Regulation of erythrocyte $Na^+/K^+/2Cl^-$ cotransport by an oxygen-switched kinase cascade

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Suilan Zheng[‡1], Nathan A. Krump[§1], Mary M. McKenna[§], Yen-Hsing Li[‡], Anke Hannemann[¶], Lisa J. Garrett[‖], John S. Gibson[¶], David M. Bodine[§2], and Philip S. Low[‡3]

From the [‡]Institute for Drug Discovery and Department of Chemistry, Purdue University, West Lafayette, Indiana 47907, the [§]Hematopoiesis Section, National Human Genome Research Institute and the [‖]National Human Genome Research Institute Embryonic Stem Cell and Transgenic Mouse Core Facility, National Institutes of Health, Bethesda, Maryland 20815, and the [¶]Department of Veterinary Medicine, University of Cambridge, Cambridge CB3 0ES, United Kingdom

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Many erythrocyte processes and pathways, including glycolysis, the pentose phosphate pathway (PPP), KCl cotransport, ATP release, Na^+/K^+-ATPase activity, ankyrin–band 3 interactions, and nitric oxide (NO) release, are regulated by changes in O_2 pressure that occur as a red blood cell (RBC) transits between the lungs and tissues. The O_2 dependence of glycolysis, PPP, and ankyrin–band 3 interactions (affecting RBC rheology) are controlled by O_2-dependent competition between deoxyhemoglobin (deoxyHb), but not oxyhemoglobin (oxyHb), and other proteins for band 3. We undertook the present study to determine whether the O_2 dependence of $Na^+/K^+/2Cl^-$ cotransport (catalyzed by $Na^+/K^+/2Cl^-$ cotransporter 1 [NKCC1]) might similarly originate from competition between deoxyHb and a protein involved in NKCC1 regulation for a common binding site on band 3. Using three transgenic mouse strains having mutated deoxyhemoglobin-binding sites on band 3, we found that docking of deoxyhemoglobin at the N terminus of band 3 displaces the protein with no lysine kinase 1 (WNK1) from its overlapping binding site on band 3. This displacement enabled WNK1 to phosphorylate oxidative stress-responsive kinase 1 (OSR1), which, in turn, phosphorylated and activated NKCC1. Under normal solution conditions, the NKCC1 activation increased RBC volume and thereby induced changes in RBC rheology. Because the deoxyhemoglobin-mediated WNK1 displacement from band 3 in this O_2 regulation pathway may also occur in the regulation of other O_2-regulated ion transporters, we hypothesize that the NKCC1-mediated regulatory mechanism may represent a general pattern of O_2 modulation of ion transporters in erythrocytes.

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This article contains Table S1 and Figs. S1–S3.

[1] These authors contributed equally to this project.
[2] To whom correspondence may be addressed: Genetics and Molecular Biology Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892. Tel.: 301-402-0902; Fax: 301-301-4929; E-mail: tedyaz@mail.nih.gov.
[3] To whom correspondence may be addressed: Dept. of Chemistry, Purdue University, West Lafayette, IN 47907. Tel.: 765-494-523; Fax: 765-494-5272; E-mail: plow@purdue.edu.

During transit from the lungs to the tissues, the human erythrocyte (red blood cell [RBC])[4] experiences changes in the partial pressure of oxygen that decrease from ~100 mm Hg on the arterial side to <5 mm Hg in metabolically active tissues (1). Although these transitions in O_2 pressure have long been known to facilitate O_2 unloading in the tissues, they have more recently been established to participate in the regulation of important RBC properties (2–4). For example, the reversible O_2-dependent association of deoxyHb with the major erythrocyte membrane protein, band 3, has been shown to constitute a molecular switch that controls the association of glycolytic enzymes with inhibitory sites on band 3, shifting the flux of glucose from glycolysis at low O_2 pressures to the pentose phosphate pathway at high O_2 pressures (5, 6). This shift in glucose metabolism is thought to be adaptive, because the resulting increase in NADPH in oxygenated conditions can help protect the erythrocyte from the oxidative stress that accompanies high O_2 pressures (5, 6). The same oxygen-dependent association of deoxyHb with band 3 has also been found to modulate the flexibility of the RBC membrane by disjoining a fraction of the band 3–ankyrin interactions at low O_2 pressure (7–9). The improved membrane deformability associated with this rupture of membrane–cytoskeletal interactions has been hypothesized to facilitate the return of deoxygenated RBCs from the microvasculature to the lungs (8, 10, 11). Finally, hypoxia has also been shown to promote ATP release from circulating erythrocytes, leading to activation of P2Y receptors on endothelial cells and the consequent vasodilation that facilitates blood flow back to the lungs (12, 13).

Changes in O_2 pressure have also been reported to modulate ion transport in human erythrocytes (14, 15). The $Na^+/K^+/2Cl^-$ cotransporter (NKCC1) catalyzes the electroneutral, passive symport of one Na^+ plus one K^+ plus two Cl^- ions across a plasma membrane, promoting an osmotically driven flow of water in the same direction (16–18). In the human erythrocyte, this cotransport is largely inactive in oxygenated cells but activated in deoxygenated RBCs (15). This O_2 regulation has been suggested to involve a kinase, because NKCC1 activation by

[4] The abbreviations used are: RBC, red blood cell; Hb, hemoglobin; deoxyHb, deoxygenated hemoglobin; NKCC1, $Na^+/K^+/2Cl^-$ cotransporter 1; WNK1, with no lysine kinase 1; OSR1, oxidative stress-responsive kinase 1; KCC, KCl cotransporter.

deoxygenation can be blocked by kinase inhibitors (19–23), although the responsible kinase (if any) has not been identified in erythrocytes. In the kidneys NKCC activity has been shown to be regulated by a WNK (With-No-K (Lys)) kinase that is inhibited by intracellular Cl^- (24) and activated by an increase in osmotic pressure. The latter somehow induces phosphorylation of the oxidative stress-responsive 1 (OSR1) kinase, which then phosphorylates and activates NKCC (25–32). Whether the osmotically regulated WNK–OSR1 signaling cascade is also involved in O_2 modulation of NKCC1 in erythrocytes remains to be examined.

