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## Interaction of deoxyhemoglobin with the cytoplasmic domain of murine erythrocyte band 3<sup>†</sup>

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### Abstract

The partial pressure of oxygen constitutes an important factor in the regulation of human erythrocyte physiology, including control of cell volume, membrane structure, and glucose metabolism. Because band 3 is thought to be involved in all three processes, and since hemoglobin (Hb) binding to the cytoplasmic domain of band 3 (cdb3) is strongly oxygen-dependent, the possibility arises that the reversible association of deoxyhemoglobin (deoxyHb) with cdb3 might constitute an O<sub>2</sub>-dependent sensor that mediates O<sub>2</sub>-regulated changes in erythrocyte properties. While several lines of evidence support this hypothesis, a major opposing argument lies in the fact that the deoxyHb binding sequence on human cdb3 is not conserved. Moreover, no effect of O<sub>2</sub> pressure on Hb-band 3 interactions has ever been demonstrated in another species. To explore whether band 3-Hb interactions might be widely involved in O<sub>2</sub>-dependent regulation of erythrocyte physiology, we undertook to characterize the effect of O<sub>2</sub> on band3-Hb interactions in the mouse. We report here that murine band 3 binds deoxyHb with significantly greater affinity than oxyHb, despite the lack of significant homology within the deoxyHb binding sequence. We further map the deoxyHb binding site on murine band 3 and show that deletion of the site eliminates deoxyHb binding. Finally, we identify mutations in murine cdb3 that either enhance or eliminate its affinity for murine deoxyHb. These data demonstrate that despite a lack of homology in the sequences of both murine band 3 and murine Hb, a strong oxygen-dependent association of the two proteins has been conserved.

Considerable evidence exists to demonstrate that multiple erythrocyte properties are regulated by the partial pressure of oxygen to which the red cells are exposed. Among the functions thought to be controlled by O<sub>2</sub> levels are glucose metabolism, cell volume and hydration, and membrane structure (1–5). Erythrocyte glucose consumption occurs primarily via glycolysis in deoxygenated cells, but upon exposure to O<sub>2</sub> a considerable fraction of the cell's glucose is channeled into the pentose phosphate pathway (6–8). The importance of this regulatory switch has been argued to lie in the heightened need for reductants during periods of elevated exposure to O<sub>2</sub> to protect the cell against oxidative stress (2, 9). Thus, by activating the pentose phosphate pathway upon erythrocyte oxygenation, the cell is assured of sufficient NADPH for both glutathione reduction and maintenance of Hb in its reduced state.

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SUPPORTING INFORMATION AVAILABLE. SDS-PAGE and CD analysis show that mouse cdb3 and mutant proteins are pure and folded correctly. Also, the raw spectra of fluorescence quenching by deoxyHb are shown in supplemental figure 3. This material is available free of charge via the Internet at <http://pubs.acs.org>.

What is the evidence for band 3-deoxyHb interactions in this regulation? Data from other labs and our own show that the glycolytic enzymes bind avidly to the NH<sub>2</sub>-terminus of band 3 (10–14). These data also demonstrate that deoxyHb (but not oxyHb) competes avidly for this enzyme binding site on human band 3 (3), and that upon red cell deoxygenation, the vast excess of deoxygenated Hb competitively displaces glycolytic enzymes from the membrane (15–16). Because the catalytic properties of the glycolytic enzymes are significantly altered upon association with band 3 (13, 17–19), reversible displacement of these enzymes by deoxyHb can explain the O<sub>2</sub>-dependent switch in red cell metabolism. However, as noted above, the lack of homology between the deoxyHb binding site on human and other mammalian band 3 orthologs raises questions about the validity of the proposed regulatory mechanism.

Evidence for the role of band 3-deoxyHb interactions in regulation of red cell volume/hydration by oxygen pressure is also mounting. To facilitate volume modulation during transit through regions of hypotonic/hypertonic stress, erythrocytes are equipped with an array of cotransporters that can reverse either cell swelling or cell shrinkage upon activation (20–22). Importantly, the K<sup>+</sup>/Cl<sup>−</sup> cotransporter (KCC) in human erythrocytes increases in activity ~20-fold during erythrocyte oxygenation (23). Moreover, this O<sub>2</sub>-dependent regulation occurs only in whole cells and Hb-containing ghosts, but not in white ghosts or whole cells treated with CO to block O<sub>2</sub> binding (24–25). Together with data showing a sigmoidal dependence of K<sup>+</sup>/Cl<sup>−</sup> cotransport on O<sub>2</sub> pressure (i.e. similar to the sigmoidal dependence of Hb saturation on O<sub>2</sub> pressure), the results suggest that Hb must participate in the O<sub>2</sub>-dependent switch in KCC activity (22, 26). Because band 3 constitutes the only established binding site for deoxyHb on the membrane (3), participation of band 3 in the O<sub>2</sub>-triggered KCC regulation has frequently been proposed (21–22, 27). Similarly, an O<sub>2</sub>-dependent change in sickle cell cation transport (termed P<sub>sickle</sub>) has been observed, as have O<sub>2</sub>-triggered changes in the activities of the Na<sup>+</sup>/K<sup>+</sup>/2Cl<sup>−</sup> cotransporter and the Na<sup>+</sup>/H<sup>+</sup> antiporter (28–30). However, once again, the absence of homology in the critical band 3-deoxyHb binding site casts doubt on the universality of the participation of band 3 in the proposed mechanism.

Finally, evidence is emerging that human erythrocytes might also modulate their membrane structural properties in response to changes in O<sub>2</sub> tension. During their ~120 day lifespan, red blood cells continuously squeeze through capillaries or sinusoids which are less than half their cell diameters. Their ability to recover their biconcave shape following exit from these depends at least in part on interactions between the plasma membrane and its underlying spectrin-based membrane skeleton (31). Importantly, band 3 constitutes a major anchor for the spectrin skeleton on the membrane and ankyrin performs the major bridging function that connects band 3 to spectrin (32). Since the band 3-ankyrin interaction has recently been shown to be O<sub>2</sub>-sensitive (Stefanovic M. and Low P. unpublished data), the possibility arises that displacement of ankyrin from band 3 by deoxyHb might serve as an O<sub>2</sub> switch that can confer O<sub>2</sub> sensitivity on red cell rheology.

