

A human embryonic hemoglobin inhibits Hb S polymerization *in vitro* and restores a normal phenotype to mouse models of sickle cell disease

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The principle that developmentally silenced globin genes can be reactivated in adults with defects in β -globin gene expression has been well established both *in vitro* and *in vivo*. In practice, levels of developmental stage-discordant fetal γ globin that can be achieved by using currently approved therapies are generally insufficient to fully resolve typical clinicopathological features of sickle cell disease. The therapeutic potential of another developmentally silenced globin—embryonic ϵ globin—has been difficult to evaluate in the absence of a convenient expression system or an appropriate experimental model. The current work analyzes the antisickling properties of an ϵ -globin-containing heterotetramer (Hb Gower-2) both *in vitro* as well as *in vivo* in a well-established mouse model of sickle cell anemia. These animals, expressing 100% human Hb S, display a chronic hemolytic anemia with compensatory marrow and extramedullary erythropoiesis, abundant circulating sickled erythrocytes, and chronic tissue damage evidenced by parallel histopathological and functional deficits. By comparison, related mice that coexpress Hb S as well as Hb Gower-2 exhibit normal physiological, morphological, histological, and functional attributes. Subsequent *in vitro* analyses substantiate results from whole-animal studies, indicating that the polymerization of deoxygenated Hb S can be significantly slowed by relatively small quantities of Hb Gower-2. Together, the *in vivo* and *in vitro* analyses suggest that reactivation of ϵ -globin gene expression would be therapeutically beneficial to adults with sickle phenotypes, and provide a rationale for detailed investigations into the molecular basis for its developmental silencing.

Human β -like globins are encoded by five homologous genes ($5'$ - ϵ - γ - δ - β - $3'$) clustered on the short arm of chromosome 11 (1). Transcriptional control elements positioned within (2) and upstream (3, 4) of this region coordinate the sequential activation and silencing of these genes during three defined periods of human development: embryonic ϵ globin (gestational weeks 4–8), fetal γ globin (gestational week 8 through parturition), and adult β and δ globins (from birth onward) (5).

Although generally predictable, both the timing and the efficiency of globin gene switching can be disrupted by specific congenital and acquired conditions. Well-described deletional and nondeletional mutations within the β -globin cluster mediate lifelong expression of γ globin (hereditary persistence of fetal hemoglobin or HbF) (6), whereas a delay in the γ -to- β transition in newborns of diabetic mothers appears to be mediated by elevated levels of butyric acid (7). In both cases, developmental stage-discordant γ -globin subunits assemble into $\alpha_2\gamma_2$ heterotetramers (HbF) that differ from normal adult $\alpha_2\beta_2$ heterotetramers (HbA) only in the identity of their β -like subunits. Fetal γ globin can also be reactivated by pharmacological methods that have proved to be extraordinarily useful for the clinical management of sickle cell anemia (8), a congenital condition defined by homozygosity for a single-nucleotide trans-

version in the β -globin gene that results in a codon 6 Glu \rightarrow Val substitution (β^S globin) (9). The sickle phenotype results from the intracellular polymerization of deoxygenated $\alpha_2\beta^S_2$ heterotetramers (HbS) into extended 14-strand fibers that disrupt both the shape and the function of the mature erythrocyte (10). Human HbF (containing γ -globin subunits) recombines with HbS into $\alpha_2\gamma\beta^S$ heterotetramers that are excluded from the polymer, lowering the effective intracellular concentration of HbS and consequently slowing its rate of self-assembly. Although highly effective, the levels of HbF required to fully ablate the sickle phenotype are rarely achieved through use of approved (8) or investigational HbF-inducing agents (11).

Like fetal γ globin, the expression of embryonic ϵ globin is normally silenced in adults despite the fact that its encoding gene remains structurally intact. ϵ -globin chains expressed from reactivated genes would be anticipated to assemble into $\alpha_2\epsilon_2$ heterotetramers (HbGower-2) (12). However, elucidation of its fundamental properties has been prevented by the fact that HbGower-2 is difficult to obtain in quantity from primary primitive erythroid cells (13), is expressed at extremely low levels in definitive erythrocytes (14, 15), and is difficult to generate by using conventional expression systems (16, 17). To remedy this situation, transgenic mouse lines were recently generated that express high levels of human α - and ϵ -globin subunits (12, 18, 19). These mice were then mated with each other and with animals carrying targeted deletions of their endogenous murine α - or β -globin genes (20, 21) to generate complex transgenic knockout animals expressing high levels of human HbGower-2. Subsequent *in vitro* analysis indicated that HbGower-2 ($\alpha_2\epsilon_2$) and HbA ($\alpha_2\beta_2$), differing only in the identity of their β -like subunits, possess related O₂ affinities, equivalent 2,3-BPG binding characteristics, and nearly identical Bohr properties (12). This analysis strongly supports a potential therapeutic role for HbGower-2 as an O₂-transport protein in thalassemic erythrocytes that are pathologically deficient in HbA.