In this paper, we explore the mechanism of O_2-dependent control of NKCC1 by investigating the impact of oxygen pressure on NKCC1 activity in erythrocytes from transgenic mice that express different mutations in the deoxyHb-binding site on erythrocyte band 3. We demonstrate that deoxyHb (but not oxyHb) binds to the cytoplasmic domain of band 3 and displaces the WNK1 kinase from its docking site on band 3. We further show that this displaced WNK1 kinase activates OSR1, which in turn phosphorylates and activates NKCC1, leading to an influx of NaCl and KCl into the cell whenever O_2 levels decrease. In this manner, the O_2 content of the erythrocyte can modulate red cell volume during RBC transit in the vasculature.

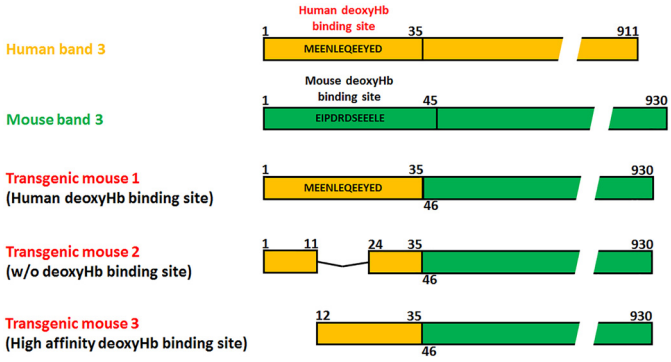

Figure 1. Design of erythrocyte band 3 proteins with altered deoxyHb-binding sites. To evaluate the role of the reversible O_2-dependent binding of deoxyHb to the N terminus of the membrane-spanning protein, band 3, transgenic mice containing sequences from human (*yellow*) and murine (*green*) band 3 were generated as described under "Experimental procedures." As noted elsewhere (34), WT mice and mice containing the human deoxyHb-binding site substituted for the similar mouse deoxyHb site displayed similar affinities for deoxyHb. In contrast, mice lacking a deoxyHb site (missing residues 12–23) exhibited no affinity for deoxyHb, and mice lacking amino acids 1–11 of band 3 surprisingly displayed extraordinarily high affinity for band 3. Together, these transgenic mice allow for analysis of the effect of the affinity of deoxyHb for band 3 on O_2 regulation of NKCC1 transport.

Results

Generation of transgenic murine erythrocytes with altered deoxyHb-binding sites on band 3

To evaluate the role of the band 3– deoxyHb interaction in regulating $Na^+/K^+/2Cl^-$ cotransport through the NKCC1 transporter, we introduced mutations into band 3 that were known to affect its association with deoxyHb. Based on previous studies that mapped the deoxyHb-binding site on the isolated cytoplasmic domain of band 3 (33, 34), we generated a transgenic mouse in which the sequence encompassing the deoxyHb-binding site (residues 1–45) on murine band 3 was replaced with the homologous sequence from human band 3 (residues 1–35; *i.e.* which includes the human deoxyHb-binding site). The resulting mouse provided us with a humanized model for analysis of the regulatory role of deoxyHb binding to band 3 in RBCs (34). In a second transgenic mouse, the same human sequence was inserted into murine band 3, except the amino acids responsible for deoxyHb binding (residues 12–23) were deleted, allowing us to determine how the lack of a deoxyHb-band 3 affects O_2 regulation of RBC properties. In the present study, we introduced a third mutation into murine band 3 (deletion of residues 1–11) that endows band 3 with a significantly higher affinity for deoxyHb (33, 35) (Fig. 1). The representative schematic strategy is shown in Fig. S1. Hematologic analyses of these transgenic mice revealed that their RBCs display essentially normal indices, except their sodium contents are slightly elevated, and their potassium contents are somewhat reduced (Table S1). Further analyses of their osmotic fragilities indicate that they also are slightly elevated (Fig. S2), which we interpret to derive from reduced retention of band 3 in the mutant membranes, as shown in Fig. S3. Although RBC membranes from a human patient in which band 3 residues 1–11 are similarly missing also contain reduced levels of band 3 (*i.e.* analogous to the mouse with the high-affinity deoxyHb-binding site on band 3), the mutation in the human RBCs appears to cause much greater membrane destabilization, perhaps because it promotes retention of much less band 3 than seen in the murine RBC membranes (36).