Figure 1A shows the sequence on human band 3 that was previously demonstrated to bind human deoxyHb (boxed residues). Not only does deletion of this sequence abrogate deoxyHb binding to human band 3, but mutations on either side of this sequence have also been found to elevate the affinity of deoxyHb for human band 3 so dramatically that deoxyHb remains bound even at supraphysiological O<sub>2</sub> pressures (3). Thus, the deoxyHb binding site appears to enable the reversible O<sub>2</sub>-dependent association of band 3 and deoxyHb precisely over the physiological range of O<sub>2</sub> pressures. To explore whether this O<sub>2</sub>-dependent binding function might have been maintained in other mammalian species despite obvious differences in primary structure, we have examined whether murine band 3 might display preferential affinity for murine oxyHb or deoxyHb. We have also mutated

many of the residues within the NH<sub>2</sub>-terminus of murine band 3 to determine whether murine Hb affinity might be affected. We report here that a nonhomologous sequence near the NH<sub>2</sub>-terminus of murine band 3 selectively binds murine deoxyHb and that mutations within and adjacent to this sequence generate band 3 mutants with either high or very low affinity for mouse deoxyHb.

## EXPERIMENTAL PROCEDURES

### Materials

Protease inhibitors were purchased from Research Products International Corp. All protein concentration steps were performed by ultrafiltration using Vivaspin tubes from GE Healthcare Life Sciences. Dialysis was performed with dialysis bags from Spectrum Laboratories Inc. Protein concentration was measured with MicroBCA protein assay from Thermo Scientific. All others materials and reagents were purchased from SIGMA Aldrich.

### GFP fusion constructs

One assay for measuring the affinity of murine deoxyHb for murine band 3 involved preparation of a fusion protein between the cytoplasmic domain of band 3 (cdb3) and GFP, and measuring the quenching of this fusion construct upon its association with Hb. The GFP fusion vector employed was a generous gift from Dr. David Thompson, Purdue University. GFP, containing a histidine tag (His<sub>8</sub>) tag attached to its own COOH-terminus was fused to the COOH-terminus of wild type and mutant cdb3 proteins. Murine cdb3 DNA (encoding amino acids 1 to 398) was PCR-amplified using a forward primer containing an NdeI cleavage site followed by a start codon (5'-cat atg ggg gac atg cgg gac cac-3') and kidney cdb3 (lacking first 79 amino acids) using this forward primer 5'-gcg cat atg gac cag agg aac cag-3'; the reverse primer was: 5'-ctc gag aaa gat ccg gcc tgt gcg-3'. Because the GFP gene has an additional NdeI cleavage site in its sequence, we mutated this site with conservative substitutions, yielding a final construct containing only the single NdeI site in the multiple cloning site. The wild type and kidney murine cdb3 sequences were fused to the NH<sub>2</sub>-terminus of GFP proteins between the NdeI and XhoI enzymatic sites. The amplified DNA product was first ligated into a pGEM-T easy vector (Promega) and then excised with NdeI and XhoI from the pGEM-T. After ligating the product into the GFP-fusion vector, the final construct was sequenced to assure the absence of unwanted mutations.

Wild-type murine cdb3 cDNA lacking GFP and His tags was prepared by PCR amplification using the same parent cDNA described above and inserted in pGEM-T vector. After digestion with NdeI and HindIII, the sequence was inserted into a pT-7.7 vector for protein expression.

Site-directed mutagenesis. All mutated proteins were prepared using a QuickChange® mutagenesis kit (Stratagene) following the manufacturer's instructions. The sense primers used in this study were as follows: for deletion of residues 1-51 (del 1-51): 5'-gga gat ata cat atg aca gcc aca gac tac gtc-3'; for deletion of residues 1-38 (del1-38): 5'-ggg tcc tgc atc tcg gtc ata tgt ata tct cc-3'; for deletion of residues 1-21 (del 1-21): 5'-gga gat ata cat atg ctg gag aac ata ata gga cag ata gc-3'; for deletion of residues 1-10: 5'-gat ata cat atg ctg gag atc ccag-3'; for deletion of residues 19-23: 5'-gat cga gac agc aac ata ata ggac-3'; for deletion of residues 21-33: 5'-cga gac agc gaa gaa cta acc atcc-3'; for deletion of residues 39-66: 5'-cta acc atc cct gt g agc tcc ggt ca-3'; for substitution of residues 19-23: 5'-cca gat cga gac agc caa caa caa tcg cag aac ata ata gga cag-3'; for substitution of residues 12-23: 5'-gga agt gct gca gat ccc aaa tcg aaa cag cca aca ac-3'; for substitution of residues 25-31: 5'-gaa ctg gag aac gaa gaa gga cag ata gaa gat aga gac cta acc-3'. The 12-23 substitution mutant was prepared using the 19-23 substitution mutant DNA as the template. All other constructs were made

using the wild-type cdb3–GFP fusion DNA as a template. Sequences of the final products were all confirmed by DNA sequencing.

### Expression and purification of proteins

GFP fusion proteins were expressed using BL21(DE3)pLysS bacteria strains (Invitrogen) in 2XYT media at 28°C for 2 to 3 hours. The collected pellet was stored overnight at –80°C and then thawed in lysis buffer (20mM NaH<sub>2</sub>PO<sub>4</sub>, 500mM NaCl, 15mM imidazole, 1 μM AEBSF, pH 7.5) at room temperature and lysed using a French press (SLM-Aminco). The bacterial lysate was clarified by centrifugation at 17,000xg on a Sorval SS-35 rotor for 30 minutes, and the supernatant was filtered and loaded onto a nickel-affinity column equilibrated in lysis buffer (GE Healthcare Life Sciences). The column was first washed with lysis buffer containing 40mM imidazole and then eluted with the same buffer containing 250mM imidazole.