In sickle cell disease, the potential therapeutic value of HbGower-2 is less dependent on its O₂-binding characteristics than on its antipolymerization activity. Mutational analyses and crystallographic studies demonstrate that pairing of deoxygenated HbS filaments is facilitated by so-called "lateral interactions" between the pathological 6Val in the β_2 subunit and a hydrophobic pocket (comprising residues 73, 84, 85, 87, and 88) on the β_1 subunit of a neighboring heterotetramer (22–24). As ϵ globin contains a polar Lys at position 87, it is anticipated that complex Hb heterotetramers of the form $\alpha_2\epsilon\beta^S$ would be excluded from this process, reducing the effective intracellular HbS concentration and, consequently, its rate of polymerization. This prediction is supported by *in vitro* analyses of β^S -globin chains

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carrying defined mutations at position 87 (23) and by the observed antipolymerization properties of β -like γ - and δ -globin subunits, each containing a polar Gln at position 87. Consequently, although the antipolymerization characteristics of Hb Gower-2 have never been established, its assembly from reactivated ϵ globin would be anticipated to significantly benefit individuals with sickle cell disease.

Here, we investigate the therapeutic potential of Hb Gower-2 for individuals with sickle cell disease and pathophysiologically related hemoglobinopathies. Complex transgenic knockout mice were generated that express 100% human Hb S or a mixture of Hb S and Hb Gower-2, which were subsequently analyzed by using informative morphological, hematological, chemical, histological, and functional methods. These studies indicate that the hemolytic anemia and characteristic end-organ damage displayed by sickle mice is largely reversed in animals that coexpress Hb Gower-2. Additional results from *in vitro* delay-time analyses suggest that benefits may accrue in animals—or humans—expressing relatively small quantities of Hb Gower-2. Based on these studies, we conclude that reactivation of ϵ -globin gene expression would provide significant benefit to individuals with sickle cell disease, either alone or in combination with conventional γ -globin inducing therapies.

Methods

Animals. All animal studies were compliant with protocols approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. The generation and propagation of mice expressing human Hb Gower-2 has been described (12); high-level developmental stage-discordant expression of transgenic human ϵ -globin is achieved by flanking its transcribed region with the human β -globin promoter and enhancer elements (18). Complex transgenic knockout mice expressing human Hb S were graciously provided by N. Mohandas (Univ. of California, Berkeley) (25). Globin phenotypes were established from mouse hemolysate by using denaturing Triton-acid-urea gel and/or nondenaturing cellulose acetate membrane electrophoresis (26, 27).

Clinical Labs. Complete blood counts were performed on citrate-anticoagulated blood by using a calibrated Hemavet analyzer (CDC Technologies, Oxford, CT). Total bilirubin levels were determined by using a standardized method recommended by the manufacturer (Sigma catalog nos. 550A and 550-11) carried out in 96-well microtiter plates to permit simultaneous analysis of test and control samples (unpublished data).

Morphological and Histopathological Studies. Wright-stained peripheral blood smears were examined by using a DMLS microscope fitted with a $\times 100$ N-Plan oil-immersion objective (Leica). Hematoxylin/eosin stained sections of formalin-fixed mouse tissues were prepared by American Histolab (Gaithersburg, MD). High-resolution photomicrographs were taken through an eyepiece adapter using a digital Twin-Cam (Camdek, Frederick, MD).

Urine Osmolalities. Urine samples collected at baseline and after a 6-h period of water deprivation were analyzed in duplicate on calibrated osmometers (Advanced Instruments) by the Clinical Laboratories at The Children's Hospital of Philadelphia.

Analysis of Erythrocyte Oxygen Affinity. Oxygen equilibrium curves were established by HEMOX analysis (TCS Medical Products, Southampton, PA) using 40 μ l of fresh heparinized blood obtained from sickle and sickle-gower mice. Samples were studied at 37°C in TES buffer (30 mM, pH 7.6) containing 134 mM NaCl, 5 mM KCl, 8 mM glucose, 0.1% BSA, and 0.01% antifoam (SAG-10, Union Carbide) (12, 28).

