2, respectively, without identifying any mo\( _{1} \)/m\( _{\beta} \)-like globins. \( \textit{18,31,32} \) We reasoned that the assembly of hemoglobin heterotetramers from transgenic human \( \alpha \)-like and \( \beta \)-like globins would be similarly enhanced in mice carrying both mo- and m\( _{\beta} \)-globin knockout alleles.

**Embryonic and semi-embryonic hemoglobins exhibit elevated \( \text{O}_2 \) affinities**

The \( \text{O}_2 \)-binding affinities of human Hbs \( \zeta\varepsilon_2 \), \( \alpha\varepsilon_2 \), and \( \zeta\beta_2 \) were determined on 3 or more occasions under standard conditions by
Table 1. Properties of human hemoglobins

| Hemoglobin | Structure | $P_{50}$ | Hill | Bohr | 2,3-BPG
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<tr>
<td>Gower-1</td>
<td>$\zeta_2\epsilon_2$</td>
<td>1.4 ± 0.06</td>
<td>1.7 ± 0.24</td>
<td>−0.25</td>
<td>0.09</td>
</tr>
<tr>
<td>Gower-2</td>
<td>$\alpha_2\epsilon_2$</td>
<td>2.7 ± 0.10</td>
<td>2.3 ± 0.02</td>
<td>−0.51</td>
<td>0.17</td>
</tr>
<tr>
<td>Portland-2</td>
<td>$\zeta_2\beta_2$</td>
<td>1.9 ± 0.17</td>
<td>1.6 ± 0.06</td>
<td>−0.10</td>
<td>0.30</td>
</tr>
<tr>
<td>A</td>
<td>$\alpha_2\beta_2$</td>
<td>3.2 ± 0.14</td>
<td>2.9 ± 0.36</td>
<td>−0.54</td>
<td>0.29</td>
</tr>
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*torr; †$\log(Y/1 – Y)/\log P_{O_2}$; ‡$\log P_{O_2}/\Delta P_{O_2}$ in the alkaline region; §Apparent binding constant (mM) calculated from half-saturation point.

The P$_{50}$ values of embryonic and semi-embryonic hemoglobins display disparate responses to changes in pH and [2,3-BPG]

The effect of pH on the O$_2$-binding affinities of the 3 human embryonic and semi-embryonic hemoglobins was determined by measuring the P$_{50}$ values for each in a series of buffers with defined pH levels ranging from 6.0 to 8.2 (Figure 4A; Table 1). The 2 human embryonic hemoglobins containing $\zeta$-globin subunits displayed attenuated Bohr effects [Hbs $\zeta\epsilon_2$ (−0.10 $\Delta\log P_{O_2}/\Delta P_{O_2}$) and $\zeta\beta_2$ (−0.25 $\Delta\log P_{O_2}/\Delta P_{O_2}$)], whereas the Bohr effect of Hb Gower-1 ($\alpha_2\epsilon_2$) and control Hb A ($\alpha_2\beta_2$) were nearly identical (−0.51 vs −0.54 $\Delta\log P_{O_2}/\Delta P_{O_2}$, respectively). The apparent binding constant of 2,3-BPG for each hemoglobin was estimated by establishing the half-saturation point using buffers with defined concentrations of the allosteric modifier (Figure 4B; Table 1). 2,3-BPG appears to bind to Hb Portland-2 ($\zeta\beta_2$) and control Hb A with equal affinity (0.30 mM and 0.29 mM, respectively) while binding to Hbs Gower-1 ($\zeta\epsilon_2$) and Gower-2 ($\alpha_2\epsilon_2$) with substantially higher avidity (0.09 mM and 0.17 mM, respectively). These results indicate that the inclusion of embryonic globin subunits may have an impact on the biochemical function of intact heterotetramers, permitting speculation on the evolutionary pressures favoring the conservation of globin gene switching.

Human embryonic and semi-embryonic hemoglobins display different physical stabilities

Hbs Gower-1, Gower-2, and Portland-2 were assessed for their stability in the setting of defined mechanical, chemical, and thermal stresses. Hbs A and S were evaluated in parallel as stable and unstable hemoglobin controls, respectively (Figure 5). The stabilities of the various hemoglobins by each of the 3 methods were in general agreement: Hbs Gower-1 ($\zeta\epsilon_2$) and Portland-2 ($\zeta\beta_2$) were equally or less stable than Hb S, whereas the stability of Hb Gower-2 ($\alpha_2\epsilon_2$) was generally intermediate between the 2 control hemoglobins. Hence, the low stability of Hb Gower-1 appears to be well adapted to the short survival of primitive erythrocytes, whereas the higher stability of Hb Portland-2 may facilitate its expression in long-lived definitive erythrocytes.
Application of the analyses of Hbs Gower-2 and Portland-2 stems from the possibility that reactivation of the $\zeta$ and $\epsilon$ genes would produce globins that could substitute for deficient or abnormal $\alpha$- or $\beta$-globin chains, respectively, in individuals with defined thalassemias and hemoglobinopathies. The implications of the current work on each of these 3 issues are considered below.