Proteins lacking GFP fusion constructs and affinity tags were expressed in BL21(DE3)pLysS bacteria (Invitrogen) in 2XYT media at 29°C for 3 to 4 hours. Bacterial pellets were lysed in 20mM Tris, 1mM EDTA, 0.2% Triton X-100, 0.2% β-mercaptoethanol, pH 7, and wild type and mutated cdb3 proteins were purified by anion exchange chromatography and hydrophobic column chromatography, as described previously (3).

### Hemoglobin purification and handling

Hemoglobin for all cdb3-GFP binding studies was obtained from erythrocytes of C57BL/6J mice, as described previously (33). Hemoglobin from C57BL/6 mice is highly homogeneous and does not polymerize when stored as other murine hemoglobins do (34). When larger quantities of blood were required for the HEMOX analyses, hemoglobins from C57BL/6J and BALB/cJ were mixed in a 1.2:1 ratio prior to analysis.

For Hb isolation, whole blood was pelleted and the buffy-coat and plasma were removed by repeated washing in PBS (137mM NaCl, 2.7mM KCl, 8.1mM NaH<sub>2</sub>PO<sub>4</sub>, and 1.5mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4). The packed red blood cells were stored at 4°C for one week to begin depletion of 2, 3- diphosphoglycerate (2, 3-DPG), after which the cells were lysed in 5 volumes of cold water and spun at 20,000xg. To remove any residual 2,3-DPG, the supernatant was first dialyzed for 12h in 1M NaCl, then 0.1M NaCl, and finally in 10mM BisTris acetate, pH 6.5. Hemoglobin concentration was determined by measuring absorbance at 540nm using an extinction coefficient of 14.17 (35). Deoxygenation was performed by flushing the Hb solution with humidified argon gas until the change in absorption spectrum confirmed deoxygenation of the sample.

### Fluorescence resonance energy transfer analysis of deoxyHb affinity for band 3

All fluorescence measurements were obtained on an AMINCO-Bowman luminescence spectrometer. The excitation wavelength for analysis of cdb3-GFP fusion protein solutions was set at 386nm and the emission spectrum was scanned from 470 to 550 nm. Both murine cdb3 mutants (05μM) and hemoglobin (1 μM) were dialyzed against 10mM BisTris acetate buffer, pH 6.5 at room temperature before analysis of binding. Samples were incubated for 5 min at room temperature prior to measurement of the GFP quenching by any bound oxyHb. The emission spectrum of a fully oxygenated solution of cdb3-GFP and Hb was recorded first, after which the solution was deoxygenated and the emission spectrum recorded again. In most cases, reversibility of the process was confirmed by re-oxygenating the sample. In addition, a control sample containing pure GFP (not fused to cdb3), was analyzed to allow subtraction of the inner filter effect using the following formula:

$$\%Hb\ quenching = \left\{ 1 - \frac{F_{protein+dHb}}{F_{gfp+dHb}} \right\} \times 100$$

where F is the fluorescence of the GFP fused protein or pure GFP in the presence of deoxyHb.

### Equilibrium binding analysis of deoxyHb affinity for band 3

To measure the equilibrium dissociation constant for the interaction of deoxyHb with different mutated forms of cdb3-GFP, increasing concentrations of deoxyHb were incubated for 45 min at room temperature with cdb3-GFP (or one of its mutants) in BisTris acetate buffer, after which the intensity of GFP fluorescence was measured as described above. Fluorescence quenching was then calculated using the above equation on three separate replicates at each deoxyHb concentration (on two different days), and the mean and standard deviations were determined at each deoxyHb concentration. GraphPad Prism software version 4.00 for Windows was then used to fit the plot of fluorescence quenching as a function of deoxyHb concentration to a simple single site noncooperative binding curve and calculate the equilibrium dissociation constant.

### Analysis of deoxyHb affinity for band 3 by measurement of oxygen dissociation curves

Preferential binding of deoxyHb over oxyHb to cdb3 can be assessed by measuring the shift in the oxygen dissociation curve of Hb in the presence of cdb3. For this purpose, hemoglobin and wild-type or mutant cdb3 were dialyzed against 10 mM BisTris acetate buffer, pH 6.5, and twenty nmol of Hb were incubated with 10 nmol cdb3 for 1 min at 37°C. 20 µl of 20% BSA solution) and 10 µl antifoam solutions (TCS, Southampton, PA) were then added to the cdb3/Hb solution and oxygen-Hb dissociation curves were generated on a HEMOX analyzer (TCS) according to manufacturer's instructions. The effect of cdb3 on Hb affinity for oxygen is reported as a shift in the P<sub>50</sub> value for Hb.

### Data analysis

All average results are presented as mean ± standard error. The graphs were made using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

## RESULTS

### Murine deoxyHb exhibits higher affinity for murine cdb3 than murine oxyHb

As discussed in the Introduction, modulation of cdb3-hemoglobin interactions by O<sub>2</sub> has been proposed to account for oxygen control of several erythrocyte properties, including cell hydration, glucose metabolism and membrane structure (1–3). However, since the sequence on human band 3 responsible for Hb binding (i.e., the NH<sub>2</sub>-terminus of band 3) is not highly conserved (see Fig. 1A), the question naturally arose whether the deoxyHb-band 3 interaction could constitute the O<sub>2</sub> sensor that would mediate O<sub>2</sub> effects on erythrocyte physiology across species. To directly test this hypothesis and based on our crystallographic data (36) that showed both termini N- and C-terminus in very close proximity, we chose to generate a fusion protein between the COOH-terminus of murine cdb3 and GFP, and examine the binding of oxyHb and deoxyHb to this fusion construct by quantitating the quenching of GFP fluorescence. As seen in Fig. 2A, murine Hb displays O<sub>2</sub>-dependent binding to murine cdb3, with deoxyHb exhibiting higher affinity for cdb3 than oxyHb. As a negative control, the kidney spliceoform of murine cdb3 (kcd3) that lacks the first 79



amino acids including the Hb binding site, displays little affinity for Hb under either oxy- or deoxygenated conditions. These results indicate that O<sub>2</sub> tension constitutes a modulator of cdb3-Hb interactions in the mouse despite the lack of significant homology in the region previously shown to bind Hb in the human.