Delay Time Analysis. Purified CO-Hb S was generously provided by K. Adachi (The Children's Hospital of Philadelphia). CO-Hb Gower-2 and CO-Hb A were prepared from the hemolysates of phenotypically appropriate mice (12) and one of the authors (J.E.R.), respectively, as described (12). CO-Hb F was prepared from hemolysate provided by an informed, consenting donor with a known HPFH determinant (29), in compliance with a protocol approved by the University of Pennsylvania Institutional Review Board. The CO-hemoglobins were converted to the oxy form by photolysis under 100% O₂ using an ice-cooled rotary condenser (12). Conversion to the oxyhemoglobin form was judged complete when the A₅₄₀/A₅₇₆ ratio was reduced to less than 0.95. Reactions (100 μ l) containing defined quantities of each hemoglobin in 1.8 M potassium phosphate (pH 7.3) were transferred to a polystyrene 96-well microtiter plate, overlaid with 100 μ l of optically inert TW oil (Inland Vacuum, Churchville, NY), and supplemented with 14.35 mM sodium dithionite. After a 30-min incubation on ice, the plate was transferred to a Spectromax 250 spectrophotometer (Molecular Devices) preheated to 30°C, and the A₇₀₀ of individual wells was determined at 10-s intervals until the polymerization reaction was complete.

Results

Generation of Mice Coexpressing Human Hbs S and Gower-2. Structural considerations suggested that Hb Gower-2 would likely exhibit antipolymerization properties *in vitro* and corresponding antisickling characteristics *in vivo* in mouse models of sickle cell disease. The experimental test of this hypothesis required comparative analyses of mice expressing human Hb S either with or without human Hb Gower-2. To generate these animals, complex transgenic knockout mice expressing 100% human Hb S ("sickle" mice) (25) were mated with independently generated "gower" mice (12) that were heterozygous for deletion of their endogenous murine (m) α -globin genes (genotype $m\alpha^{+/-}$), nullizygous for their endogenous $m\beta$ globin genes (genotype $m\beta^{-/-}$), and carried independent transgenes encoding human α and ϵ globins (Fig. 1A). The globin phenotypes of offspring were established and verified by using a combination of denaturing and nondenaturing electrophoretic methods (26, 27) (Fig. 1B). Doubly nullizygous $m\alpha$ and $m\beta$ animals expressing human Hb S, with or without human Hb Gower-2 ("sickle-gower" and "sickle" mice, respectively), were either studied directly or interbred to generate additional mice for analysis. In contrast to sickle females, sickle-gower females carried pregnancies to term and effectively nurtured their offspring, facilitating rapid generation of phenotypically desirable mice for study.

Sickle Erythrocytes Are Not Detected in Blood from Sickle-Gower Mice. All clinically significant sickle syndromes—including sickle cell disease, sickle- β thalassemia, and hemoglobin SC disease—are characterized by the presence of sickled cells (drepanocytes) in the peripheral blood. The effect of Hb Gower-2 on the morphology of sickle erythrocytes was assessed by comparing Wright-stained peripheral blood smears prepared from sickle and sickle-gower mice (Fig. 2). The abundance of drepanocytes observed in smears from sickle mice contrasted sharply with the complete absence of these cells in smears prepared from sickle-gower animals. Peripheral smears from mice with both phenotypes displayed a mild anisopoikilocytosis consistent with an imbalance in the expression of α - and non- α globin chains. As the sickle phenotype in humans is invariably predicted by the presence of drepanocytes, and only slightly altered by globin chain imbalance, the observed difference in erythrocyte morphologies indicated that the physiological benefit of Hb Gower-2 *in vivo* derives primarily from its antisickling properties.