O_2 regulation of NKCC1 is inhibited by altered deoxyHb–band 3 interactions

Although the activity of NKCC1 has been shown to be O_2-regulated in erythrocytes from several vertebrate species, including humans (1, 14, 15), O_2 regulation has not been reported in mice. As shown in Fig. 2*A*, cotransport of NaCl and KCl in WT mouse erythrocytes is elevated ~2.5-fold upon deoxygenation, *i.e.* comparable with the effect reported in human RBCs (15). A similar increase in NKCC1 activity is seen in murine erythrocytes in which the human deoxyHb-binding sequence has been substituted for the homologous mouse sequence, suggesting that the human and murine sequences interact similarly with deoxyHb. In contrast, when the murine deoxyHb-binding sequence is either deleted or replaced with a high-affinity sequence that prevents reversible deoxyHb binding over the physiological range of O_2 pressures, oxygen regulation of NKCC1 activity is abrogated. These data suggest that reversible association of deoxyHb with the cytoplasmic domain of band 3 constitutes the molecular switch that controls NKCC1 activity in erythrocytes and that mutations that either decrease or increase the affinity of deoxyHb for band 3 inhibit this regulation of NaCl plus KCl cotransport.

Although this interpretation of O_2 regulation is consistent with both the transport data and the previously established mechanism for O_2 regulation of glycolysis and ankyrin binding (34), a replotting of the data showing absolute rather than relative $Na^+/K^+/2Cl^-$ transport rates argues for a more complex interpretation. As seen in Fig. 2*B*, RBCs in which deoxyHb is always bound (high-affinity mutant) or always dissociated (Hb site deletion mutant) both display elevated transport rates,

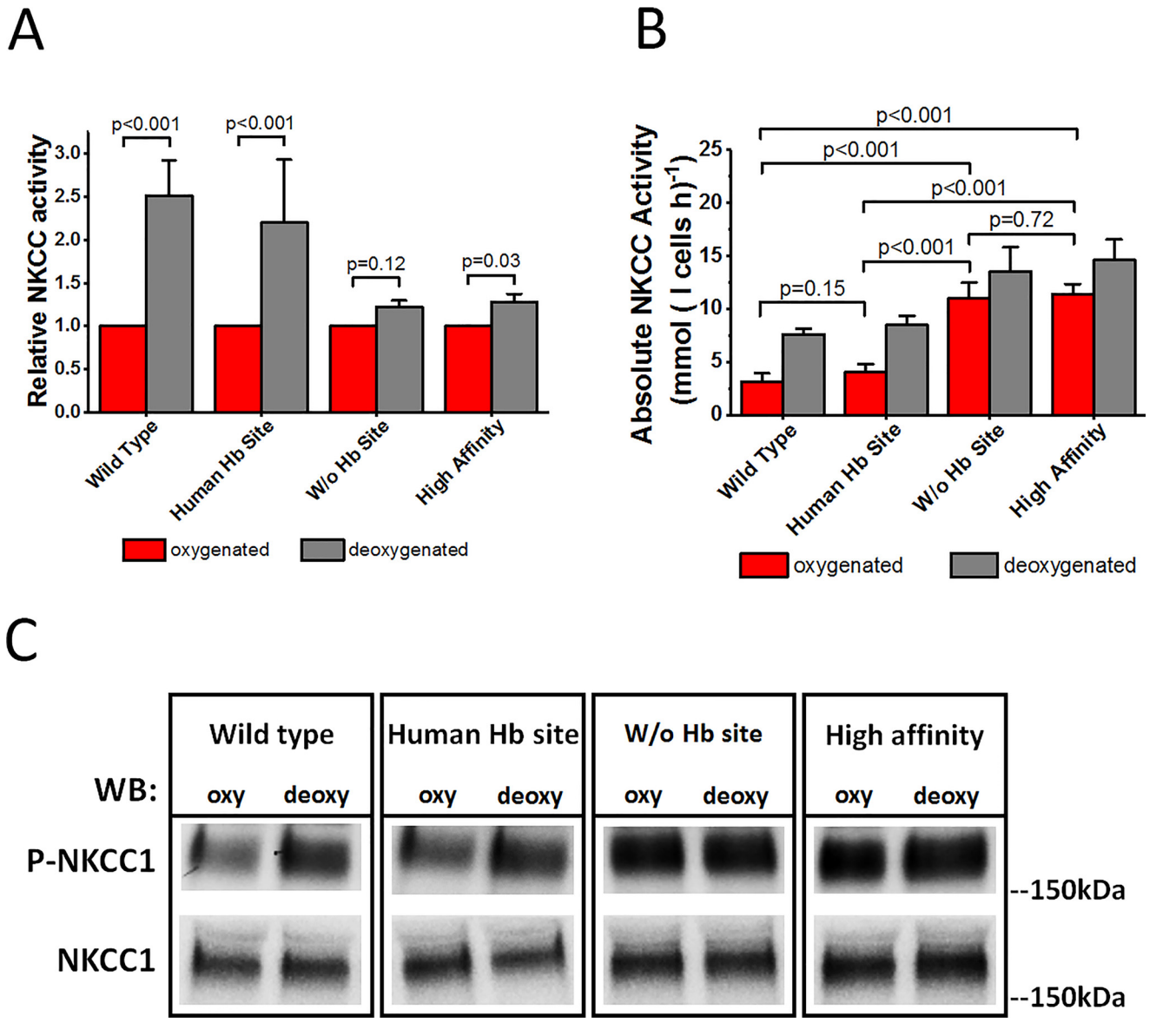

Figure 2. Effect of deoxygenation on murine erythrocyte NKCC1 activity and phosphorylation level. *A*, analysis of NKCC1 activity of deoxygenated erythrocytes from WT and mutated RBCs relative to the rates of NKCC1 activity in their oxygenated counterparts. NKCC1 transport rates from all oxygenated erythrocytes were set as 1.0 and compared with the NKCC1 transport rates of deoxygenated erythrocytes (means of four experiments ± S.D.). *B*, analysis of the absolute transport rates of NKCC1 in oxygenated and deoxygenated WT and transgenic murine erythrocytes (means of four experiments ± S.D.). In both studies, bumetanide (NKCC specific inhibitor)-sensitive K^+ flux was used as a measure of NKCC1 transport. *C*, effect of RBC oxygenation on the phosphorylation of NKCC1 (*P-NKCC1*) on residues Thr^{206} and Thr^{211} in intact WT and mutated murine RBCs. Total NKCC1 content is used as a gel loading control in the lower row of each immunoblot (representative of blots from four experiments). *deoxy*, deoxygenated; *oxy*, oxygenated; *WB*, Western blot.