### **Deoxyhemoglobin binding site on mouse cdb3 is primarily located within the first 21 residues of the anion transporter**

The sequence alignment showed residues 21-33 of murine cdb3 to be most similar to amino acids 12-23 of human cdb3, i.e. the deoxyHb binding site on human cdb3 (see ref. 3, as indicated by the box in Fig. 1A). To test whether residues 21-33 of murine cdb3 indeed comprise the deoxyHb binding site on murine cdb3, these residues were deleted and deoxyHb binding affinity was analyzed by measuring O<sub>2</sub> dissociation curve. As seen in Fig. 2B only a small reduction in deoxyHb binding ensued, suggesting that residues 21-33 of murine cdb3 do not contain the major residues involved in Hb binding.

To begin to identify those residues that might be critical for deoxyHb binding in the mouse, we prepared a series of deletion and substitution mutants near the NH<sub>2</sub>-terminus of murine cdb3 (Fig. 1B) and re-examined each mutant's affinity for deoxyHb. As seen in Fig. 3A (see also the raw data in Fig. S3), deletion of either amino acids 21-33 or 39-66 exerts little effect on GFP fluorescence, suggesting that residues 21 to 66 do not participate significantly in deoxyHb binding. In contrast, a dramatic decrease in fluorescence quenching is seen upon deletion of any NH<sub>2</sub>-terminal fragment containing residues 1-21. These data suggest that the murine deoxyHb binding site resides within the first 21 amino acids of the cdb3 polypeptide (Fig. 3A).

### **The deoxyhemoglobin binding site on murine cdb3 resides between amino acids 12 and 23**

Based on the fact that cdb3-deoxyHb binding is predominantly electrostatic in nature (14, 37), and since the negatively charged amino acids E<sup>19</sup>EYED<sup>23</sup> were shown to play a critical role in human deoxyHb binding to human cdb3 (3), we next decided to explore whether the murine sequence E<sup>19</sup>ELEE<sup>23</sup> might be similarly involved in murine deoxyHb binding. For this purpose, we either deleted the sequence (del19-23) or replaced each acidic residue with its uncharged amide counterpart (subst19-23, Fig. 1B). Moreover, because the first 10 amino acids in murine cdb3 also contain four acidic residues (see Fig. 1B), we also investigated whether deletion of these residues might similarly reduce deoxyHb binding (del 1-10). The results of these studies are presented in Fig. 3B. As shown in the bar graphs, removal or replacement of acidic amino acids between amino acids 19 and 23 (see del19-23, and subst19-23) yields a cdb3 with significantly reduced affinity for deoxyHb, suggesting that negatively charged residues within the 19-23 peptide contribute prominently to deoxyHb binding. Surprisingly, however, deletion of residues 1-10 with their accompanying four acidic residues exerts little effect on deoxyHb affinity (Fig. 3B, del1-10). Taken together, these findings suggest that the binding site of murine deoxyHb on murine cdb3 does not reside within the first 10-mer fragment, but rather is centered in the directly adjacent region comprising residues 19-23 of mouse cdb3.

Because removal or replacement of the four acidic amino acids between residues 19 and 23 resulted in only 60% reduction in deoxyHb binding (Fig. 3B), and since the deoxyHb binding peptide in human band 3 comprised 7-12 amino acids, we elected to determine whether elimination of additional negative charges near the NH<sub>2</sub>-terminus of cdb3 might further reduce deoxyHb affinity. For this purpose, all 7 acidic amino acids between residues 12 and 23 were converted to their corresponding amides (Fig. 1B, subst12-23), and deoxyHb binding was again examined. As seen in Fig. 3C, removal of the aforementioned additional negative charges further lowered cdb3 affinity for deoxyHb to 15% of normal. These data

suggest that the major docking site of murine deoxyHb on murine cdb3 resides primarily between amino acids 12 and 23.

### **Identification of a mutant murine cdb3 with higher affinity for murine deoxyHb than wild type murine cdb3**

The finding that certain mutations in human cdb3 led to a polypeptide with little affinity for deoxyHb, while other mutations generated a band 3 with such high affinity that even supraphysiological pressures of O<sub>2</sub> could not promote its dissociation from deoxyHb (3) suggests that the O<sub>2</sub> dependence of Hb-cdb3 binding occurs precisely over the physiological range of oxygen pressures. To determine whether murine cdb3 might have a similar sensitivity to physiological O<sub>2</sub>, we looked for mutations in cdb3 that might elevate its deoxyHb affinity to a level that would prevent deoxyHb dissociation even at supraphysiological O<sub>2</sub> pressures. Identification of such a mutation would support the contention that O<sub>2</sub> regulation of Hb binding in the mouse was also tuned to occur over physiologically relevant O<sub>2</sub> pressures. Initial attempts to generate a murine cdb3 with elevated deoxyHb affinity were guided by the mutations in human cdb3 that produced the undesirable excessive affinity. Surprisingly, deletion of neither of the murine sequences (del1-10, del39-66), that corresponded with the human sequences (i.e. residues 1-10 or 29-52) whose deletion yielded a cdb3 with extraordinarily high affinity for deoxyHb, generated a murine cdb3 with elevated affinity for deoxyHb. However, guided by the fact that the interaction between cdb3 and deoxyHb is electrostatic in nature, we hypothesized that replacement of some of the amino acids in the sequence 25-31 with negative residues might generate a cdb3 with increased affinity for deoxyHb (Fig. 1B, subst25-31). As seen in Fig. 3C, deoxyHb binding to the subst25-31 mutant yielded the strongest fluorescence quenching yet observed with murine proteins, indicating that addition of these charged residues promotes an unusually high affinity for murine deoxyHb. The apparent absence of a similar sequence in nature suggests that higher cdb3-deoxyHb affinity is not functionally desirable.