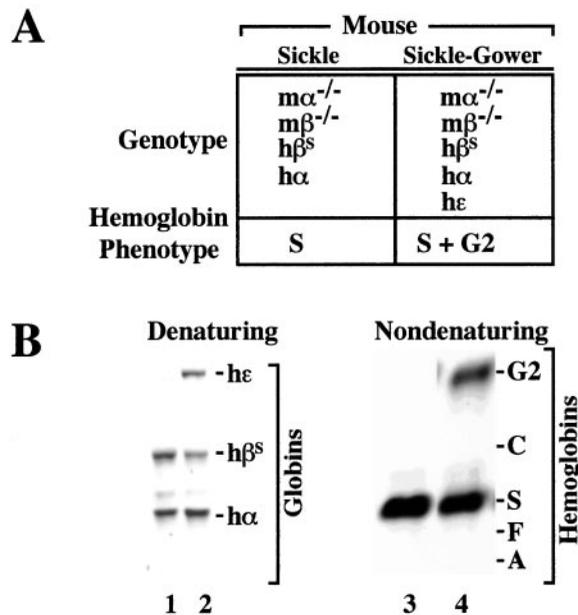


Fig. 1. Generation of mice expressing human Hb S with or without Hb Gower-2. (A) Genotypes and hemoglobin phenotypes of selected mice used in this study. m, mouse; h, human; $-/-$, nullizygous; $\beta^S = \beta^{\text{Glu} \rightarrow \text{Val}}$; S = Hb $\alpha_2\beta_2^S$; G2 = Hb $\alpha_2\epsilon_2$. (B) Electrophoretic analysis of globin phenotypes. Hemolysate from sickle (lanes 1 and 3), and sickle-gower mice (lanes 2 and 4) was resolved by denaturing Triton-acid-urea gel electrophoresis (Left) and nondenaturing cellulose acetate electrophoresis (Right). The positions of individual denatured globins and nondenatured hemoglobin heterotetramers are indicated to the right of the two gels, respectively.

Coexpression of Human Hb Gower-2 Corrects the Hemolytic Anemia Characterizing Sickle Mice. The chronic hemolysis of sickle cell disease in humans reflects accelerated clearance of morphologically and functionally abnormal erythrocytes both in the bone marrow and in the peripheral blood. To define the effect of Hb Gower-2 on this process, we performed complete blood counts on nine sickle mice and seven sickle-gower mice (Fig. 3A). By comparison to sickle mice, sickle-gower animals exhibited a 2-fold increase in hemoglobin levels, as well as more modest elevations in red cell count, hematocrit, and mean corpuscular hemoglobin. Of interest, the mean corpuscular volume was nearly identical in both sickle and sickle-gower mice, confirming that the benefit of Hb Gower-2 in sickle cell anemia relates to its antisickling properties and not to secondary effects on globin chain balance. The beneficial effect of Hb Gower-2 on the survival of sickle erythrocytes was subsequently assessed by using serum bilirubin as an indicator of ineffective erythropoiesis and peripheral hemolysis (Fig. 3B). Sickle mice displayed significantly elevated bilirubin levels consistent with accelerated erythrocyte destruction. By comparison, bilirubin levels in

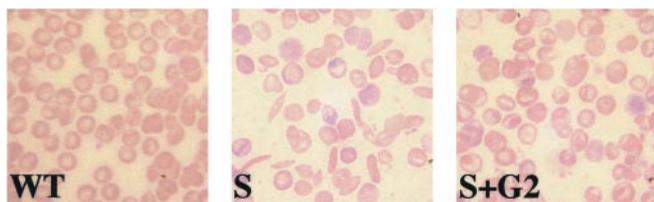


Fig. 2. Peripheral blood smears from sickle mice that coexpress Hb Gower-2 do not contain drepanocytes. Wright-stained peripheral blood smears from wild type (WT), sickle (S), and sickle-gower (S+G2) mice are illustrated. (Original magnification $\times 1,000$.)