regardless of O_2 pressure. In contradistinction, data from the same study in WT mice demonstrate that deoxyHb binding to band 3 enhances $Na^+/K^+/2Cl^-$ transport, whereas dissociation of deoxyHb from band 3 reduces $Na^+/K^+/2Cl^-$ transport, suggesting that erythrocytes in which the deoxyHb site has been deleted should have shown reduced rather than elevated NKCC1 activity.

O_2-dependent regulation of NKCC1 activity involves NKCC1 phosphorylation

To reconcile the above data, we explored whether the pathways of NKCC regulation present in other tissues are similar to the O_2 regulation seen in RBCs. As demonstrated by Moriguchi *et al.* (25) and Vitari *et al.* (26), regulation of NKCC in the kidneys involves a signaling cascade in which an increase in osmotic pressure activates WNK kinase, which in turn phosphorylates OSR1 kinase. Activated OSR1 then phosphorylates NKCC on threonines 212 and 217, leading to NKCC activation and the consequent volume changes that protect the kidneys from osmotic stress and facilitate transepithelial salt resorption (37).

To determine whether changes in O_2 pressure might similarly induce changes in NKCC1 phosphorylation in murine erythrocytes, we examined the O_2 dependence of NKCC1 phosphorylation in murine RBCs using an antibody directed against phosphothreonines 212 and 217 of human NKCC1 (equivalent to Thr^{206}/Thr^{211} on murine NKCC1) that are involved in