### **Confirmation of the deoxyHb binding site on murine cdb3 by analysis of the equilibrium dissociation constant**

Fluorescence resonance energy transfer is strongly dependent on the distance and orientation between the fluorescence donor and the associated quencher. For example, if the distance between donor and quencher were to decrease as a consequence of a mutation in cdb3, an increase in quenching might be expected even in the absence of a change in affinity. To confirm that alterations in donor to quencher distance and/or orientation did not bias the above data on cdb3-deoxyHb relative affinity, we measured the equilibrium dissociation constants of the three most critical forms of cdb3 for murine deoxyHb: namely, i) wild-type cdb3, ii) the cdb3 mutant with the lowest affinity for deoxyHb (subst 12-23), and iii) the cdb3 mutant with the highest affinity for deoxyHb (subst 25-31). As shown in Figure 4, the cdb3 mutant that previously showed no affinity in the GFP quenching assay also showed no affinity in the equilibrium binding study. Moreover, the mutant that displayed the highest affinity for deoxyhemoglobin in the quenching assay also displayed the highest affinity in the equilibrium binding assay, lowering the equilibrium dissociation constant for deoxyhemoglobin from 1.04  $\mu$ M (wild type band 3) to 0.39  $\mu$ M for the mutated band 3. These data confirm that the relative affinities of the three major forms of cdb3 for deoxyHb correspond to their relative extents of quenching upon addition of deoxyHb.

### **Confirmation of the deoxyHb binding site on murine cdb3 by analysis of Hb-oxygen dissociation curves**

To confirm the above deoxyHb binding site analyses, we determined the binding affinity of our various murine cdb3 mutants using a second and independent method. In this method,



the ability of cdb3 to shift the oxygen dissociation curve for Hb to higher O<sub>2</sub> pressures is used as a measure of the affinity of cdb3 for deoxyHb (3). Thus, the greater the shift in the P<sub>50</sub> values of Hb (i.e. the O<sub>2</sub> pressure where Hb is half saturated with oxygen) to higher O<sub>2</sub> pressures, the greater the affinity of the mutant cdb3 for deoxyHb (3, 38). In agreement with results from the FRET assay, the low affinity mutant, subst12-23, did not affect the O<sub>2</sub> dissociation curve of Hb, but rather displayed the same P<sub>50</sub> value as Hb alone (Fig. 5). In contrast, the mutant that exhibited the greatest quenching capacity in the FRET assay also showed the largest right shift of the Hb-O<sub>2</sub> dissociation curve, confirming its exaggerated affinity for cdb3. As anticipated from the FRET data, removal of the first 10 amino acids from the NH<sub>2</sub>-terminus of mouse cdb3 did not induce an elevated affinity for mouse Hb; i.e. in contradistinction to the impact of the same deletion in human cdb3.

## DISCUSSIONS

Considerable effort has been devoted to characterizing the interaction between human deoxyHb and human band 3 (3, 14, 38–40), even to the point of mapping the amino acids involved in binding (3) and solving the crystal structure of a complex of deoxyHb with the NH<sub>2</sub>-terminus of band 3 (38). The results collectively suggest that the NH<sub>2</sub>-terminus of band 3 inserts into the 2, 3-DPG binding cavity in the center of the deoxyHb tetramer, with residues 12-23 of band 3 establishing the major contact with amino acids along the walls of the deoxyHb cleft (41–42). Since exposure to O<sub>2</sub> induces a “T” to “R” state transition that closes the central cationic cavity in deoxyHb, oxygenation of Hb leads to ejection of band 3 from the cavity, causing dissociation of oxyHb from the membrane (38). We and others have hypothesized that this reversible association of deoxyHb with band 3 might directly or indirectly (via an induced conformational change in the anion transporter) alter the functional properties of proteins associated with band 3 (1, 21–22). Because deoxyHb competes directly with glycolytic enzymes (19) and ankyrin (Stefanovic M. and Low P. unpublished data) for binding to cdb3, a mechanism whereby changes in O<sub>2</sub> might modulate both erythrocyte metabolism and membrane structural properties can be readily envisioned. However, an explanation of how deoxyHb binding could regulate K<sup>+</sup>/Cl<sup>−</sup> and Na<sup>+</sup>/K<sup>+</sup>/2Cl<sup>−</sup> cotransport, Na<sup>+</sup>/H<sup>+</sup> antiport, or P<sub>sickle</sub> is much more difficult to perceive. While a variety of pathways can be proposed, we offer the following hypothesis as one that is supported by some experimental data. We propose that binding of deoxyHb to the cytoplasmic domain of band 3 induces a conformational change in the protein’s membrane-spanning domain and that this conformational change is communicated to associated ion transporters via interfacial interactions. Evidence that significant communication occurs between the cytoplasmic and membrane-spanning domains of band 3 derives from studies showing that: i) association of Hb with cdb3 affects stilbene disulfonate binding to an externally exposed site on the membrane-spanning domain of band 3 (43–44), ii) binding of Hb (45) or ankyrin (46) to cdb3, or phosphorylation of cdb3 (47) alters anion transport through the membrane-spanning domain of the polypeptide, and iii) hemichrome binding to cdb3 induces autologous antibody binding to an external epitope on band 3 (48–50). If the conformational changes that mediate these functional alterations in the membrane-spanning domain of band 3 are also “sensed” by associated ion transporters, O<sub>2</sub> regulation of K<sup>+</sup>/Cl<sup>−</sup> and Na<sup>+</sup>/K<sup>+</sup>/2Cl<sup>−</sup> cotransport, Na<sup>+</sup>/H<sup>+</sup> antiport, and P<sub>sickle</sub> might be envisioned.