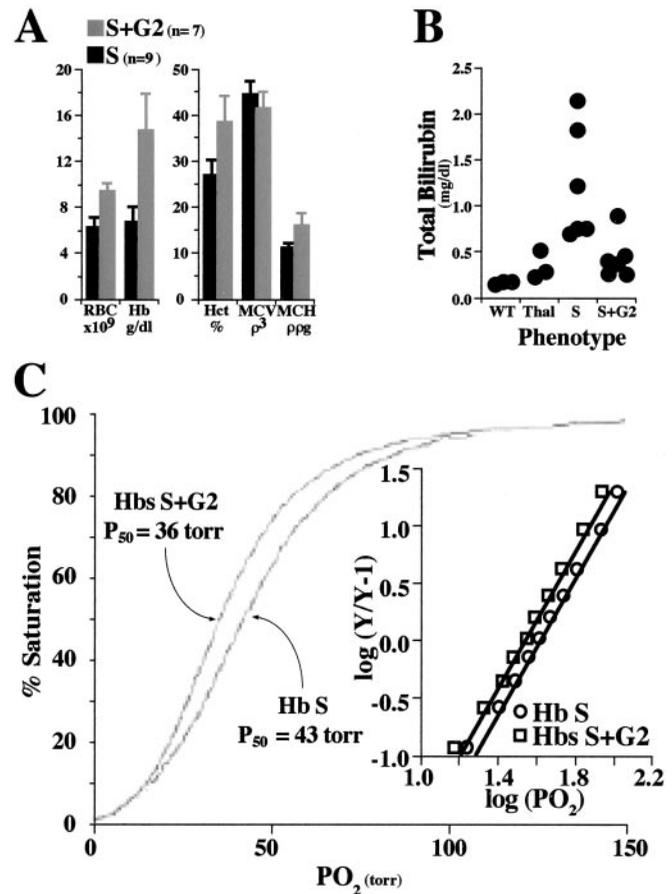


Fig. 3. Hb Gower-2 corrects the anemia in sickle mice. (A) Normalization of blood counts in sickle-gower mice. Complete blood counts were performed on 9 sickle (S; black bars) and 7 sickle-gower animals (S+G2; gray bars); the average \pm SD value for each hematological parameter is displayed. RBC, red cell count; Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin. (B) Coexpression of Hb Gower-2 corrects the hyperbilirubinemia of sickle hemolysis. Total bilirubin was determined in serum prepared from 6 sickle (S), and 7 sickle-gower mice (S+G2). Serum from 3 wild-type mice (WT) and 3 mice containing heterozygous knockouts of their endogenous β -globin genes (Thal) were studied in parallel as controls. (C) Intact erythrocytes from sickle-gower mice exhibit elevated O_2 affinity. Oxygen equilibrium curves for intact cells from sickle (S) and sickle-gower (S+G2) mice are illustrated, along with calculated P_{50} values (36 and 43 torr, respectively; 1 torr = 33 Pa). Hill plots of the data (Inset) indicate an n of 2.9 for intact cells from both animals.

sickle-gower mice corrected to levels observed in β -thalassemic animals, verifying that the hematological benefit of Hb Gower-2 derives primarily from its mitigating effect on sickle hemolysis.

As Hb Gower-2 displays an elevated O_2 affinity in solution (12), we also considered the possibility that Hb Gower-2 might elevate hemoglobin levels in sickle-gower mice through its effects on tissue O_2 delivery. Consequently, we studied the O_2 -binding characteristics of intact erythrocytes obtained from sickle and sickle-gower mice (Fig. 3C). Oxygen equilibrium curves constructed from these studies indicated that sickle-gower erythrocytes bound O_2 more tightly than erythrocytes containing only Hb S (P_{50} s of 36 and 43, respectively). Results from Hill analyses were also consistent with previous *in vitro* studies (12), indicating that the identity of the β -like globin has little effect on hemoglobin subunit cooperativity in intact cells ($n = 2.9$ for cells from mice with both hemoglobin phenotypes). The *in vivo* data suggest that the high hemoglobin levels in

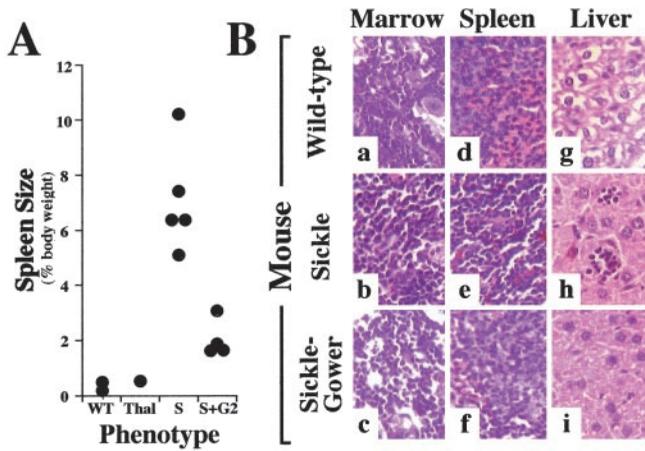


Fig. 4. Resolution of compensatory erythropoiesis in sickle-gower mice. (A) Coexpression of Hb Gower-2 reverses the splenomegaly characterizing sickle mice. The spleen sizes from adult wild-type ($n = 2$), thalassemic ($n = 1$), sickle ($n = 5$), and sickle-gower mice ($n = 4$) are displayed as a percentage of total body weight. (B) Coexpression of Hb Gower-2 reverses the extramedullary erythropoiesis present in sickle mice. Representative fields from hematoxylin/eosin-stained sections prepared from marrow, spleen, and liver of wild-type, sickle, and sickle-gower mice. Foci of extramedullary erythropoiesis and hemosiderin stained phagocytic cells are observed in *h*. (Original magnification $\times 400$.)

sickle-gower mice result in part from a physiological response to the elevated affinity of O_2 for Hb Gower-2.