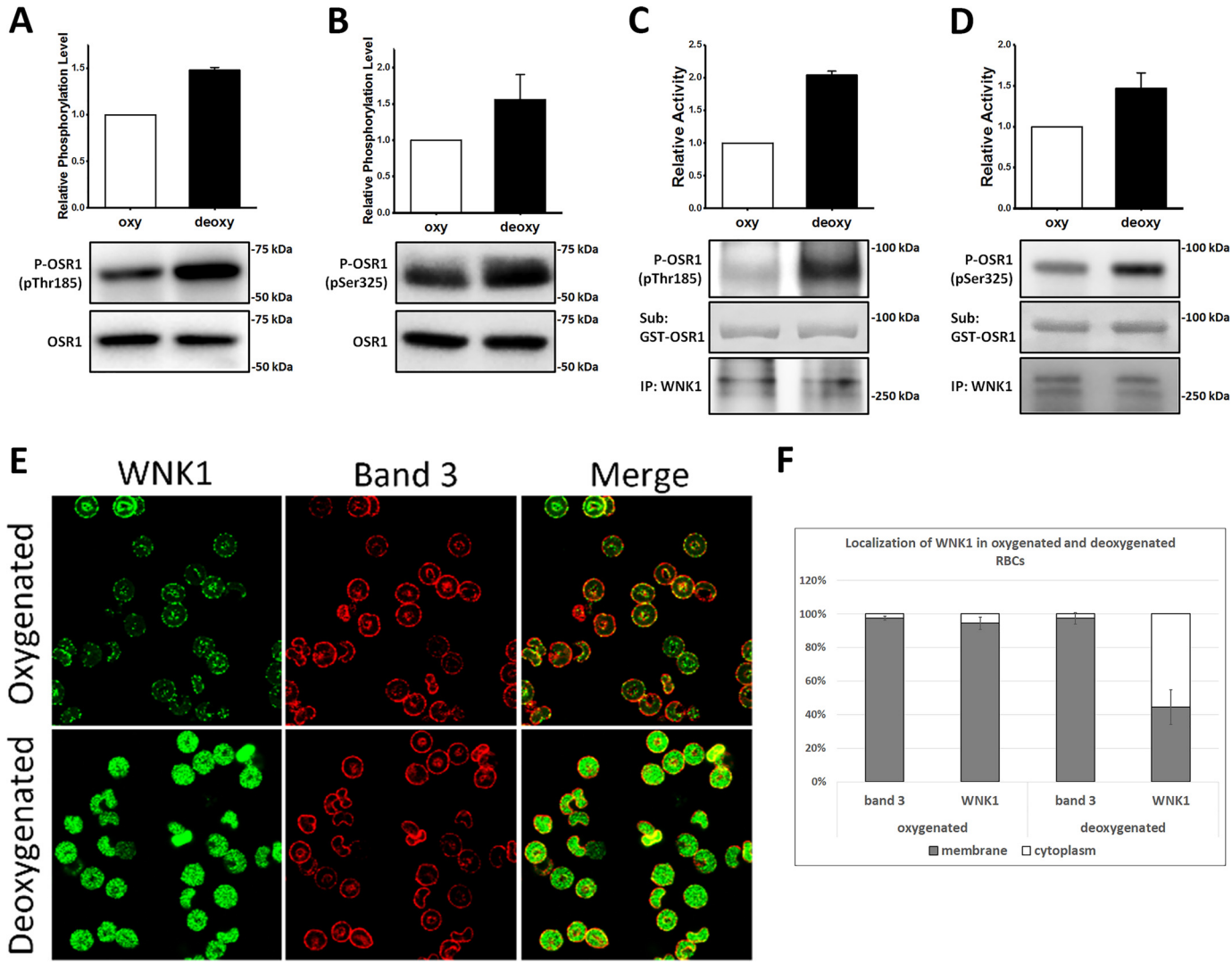

Figure 3. Effect of deoxygenation on activation of WNK1 and OSR1. *A*, comparison of the level of phospho-OSR1 (pThr[185]) in oxygenated and deoxygenated murine RBCs. Intact RBCs were either oxygenated or deoxygenated prior to lysis in Triton X-100 and immunoblotting with anti-phospho OSR1 (P-OSR1) or total OSR1. The antibody to P-OSR1 recognizes only phosphothreonine 185. The ratios of P-OSR1 to total OSR1 from oxygenated RBCs was set as 1.0 and compared with those from the deoxygenated cells. Quantitative results are shown as means of three experiments ± S.D. *B*, similar to *A* except the antibody to P-OSR1 recognizes only phosphoserine 325. *C*, WNK1 was immunoprecipitated from the aforementioned whole cell extracts using anti-WNK1 antibody, and WNK1 kinase activity was determined in the pellets by analysis of their ability to phosphorylate GST-OSR1 on Thr[185] using the antibody in *A* (*P-OSR1*). Kinase activity of WNK1 from oxygenated erythrocytes was set as 1. Total WNK1 in the pellet was also quantitated by immunoblotting (*IP: WNK1*). *D*, same as in *C*, except the anti–P-OSR1 used recognizes only phosphorylated Ser[325]. Kinase activity of WNK1 from oxygenated erythrocytes was set as 1. *E*, translocation of WNK1 from the RBC membrane to cytosol was detected by immunofluorescent staining of WNK1 in oxygenated and deoxygenated whole murine erythrocytes. Changes in WNK1 staining intensity are due to differences in epitope accessibility between WNK1 on the membrane and WNK1 in the cytosol (see Ref. 32 for examples of other RBC proteins exhibiting similar behavior). To visualize the location of the membrane in the same focal plane as WNK1, band 3 was costained in *red* (*center panels*). The images were taken under a Fv1000 confocal microscope (Olympus) using a 60× oil-immersion objective lens. *F*, quantitative of WNK1 localization to the membrane in oxygenated and deoxygenated murine erythrocytes. ImageJ analysis was used to compare the location of WNK1 with band 3, where band 3 was assumed to be 100% localized to the membrane in both oxygenated and deoxygenated erythrocytes (means of 20 cells ± S.D.). *deoxy*, deoxygenated; *oxy*, oxygenated.

NKCC1 activation in response to osmotic stress in the kidneys (38). As shown in Fig. 2*C*, deoxygenation of erythrocytes containing a functional band 3 deoxyHb-binding site (*i.e.* either WT or humanized) promotes a ~2-fold increase in phosphorylation of threonines 206 and 211, whereas deoxygenation of RBCs either lacking this site or containing the high-affinity deoxyHb site has no significant effect on NKCC1 phosphorylation. This O_2 dependence correlates with the O_2-insensitive and constitutively activated NKCC1 activity that we observed in RBCs containing either the high deoxyHb affinity mutant and the deoxyHb site deletion mutant, *i.e.* the two mutant erythrocytes that display O_2 independent and constitutively high phosphorylation of threonines 206 and 211 (Fig. 2*C*).

Deoxygenation of erythrocytes activates both WNK1 and OSR1

To determine whether the catalytic activity of WNK1 and/or OSR1 might be regulated by O_2 in RBCs, we lysed oxygenated and deoxygenated murine RBCs in Triton X-100 and immunoblotted the lysates with antibodies to OSR1 phosphorylated on threonine 185 and serine 325 (26, 39, 40), *i.e.* two conserved residues on OSR1 known to be phosphorylated by WNK1 (25, 26). As

shown in Fig. 3*A*, similar levels of total OSR1 were present in both oxygenated and deoxygenated cells; however, the level of OSR1 phosphorylation on Thr^{185} and Ser^{325} was much higher in deoxygenated than oxygenated RBCs. These data suggest that deoxygenation promotes OSR1 phosphorylation/activation.

To determine whether WNK1 might catalyze the above O_2-dependent OSR1 phosphorylation as it does osmotically induced OSR1 phosphorylation in kidneys, we immunoprecipitated WNK1 from both oxygenated and deoxygenated RBCs and examined its ability to phosphorylate exogenously added OSR1 on Thr^{185} and Ser^{325}. As shown in Fig. 3 (*C* and *D*), respectively, phosphorylation of OSR1 on Thr^{185} and Ser^{325} was significantly higher when performed with WNK1 immunoprecipitates from deoxygenated than oxygenated cells. Importantly, more than 90% of WNK1 proteins were localized to the membrane in oxygenated erythrocytes, whereas more than 50% of WNK1 was translocated into the cytoplasm in deoxygenated erythrocytes (Fig. 3, *E* and *F*). These data suggest that WNK1 is predominantly membrane-bound and inactive in oxygenated RBCs but cytosolic and more active in deoxygenated RBCs, similar to behavior observed for glycolytic enzymes (41, 42).