To evaluate whether the same O<sub>2</sub> regulatory mechanism might apply broadly among mammalian species, we explored whether the O<sub>2</sub>-dependent Hb binding in humans might also occur in mice. Based on the alignment of the NH<sub>2</sub>-termini of human and mouse cdb3s, the predicted murine Hb binding site for deoxyHb was predicted to reside between residues 21 and 33 (Fig. 1A). However, when this sequence was deleted from murine cdb3, deoxyHb was found to display normal affinity for cdb3. In contrast, when residues 12-23 were mutated, Hb affinity was significantly compromised. Thus, although the binding site of Hb

on murine cdb3 differs from that in humans, the ability of physiological changes in O<sub>2</sub> pressure to modulate Hb binding has been conserved. This suggests that O<sub>2</sub> modulation of red cell properties might constitute an important regulatory mechanism that is required for adequate function of mammalian erythrocytes.

Curiously, mutations in human band 3 that generated a cdb3 with abnormally high affinity for deoxyHb were also not conserved in the mouse. Thus, whereas deletion of residues 1-10 or 29-52 in human cdb3 promoted such affinity for deoxyHb that its dissociation could not be induced even at supraphysiological O<sub>2</sub> pressures, mutation of the same residues in the mouse had little effect on deoxyHb binding. In contrast, introduction of negatively charged residues directly adjacent to the Hb binding site on murine cdb3 was found to yield a cdb3 with greatly elevated Hb affinity, as demonstrated by both FRET and oxygen dissociation curve analysis (Fig. 3C and 4, subst25-31). Thus, the murine erythrocyte like the human erythrocyte appears to have a finely tuned O<sub>2</sub> regulatory system that responds to changes in O<sub>2</sub> pressure precisely over the physiological range of O<sub>2</sub> tensions.

Finally, the question naturally arises whether malfunction of this O<sub>2</sub>-triggered switch in erythrocyte properties might have medical consequences. That is, if a pathological condition or mutation were to cause sustained association of deoxyHb with band 3, would an observable “constitutively on” phenotype emerge? While insufficient information currently exists to provide an unequivocal answer to this question, several observations argue that it may ultimately be answered in the affirmative. Thus, a diversity of mutations in the central cavity of deoxyHb, where acidic amino acids are replaced with neutral or cationic residues, have been reported to lead to an activation in K<sup>+</sup>/Cl<sup>-</sup> cotransport (51–52). If the consequent change in cdb3 affinity were to be communicated through the membrane-spanning domain of band 3 to the KCC, the change in K<sup>+</sup>/Cl<sup>-</sup> cotransport might be explained. Similarly, patients with mutant hemoglobins characterized by reduced O<sub>2</sub> affinities have been reported to have elevated 2,3-DPG levels in their erythrocytes (53). While other explanations are again possible (54), it is also conceivable that increased binding of deoxyHb to cdb3 might lead to the constitutive displacement of glycolytic enzymes from band 3 and the consequent production of elevated 2,3-DPG. Along the same lines, patients with compromised erythrocyte oxygenation due to poor gas exchange in the lungs (e.g. chronic obstructive pulmonary disease (55), cystic fibrosis (56), asthma (57), and chronic cardiac insufficiency (58)) have been frequently reported to have erythrocytes with atypical deformabilities and/or morphologies. While other explanations are again possible, it should not be ignored that deoxyHb displacement of ankyrin from band 3 might contribute to these structural abnormalities. Finally, humans and dogs that have been exposed to high altitude/hypoxia are commonly observed to produce erythrocytes with prolonged filtration times (59–60). Because the slower filtration of their erythrocytes can be reversed with pentoxifylline, a drug that regulates erythrocyte hydration through control of ion transporters (61–62), it is plausible that the altered rheology of these individuals’ red cells might derive from sustained deoxyHb-band 3 interactions leading to an abnormal ion balance.

Regardless of whether any of the above pathologies are impacted by deoxyHb-band 3 interactions, the observation that an O<sub>2</sub> regulatory switch that controls these interactions is conserved in anucleate erythrocytes, despite considerable variability in the primary structure of band 3, suggests that the ability to regulate erythrocyte properties with O<sub>2</sub> is useful and that deviations from this, if they have accrued, appear to be inconsistent with life.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## The abbreviations used are

<b>cdb3</b>	cytoplasmic domain of band 3
<b>Hb</b>	hemoglobin
<b>deoxyHb</b>	deoxyhemoglobin
<b>GFP</b>	green fluorescence protein
<b>KCC</b>	K <sup>+</sup> /Cl <sup>-</sup> cotransporter
<b>AEBSF</b>	4-(2-Aminoethyl) benzenesulfonyl fluoride
<b>2,3-DPG</b>	2, 3-diphosphoglycerate