Extramedullary Hematopoiesis Normalizes in Sickle-Gower Mice. The chronic hemolysis characterizing sickle cell disease effects a compensatory erythropoiesis in both humans and mice (1, 25, 30). The prediction that the mitigating effects of Hb Gower-2 on sickle hemolysis might reverse the accompanying bone marrow erythroid hyperplasia and extramedullary hematopoiesis was subsequently assessed by examination of marrow, spleen, and liver tissue from adult sickle, sickle-gower, and control animals (Fig. 4). Splenic hematopoiesis is not observed in humans with sickle cell disease, because of splenic autoinfarction (1), but is a prominent feature of sickle cell mouse models (25, 30). The splenomegaly present in sickle mice was not observed in sickle-gower animals, suggesting a parallel reduction in extramedullary erythropoiesis as well (Fig. 4A). This hypothesis was supported by subsequent histopathological examination of hematopoietic tissues from these animals. Bone marrow from sickle animals displayed a striking erythroid hyperplasia that was largely normalized in sickle-gower animals (Fig. 4B *a-c*). In addition, normal splenic architecture, effaced in sickle spleens by an infiltrate largely comprising erythroid progenitors, was partially restored in sickle-gower animals (Fig. 4B *d-f*). Liver sections from sickle mice demonstrated abundant, large foci of extramedullary erythropoiesis well as numerous hemosiderin-stained cells (Fig. 4B*h*), whereas similar foci were rarely observed in liver sections from sickle-gower mice (Fig. 4B*i*). These histopathological findings reflect a compensatory response to chronic hemolysis in sickle mice that is physiologically unnecessary in sickle mice that coexpress Hb Gower-2.

Coexpression of Hb Gower-2 Reverses Pathological Histology in Sickle Mice. The high-osmolality, low- O_2 renal environment favors polymerization of deoxygenated cellular Hb S, resulting in characteristic renal histopathological and functional defects in both humans and mice. Analysis of renal histopathology and function in sickle ($n = 5$), sickle-gower ($n = 4$), and control animals ($n = 2$) revealed that sickle-related renal damage was prevented by coexpression of Hb Gower-2 (Fig. 5). Kidneys from sickle mice displayed an interstitial inflammatory infiltrate (Fig.

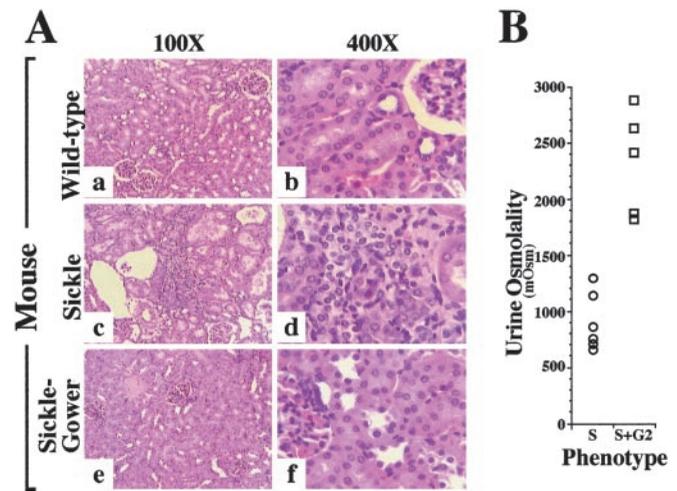


Fig. 5. Hb Gower-2 reverses functional renal defects present in sickle mice. (A) Comparison of renal histopathology. Illustrative low- and high-power fields ($100\times$ and $400\times$, respectively) from hematoxylin/eosin-stained sections prepared from kidneys of wild-type (WT), sickle (S), and sickle-gower (S+G2) mice. (B) Coexpression of Hb Gower-2 reverses the isosthenuria characterizing sickle mice. The osmolalities of urine collected from 6 sickle (S; \circ) and 5 sickle-gower mice (S+G2; \square) are displayed.

5A *c* and *d*) that was not present in sections from wild-type or sickle-gower animals (Fig. 5A *a* and *b*, and *e* and *f*, respectively). We also assessed the corresponding functional defect in urine concentrating ability (isosthenuria) known to affect humans with sickle cell anemia (1) as well as mouse models of this disease (25, 30). Urine from sickle-gower mice ($n = 6$) contained 2- to 3-fold higher solute levels than urine from sickle controls ($n = 5$), demonstrating the beneficial effects of Hb Gower-2 on renal function (Fig. 5B). The difference in urine concentrating ability was maintained after a 6-h period of water deprivation (not shown). Hence, concordance between histopathological and functional findings indicates that a substantial physiological benefit accrues to sickle mice that coexpress Hb Gower-2.