WNK1 interacts with the N-terminal of band 3

To confirm that the deoxyHb-binding site on band 3 is involved in WNK1 binding, we used an anti-WNK1 antibody to pull down the WNK1 kinase from detergent extracts of murine erythrocytes to determine whether band 3 is coprecipitated with it. As shown in Fig. 4, similar amounts of WNK1 were pelleted from WT erythrocytes and erythrocytes from all three mutant mice, suggesting that the kinase is similarly expressed in all four mice. However, the amount of band 3 copelleted with WNK1 was 2-fold higher in RBCs containing a functional deoxyHb-binding site (*i.e.* WT and humanized) than in mutant erythrocytes either lacking the deoxyHb-binding site or expressing the high-affinity binding site. These data argue that mutations in the deoxyHb site on band 3, regardless of whether they enhance or eliminate deoxyHb binding, cause a concurrent loss in WNK1 binding, confirming that at least some of the deoxyHb binding residues at the N terminus of band 3 are involved in WNK1 binding. Because displacement of WNK 1 from band 3 induces its activation (Fig. 3), these results explain why both deoxyHb-binding site mutants (*i.e.* mutants without a Hb site and mutants containing the high-affinity Hb site) exhibit a similar constitutive O_2-independent activation (Fig. 2*B*) and phosphorylation (Fig. 2*C*) of NKCC1. Taken together, our data demonstrate that RBC deoxygenation induces deoxyHb binding to band 3 (residues 12–23 (43)), which in turn promotes displacement and activation of WNK1.

To determine whether the same regulatory pathway for O_2 modulation of NKCC1 activity in the mouse might be operative in human RBCs, we expressed GST fusion constructs of the intact cytoplasmic domain of human band 3 (cdb3; residues 1–379) containing no mutation, the high-affinity deoxyHb-binding site mutation, or the deoxyHb-binding site deletion mutation and examined their relative abilities to copellet WNK1 from crude extracts of HEK293 cells expressing WNK1 (Fig. 5). Although GST pulldown of WT human cdb3 was found to copellet WNK1, none of the cdb3s containing a mutated

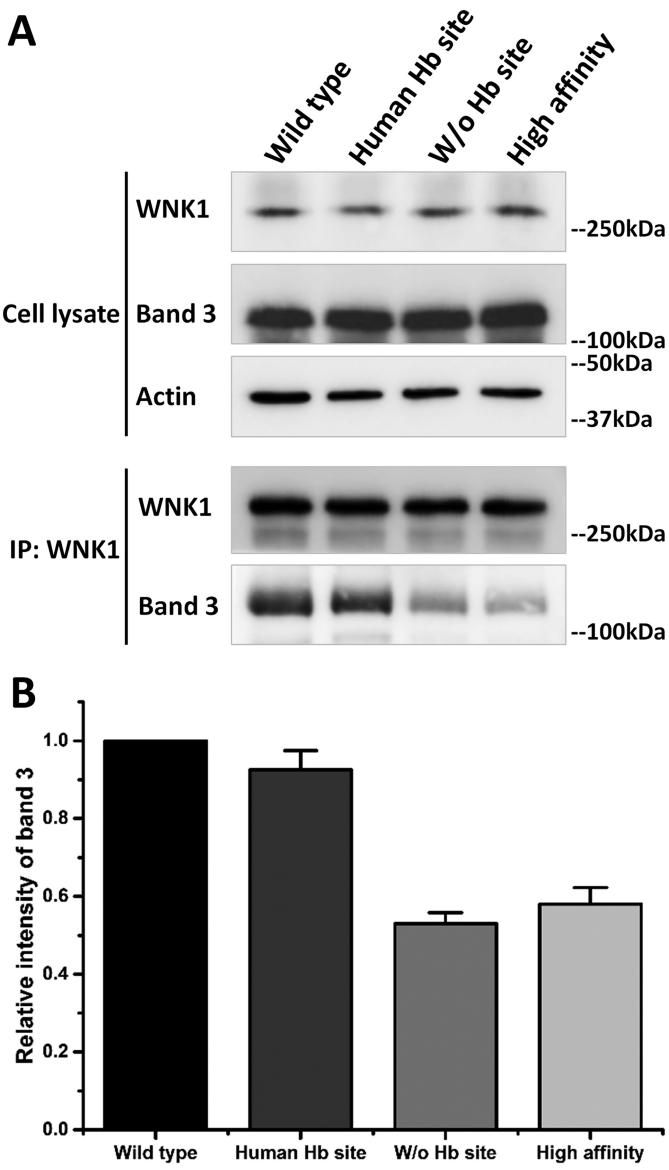

Figure 4. Effect of mutations in the deoxyHb-binding site on the interaction between WNK1 and band 3. *A*, whole-cell RBC lysates (see Fig. 3) from either WT or transgenic mice were incubated with anti-WNK1 antibody, and the amount of coprecipitated band 3 was analyzed by immunoblotting (*IP: WNK1*). *B*, densitometric analysis of coprecipitated band 3 from the transgenic murine erythrocytes. The amount of band 3 copelleted from WT murine erythrocytes was set as 1.0 and compared with the amount of band 3 copelleted from the transgenic murine erythrocytes (means of three experiments ± S.D.).

deoxyHb-binding site was able to copellet WNK1. These data confirm that deoxyHb and WNK1 share an overlapping binding site on both murine and human band 3 and that mutation of this site to either decrease or increase deoxyHb affinity leads to loss of WNK1 binding in both species.