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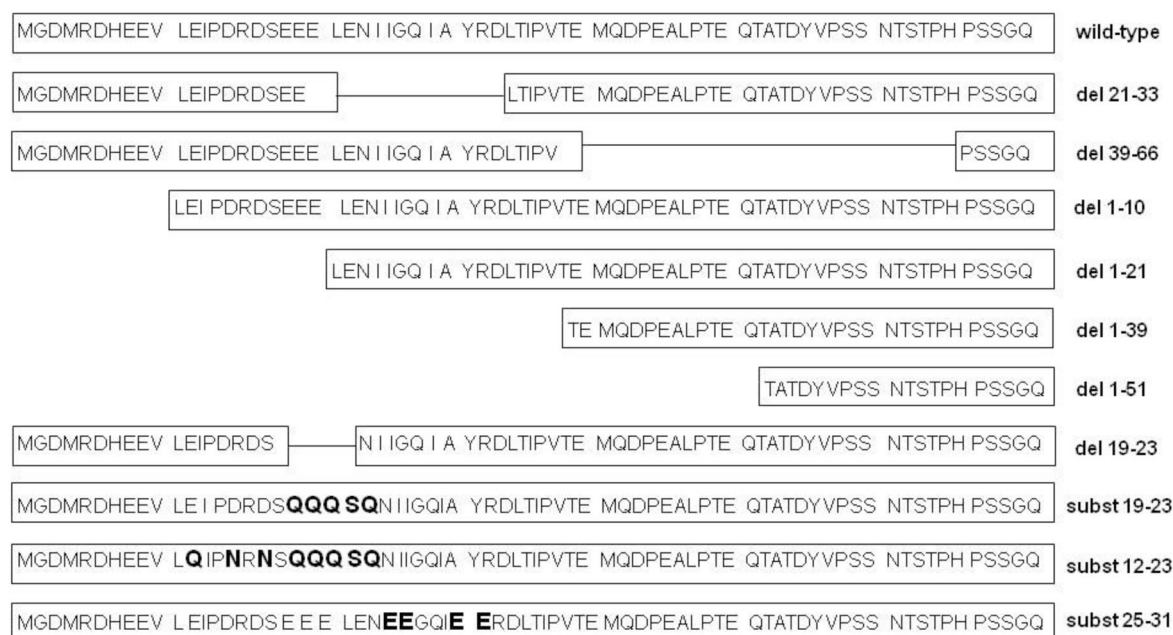
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rat        MGDMDRDHEEVLEIP-DRDSE-----EELEHVIEQI-AYRDLT---IPVTEMQ-----ESEALPTEQTATDYIPT
rabbit     -----MGDL-QEDYE-----EVLGEILEEE-EYEDPG---VMPQVE-----EPAASPFEPAPTDYHTT
dog        ---MDNQDSTMEKW-QDDHE-----EGLEDTLQE-EYEDPY---VPMQVE-----EPVAYPTEPTATDEDTV
horse      MDDSQENPEEIEVIVEEDYEGPGIITSPPPEAIMEE-DYEGPG---VMSQPE-----EPVAHPTEPTAADHHTI
cow        -----MGDP-EEYE-----DQLEETLEQK-EYEDHDSVSIPEEAEGDTIQEEEEAEARNQLTDDYHTT
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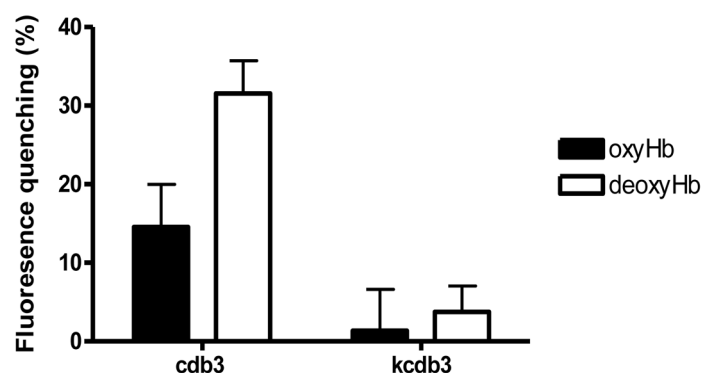
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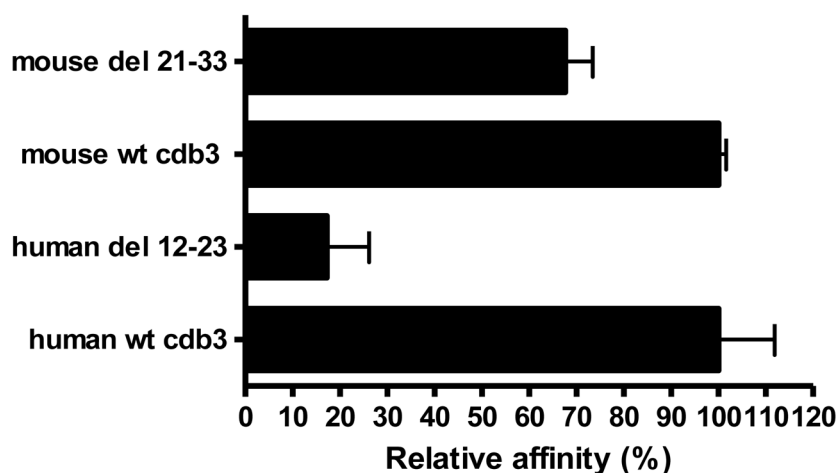
**Figure 1.**

A) Alignment of the NH<sub>2</sub>-terminus of band 3 from different mammalian erythrocytes. The experimentally established deoxyhemoglobin binding sites for human and murine band 3 are framed in boxes. The sequence alignment was performed using the CLUSTAL W program (<http://workbench.sdsc.edu/>). Asterisks (\*) indicate complete sequence conservation; (:) - conservation of strong groups; (.) - conservation of weak groups. B) Description of the mutant cytoplasmic domains of band 3 used in this study. For simplicity, only the first 70 amino acids of the 398 residue mouse cdb3 are shown. Deletions are denoted as (del) and substitutions as (subst). The substituted residues are printed in bold letters.