Detailed histopathological evaluation of other tissues (adrenal, bone, brain, esophagus, eye, gall bladder, heart, large and small intestine, lymph nodes, skeletal muscle, nerve, ovaries, pancreas, parathyroid, skin, stomach, thymus, thyroid, trachea, and uterus) did not reveal any consistent findings that could be linked to the relevant globin phenotype. The single exception was that lung sections from sickle animals exhibited an erythrocytic infiltrate in the alveolar walls, whereas lung sections prepared from sickle-gower animals more closely resembled sections from control mice (not shown). The significance of this observation is unclear, although it is tempting to speculate on a potential link to acute and chronic pulmonary disorders that develop in individuals with sickle cell disease (31, 32).

Hb Gower-2 Displays Antipolymerization Activity *in Vitro*. The capacity of Hb Gower-2 to reverse the sickle phenotype in transgenic mouse models predicts its ability to slow polymerization of deoxygenated Hb S heterotetramers in intact erythrocytes. This process is reproduced *in vitro* by using a method in which solutions containing defined quantities of deoxygenated hemoglobins are monitored for the onset of hemoglobin nucleation, the initiating step in the polymerization process (33) (Fig. 6). The time-to-nucleation (delay time) of solutions containing Hb S was significantly prolonged by the presence of Hb Gower-2, consistent with the preceding *in vivo* studies. Surprisingly, the antipolymerization characteristics of Hb Gower-2 were less pronounced than those of Hb F, and nearly identical to those of

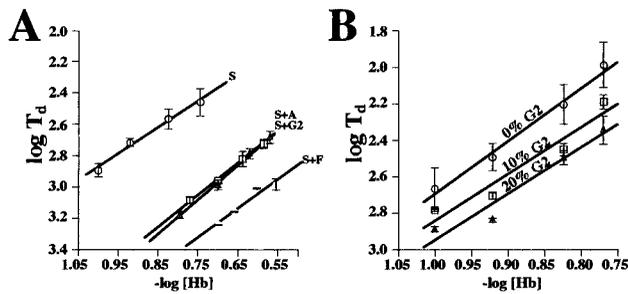


Fig. 6. Hb Gower-2 exhibits potent antipolymerization activity *in vitro*. (A) Hb Gower-2 prolongs the delay time of hemoglobin mixtures containing Hb S. The delay times of deoxygenated solutions containing human Hb S alone (open circles), or Hb S in equal proportions with either Hb Gower-2 (open squares), Hb A (open triangles), or Hb F (filled squares) are plotted as a function of total hemoglobin concentration. Each point represents the mean \pm SD of three independent measurements. (B) Low proportions of Hb Gower-2 significantly delay polymerization of Hb S solutions. The delay times of deoxygenated solutions containing 100% human Hb S (circles), 90% Hb S and 10% Hb Gower-2 (squares), or 80% Hb S and 20% Hb Gower-2 (triangles) are plotted as a function of total hemoglobin concentration. Each point represents the mean \pm SD of three independent measurements.

Hb A (Fig. 6A). Nevertheless, hemoglobin mixtures containing as little as 10% Hb Gower-2 displayed a clear increase in delay time (Fig. 6B). Together, these data indicate that small proportions of Hb Gower-2 can significantly delay nucleation of Hb S-containing solutions, and suggest a molecular basis for the beneficial role of Hb Gower-2 observed in sickle mouse models.

Discussion

Although the ontogenic basis for conservation of globin gene switching is a matter of conjecture, the principle has been established that developmentally silenced globin genes can be successfully reactivated in adults (8, 11, 34). The therapeutic benefit of this process to individuals with defects in β -globin expression requires that the reactivated gene is structurally intact and that its encoded globin protein assembles into hemoglobin heterotetramers with physiologically desirable properties. The functional adaptability of the γ -globin genes has been clearly demonstrated by naturally occurring HPFH mutations that result in its persistent expression in adult erythrocytes (6). In contrast, the absence of similarly functioning mutations up-regulating ϵ -globin expression in adults has limited parallel studies of the physiological suitability of ϵ -globin subunits in adults. Recent studies have indicated that the O_2 -transporting capacity of Hb Gower-2, although different from that of Hb A in several important respects, do not limit its application as a substitute hemoglobin in adults with thalassemia (12). In contrast, the theoretical capacity of Hb Gower-2 to prevent the molecular pathology characterizing sickle erythrocytes has not been previously addressed.