WNK1 is believed to respond to osmotic stress in the kidneys by regulating the locations and activities of multiple ion transporters and channels (27). Important to this regulatory role appears to be the translocation of WNK1 to intracellular compartments, mediated by its C-terminal domain in response to elevated osmotic pressure (27). To determine whether the same domain of WNK1 might be involved in regulating WNK1's O_2-dependent translocation in RBCs, we expressed intact

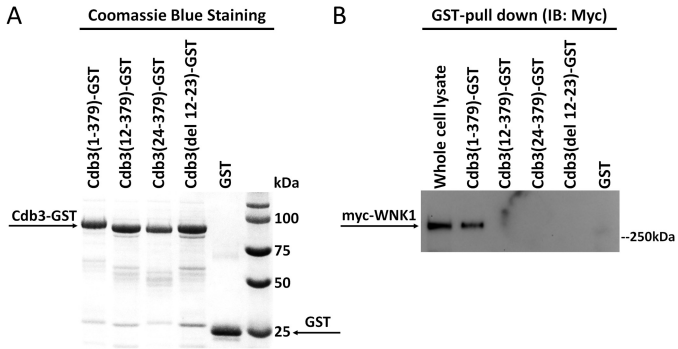

Figure 5. Effect of deoxyHb-binding site mutations in the cytoplasmic domain of band 3 on its association with WNK1. *A*, Coomassie Blue staining showing the GST fusion of WT or mutated human cdb3 used in the pull-down assay. *B*, lysates from HEK293 cells expressing Myc-tagged WNK1 were incubated with a GST fusion of WT or mutated human cdb3. Copelleted WNK1 with GST-cdb3 was then detected by immunoblotting with an anti-Myc tag antibody (*IB: Myc*). The whole cell lysate lane contains 5% of the total cell lysate. The blot shown here is representative of blots from three independent experiments. Densitometric analysis performed with ImageJ software indicates that ~9.3 ± 1.8% of WNK1 in the total lysate is pulled down with WT human cdb3.

WNK1, the WNK1 N-terminal kinase domain, and the WNK1 C-terminal "translocation domain" in HEK293 cells and examined which construct associated with a GST fusion of human cdb3. As shown in Fig. 6, both the intact and C-terminal domain of WNK1 were readily pulled down by GST-cdb3, whereas the N-terminal domain of WNK1 was not. These data argue that a similar domain and mechanism is involved in regulating osmotic translocation of WNK1 in the kidneys and O_2 translocation of WNK1 in erythrocytes.

Discussion

Orskov and others (1, 15, 22, 44, 45) have shown that several erythrocyte cation transporters are O_2-regulated. Initially, a deoxyHb-binding site competition mechanism was dismissed, because erythrocyte cation transporters are not thought to associate with band 3. The binding site competition mechanism then became even more implausible when either elimination or augmentation of deoxyHb's binding affinity for band 3 yielded the same enhancement of NKCC1 transport (Fig. 2*B*). Reconciliation of these data finally occurred when we discovered that (i) band 3 interacts directly with WNK1, (ii) deoxyHb-induced displacement of WNK1 from band 3 initiates a signaling cascade resulting in phosphorylation/activation of OSR1 and phosphorylation/activation of NKCC1 (Figs. 2 and 3), and (iii) mutations leading to either elimination or augmentation of the deoxyHb-binding site on band 3 cause dissociation of WNK1 from band 3 (*i.e.* accounting for its activation in both mutant RBCs; Fig. 4). Thus, similar to oxygen's regulation of ankyrin affinity and glucose metabolism, the molecular mechanism for oxygen's control of NKCC1 reduces to a simple competition between deoxyHb and another protein (*i.e.* WNK1) for docking on band 3. To the best of our knowledge, this report constitutes the first description of a molecular mechanism by which O_2 regulates any solute transporter.

With the O_2 switch for regulation of glycolysis, ankyrin binding, and NKCC1 activity now all involving a competition between deoxyHb and another protein for a shared binding site on band 3, the question naturally arose of whether other still uncharacterized O_2-regulated pathways (*e.g.* ATP release (13), KCl cotransport (1, 15, 46), NO release (47), the Na^+/K^+-ATPase (48, 49), and Na^+/H^+ exchanger (50, 51), etc.) might be similarly modulated by competition between deoxyHb and another band 3-binding protein for a common site on band 3. Based on the regulatory mechanism elucidated here, it seems possible that membrane-spanning proteins like Piezo-1, the KCl cotransporter, the Na^+/H^+ exchanger, and the Na^+/K^+-ATPase may not directly compete with deoxyHb for association with band 3, but rather might be regulated by other signaling proteins that do directly compete for binding to band 3. The most obvious candidate would be the KCl cotransporter (KCC), because KCl cotransport has been shown to be inhibited by a signaling pathway involving WNK1 activation of OSR1 followed by OSR1 phosphorylation of the KCC (29, 52, 53). Because the effect of O_2 on KCl cotransport is exactly the opposite of its effect on $Na^+/K^+/2Cl^-$ cotransport (1, 54–56) (*i.e.* oxygenation of RBCs activates KCl cotransport while it inhibits $Na^+/K^+/2Cl^-$ cotransport and vice versa) and because the phosphorylation sites on the KCC and NKCC are homologous (57, 58), it seems reasonable to posit that the same deoxyHb displacement of WNK1 might be responsible for the reciprocal inhibition/activation of KCl and $Na^+/K^+/2Cl^-$ cotransport, respectively. Indeed, in view of the plethora of signaling proteins reported to bind band 3 in RBCs (*e.g.* Fgr, Hck, Lyn, Syk, SHP2, and casein kinase I (59–61)), it is not inconceivable that many of the erythrocyte's known O_2-regulated processes might be controlled by a similar deoxyHb-mediated displacement mechanism involving release of a signaling enzyme from band 3.