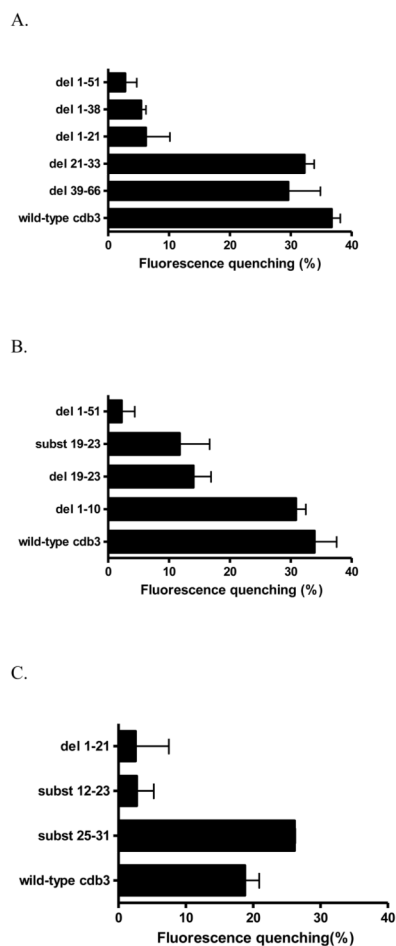
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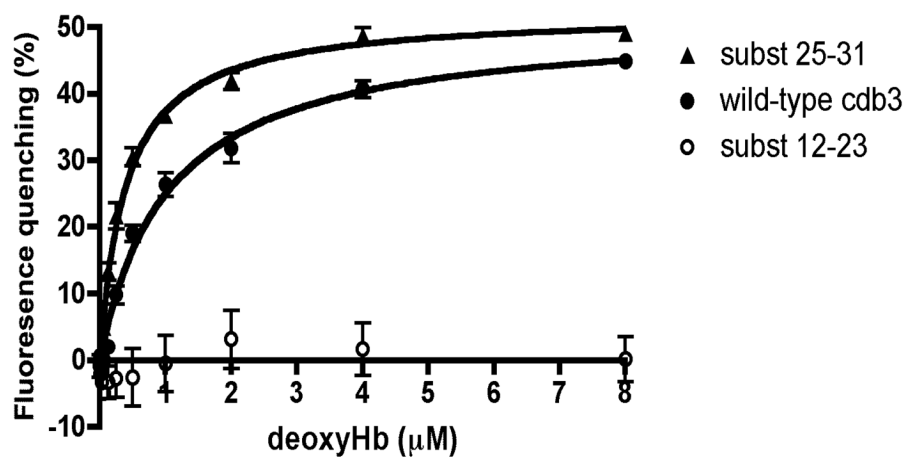
**Figure 2.**

A) Relative affinity of murine oxy- and deoxyHb (1 $\mu$ M) for murine cdb3 (0.5 $\mu$ M) determined by fluorescence quenching. Because the kidney spliceform of cdb3 (kcdb3) lacks the entire NH<sub>2</sub>-terminus (residues 1-79), it was used as a nonbinding (negative) control. B) Effect of deletion from murine cdb3 of the sequence homologous to the deoxyHb binding site on human cdb3 on the affinity of the mutated murine cdb3 for murine deoxyHb. Based on the sequence alignment in panel A, the sequence in murine cdb3 (residues 21-33) found to be most homologous to the deoxyHb binding site on human cdb3 (residues 12-23) was deleted and its relative binding affinity for murine deoxyHb was examined by measuring the shift in the oxygen dissociation curve in the presence of cdb3. For comparison, the relative affinity of human deoxyHb for human cdb3 is also shown.



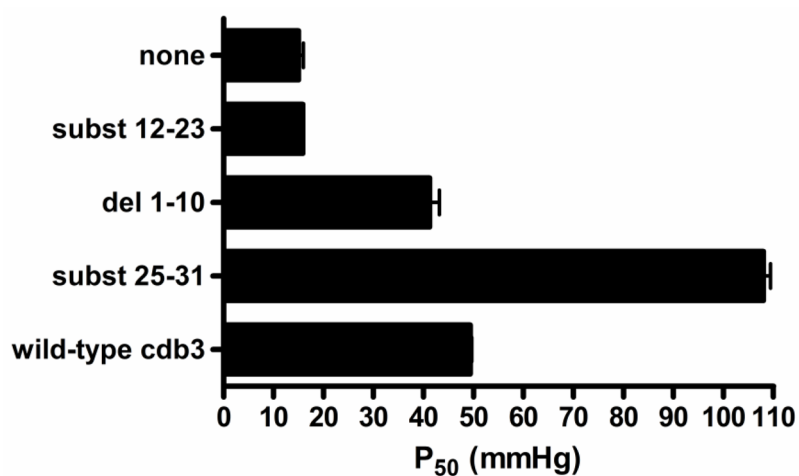
**Figure 3.**

A) Comparison of the relative affinities of different murine cdb3 mutants for murine deoxyHb. For analysis of deoxyHb binding affinity, the COOH-terminus of each cdb3 was fused to GFPuv and the binding of deoxyHb was assayed by quantitating the quenching of GFPuv fluorescence upon deoxyHb binding. In all cases, the inner filter effect due to Hb absorption of GFPuv fluorescence was subtracted (see Methods). As seen, mutant proteins that lack the first 21 amino acids do not bind deoxyHb (i.e. GFP fluorescence is high for del 1-51, del 1-38, and del 1-21), but when these first 21 residues are present (wild-type, del 21-33, del 39-66), binding affinity is high. A 1-51 deletion mutant was used as a negative control, since it exhibits no affinity for deoxyHb. See Fig. 1 for description of the mutations. B) Localization of the deoxyHb binding site on murine cdb3 to residues 12-23. C) Examination of substitution mutations within the binding region comprising residues 12-23 of cdb3.



**Figure 4.**

Evaluation of equilibrium binding affinity of deoxyHb for various isoforms of cdb3. Increasing concentrations of deoxyHb were incubated for 45 min at room temperature with cdb3-GFP or one of its mutants and the equilibrium binding constant was calculated as described in the Methods (n=3). Derived  $K_d$  values were  $1.04 \pm 0.14 \mu\text{M}$  for wild type cdb3-GFP and  $0.39 \pm 0.03 \mu\text{M}$  for the residues 25-31 substitution mutant. The 12-23 substitution mutant displayed no measurable affinity for deoxyHb.



**Figure 5.**

Confirmation of the deoxyHb binding site on murine cdb3 by evaluation of the effect of various NH<sub>2</sub>-terminal mutants of cdb3 on the Hb-O<sub>2</sub> dissociation curve. Hb-O<sub>2</sub> binding curves were measured for Hb alone, or Hb in the presence of wild-type cdb3, del(1-10), del(1-21), subst(12-23), or subst(25-31), and the affinity of deoxyHb for cdb3 was determined by analysis of the oxygen pressure where the murine Hb became 50% saturated with O<sub>2</sub> (P<sub>50</sub>).