Many well-established studies have identified functionally crucial amino acid residues in the $\alpha$- and $\beta$-globin chains. Our data generally confirm the role of these residues, but they also emphasize the importance of distant residues on specific hemoglobin functions through their likely influence on the high-order structure of the globin molecule. In general, Hb Gower-2 and Hb A function similarly, suggesting that many of the 36 amino acid differences between the $\beta$- and $\epsilon$-globin chains (of 146 total residues) cluster in functionally silent domains (Table 1). The 2 hemoglobins exhibit similar $O_2$-binding characteristics and display Hill coefficients consistent with normal hemoglobin function (Figures 3B, E), reflecting the conservation of 13 of 17 and 15 of 16 residues comprising the $\alpha\beta_1$ and $\alpha\beta_2$ interfaces, respectively. Unexpectedly, Hb Gower-2 and Hb A displayed markedly different affinities for 2,3-BPG (Figure 4B) despite their identity at all 4 residues believed to mediate this property (1Val, 2His, 82Lys, and 143His). Although the half-saturation method provides only an estimate of the strength of this interaction, it is likely that one or more differences in amino acid content elsewhere in the $\epsilon$- and $\beta$-globin chains alter the spatial arrangement, and hence the function, of the 4 conserved residues. A less likely explanation is that a fifth, unrecognized residue, differing between the $\beta$- and $\epsilon$-globin chains, directly participates in 2,3-BPG binding. Crystallographic evaluation of Hb Gower-2, available in quantity from the transgenic–knockout mice, would be useful in evaluating these 2 possibilities.

In comparison to the $\beta$-like globin chains, the 57 (of 141) amino acid differences between the $\zeta$- and $\alpha$-globin chains appear to occupy functionally sensitive regions (Table 1). Hb Portland-2 and Hb A, differing only in the identity of their $\alpha$-like chains, display substantially different $P_50$ values and Hill coefficients (Figure 3C, F) despite sharing 14 of 16 and 15 of 15 residues at the $\alpha\beta_1$ and $\alpha\beta_2$ interfaces, respectively. Remarkably, 2 of the 3 interface substitutions are conservative, emphasizing the unpredictable functional effects of changes in high-order globin subunit structure resulting from spatially distant amino acid substitutions. Although both globins share a common 122His, the $\alpha$1Val-$\zeta$1Ser exchange would be expected to substantially blunt the Bohr effect exhibited by Hb Portland-2, a prediction that is experimentally observed (Figure 4A). A direct comparison of Hb Portland-2 function with its high-resolution structure, when available, may yield important new information relative to the function of human heterotetramers.

The observation that the properties of Hb Gower-1, comprising developmental stage-concordant $\zeta$- and $\epsilon$-globin subunits, are strikingly different from those of Hb A suggests a strong evolutionary basis for Hb switching (Figures 3A, 4A-B; Table 1). A widely held, yet largely unsubstantiated, explanation for this process suggests that embryonic and fetal hemoglobins have been evolutionarily selected to facilitate the trans-placental delivery of $O_2$ during intrauterine development. This hypothesis has been challenged by studies that do not detect the predicted elevated rates of fetal wastage in pregnant women with high-affinity hemoglobins. By demonstrating that the $O_2$-binding properties of Hb Gower-1...
differ from those of Hb F, our data support the latter dissenting opinion. In contrast to Hb F, which exhibits a normal O₂ affinity (in the absence of 2,3-BPG), a relatively high Bohr effect, and poor 2,3-BPG binding, we have found that Hb Gower-1 displays a high O₂ affinity, a reduced Bohr effect, and relatively tight 2,3-BPG binding. Were their developmental roles strictly limited to O₂ transport, the O₂-binding properties of Hbs Gower-1 and F might be anticipated to be more similar. An alternative explanation for this difference is that the evolutionary basis for developmental hemoglobin switching reflects properties of Hb Gower-1 that are independent of its gaseous-exchange function. This possibility is underscored by recent reports indicating that in addition to its O₂-transporting function, Hb A may serve an important role in the regulation of vascular tone and blood flow.54 It is clear that the functional properties of Hbs Gower-1, reflecting poorly understood evolutionary demands and manifesting as phylogenic conservation of hemoglobin switching, merit additional study.

We have previously proposed that Hbs Portland-2 (ζβ₂) and Gower-2 (αε₂) might be suitable substitutes for Hb A in individuals with defined hemoglobinopathies and thalassemias.18,19 These 2 hemoglobin combinations might resemble those existing adult globin chains in adults in whom the embryonic globin genes were reactivated. The functional identity between Hb Gower-2 (αε₂) and Hb A (αβ₂) indicates that the former hemoglobin would act in a physiologically valuable manner in definitive erythrocytes. Evidence that the ε- and γ-globin genes are independently regulated27-31 raises the possibility that different classes of agents, with different toxicities, might be used to separately reactivate expression from the 2 genes. This consideration is particularly important in patients with severe β-thalassemia determinants in whom γ-globin reactivation by current agents is toxicity-limited.39,40 The observation that Hb Gower-2 will serve a physiologically important role in individuals with any of several hemoglobinopathies and thalassemias should serve as an impetus to identify agents that might be useful in this regard.

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