The current manuscript demonstrates that mice expressing human Hb S clearly benefit from coexpression of human embryonic ϵ globin. Sick mice carrying a transcriptionally active ϵ -globin transgene exhibit a correction in red cell morphology (Fig. 2), a reduction in hemolysis (Fig. 3), and resolution of the compensatory marrow and extramedullary erythropoiesis (Fig. 4). Moreover, sickle mice that coexpress Hb Gower-2 display histopathological (Fig. 5A) and functional resolution of sentinel end-organ damage (Fig. 5B), indicating a benefit to the organism as a whole. These physiological observations are consistent with *in vitro* demonstrations of antipolymerization activity for Hb Gower-2 (Fig. 6).

As reactivation of developmentally silenced β -like globin genes would be anticipated to skew the balance of α and non- α globins in favor of the latter, the current model may be an excellent one for anticipating the consequences of reactivated

ϵ -globin expression in humans with sickle cell disease. A hypothetical concern of this approach relates to the physiological consequences of expressing Hb Gower-2, which demonstrates an increase in O_2 affinity both *in vitro* (12) as well as in intact erythrocytes (Fig. 3C). This property might be anticipated to result in a relative or absolute polycythemia, which could have adverse rheological consequences on circulating erythrocytes containing high levels of Hb S. Consistent with this hypothesis, mice coexpressing Hb Gower-2 exhibited unusually high hemoglobin levels (Fig. 3A), although they appeared generally healthy and did not exhibit overt histological pathology or functional deficits. This observation parallels the experience in humans with sickle cell disease who express significant levels of high-affinity Hb F but do not exhibit a physiology that limits the therapeutic application of γ -globin inducing agents. From this perspective it appears that the benefits of Hb Gower-2 in sickle cell disease have been clearly demonstrated, whereas its potential toxicities remain largely hypothetical.

The antipolymerization characteristics of Hb Gower-2 are likely to arise from the interruption of a hydrophobic domain by a Lys residue at ϵ -globin position 87. The absence of this feature (which is present in Hb S and Hb A) is hypothesized to result in the exclusion of Hb $\alpha_2\epsilon\beta^S$ heterotetramers from the deoxygenated Hb S polymer, resulting in net slowing of the polymerization process. The importance of the hydrophobic domain to the sickling process has been clearly demonstrated by *in vitro* analyses of human β -globin variants containing informative mutations at this site (23). Consequently, we were surprised that the antipolymerization potential of Hb Gower-2 was not as substantial as we had initially anticipated. A possible explanation for our results is that other molecular interactions (e.g., the $^{73}\beta_1^{Asp-4}\beta_2^{Thr}$ lateral interaction; ref. 24), may be more important to stabilizing filaments pairs than previously recognized. It is also formally possible that the antisickling effects of ϵ - and γ -globin subunits are mediated by domains that are independent of the hydrophobic acceptor pocket. A consequence of this possibility is that reactivated expression of both of the silenced globins would result in unpredictable, but potentially synergistic antisickling activity. Our attempts to investigate this possibility *in vitro* have not indicated any such effect (not shown), although conventional *in vitro* delay time methods may be poorly suited for this type of analysis. These considerations emphasize the importance of verifying *in vitro* characteristics in independent *in vivo* cellular or whole-animal models.

Finally, the demonstration that ϵ globin can serve an important therapeutic role in β thalassemia and sickle cell anemia raises the issue of the method through which expression of the cognate developmentally silenced gene might be reactivated. *A priori*, it is likely that the ϵ - and γ -globin genes are independently regulated, as they are not coexpressed at significant levels during normal development (15). Functional studies indicate that developmental control of ϵ -globin expression is gene-autonomous (35), a claim supported by the identification of ϵ -globin gene-specific positive and negative transcriptional regulatory elements (36–38). Defining the gene-specific mechanism through which the ϵ -globin expression is silenced may have profound clinical consequences, as pharmacological reactivation of the γ - and ϵ -globin genes would likely permit the use of functionally independent agents with nonoverlapping toxicities. It is particularly important to recognize that its antisickling effect requires low levels of ϵ -globin protein, so that moderate success at reactivating its expression would likely have substantial mitigating effects on the sickle phenotype in humans.

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