It is interesting that oxygen regulation of $Na^+/K^+/2Cl^-$ transport in the erythrocyte turns out to be so similar to osmotic regulation of $Na^+/K^+/2Cl^-$ transport in the kidneys. Except for the transition from osmotic stimulation to oxygen stimulation, the signaling cascade appears to be almost identical. However, evolutionary implementation of this regulatory capability in erythrocytes may not have been mechanistically trivial. Thus, although band 3 is expressed in the kidneys, kidney band 3 lacks the first 65 amino acids present in erythrocyte band 3 (62), and these amino acids are precisely those that are required for O_2 regulation (Figs. 2 and 4). Because these additional 65 amino acids are not homologous to any other sequence reported in the protein sequence databases (except other erythrocyte band 3's), it is also unlikely that the new N terminus of band 3 could have derived from an exon normally present elsewhere in the genome. Rather, the fact that addition of these amino acids induces global changes in band 3's structure (63, 64) and function that endow band 3 with the ability to bind ankyrin, multiple glycolytic enzymes, several kinases, and deoxyhemoglobin, etc. (64–66), suggests that the added N terminus evolved to improve erythrocyte function. Although further studies will be required to define how O_2 regulation of $Na^+/K^+/2Cl^-$ transport enhances the erythrocyte's evolutionary fitness, the above considerations suggest that the ability of O_2 to modulate RBC cation content must somehow improve the red cell's function. It will be important in the future to determine how O_2-regulated changes in Na^+ and K^+ concentrations benefit the erythrocyte's performance. However, the fact that

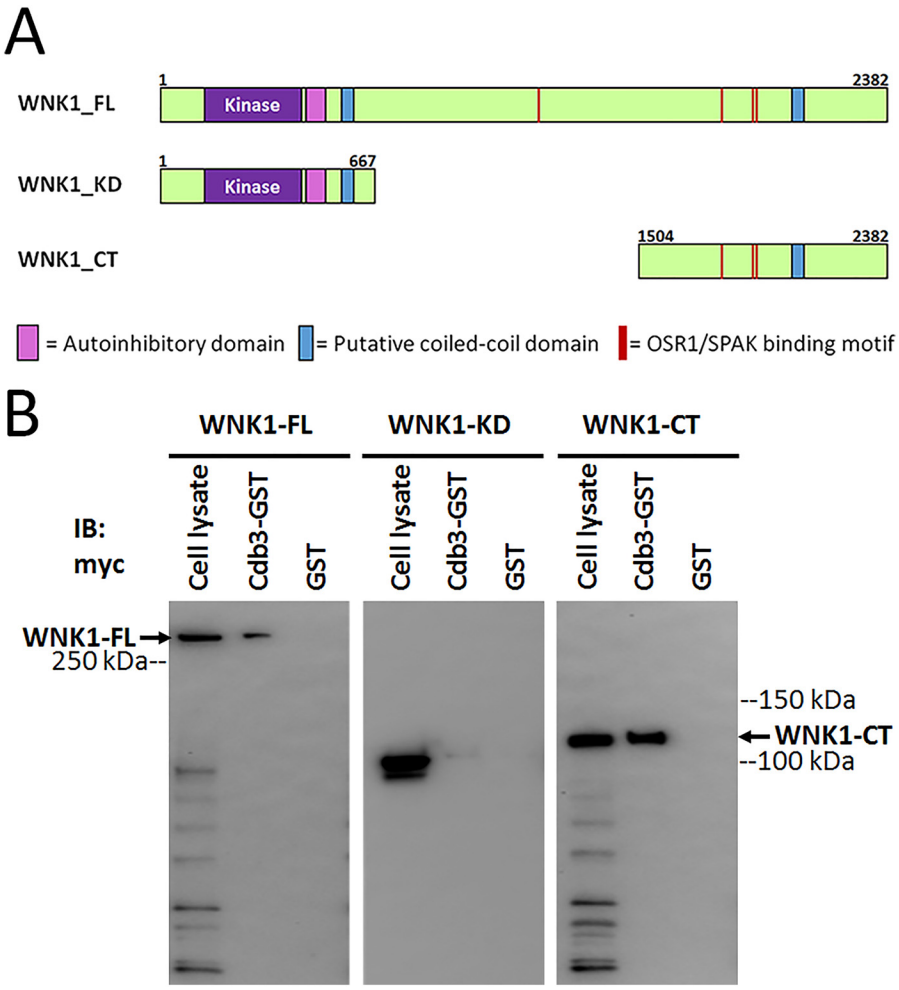

Figure 6. Mapping of the interaction between WNK1 and band 3. *A*, schematic representation of WNK1 fragments used in pulldown assay of *B*. *B*, HEK293 cell lysates expressing Myc-tagged full-length WNK1 (*WNK1-FL*), the kinase domain of WNK1 (*WNK1-KD*), or the C-terminal fragment of WNK1 (*WNK1-CT*) were mixed with a GST fusion of human cdb3. Copelleted proteins were separated by SDS-PAGE, and WNK1 fragments were detected by immunoblotting using an anti-Myc tag antibody. The blots shown here are representative of blots from three independent experiments.

catecholamine's have been found to regulate NKCC activity in avian RBCs (21, 67) but not in mammalian erythrocytes) and that catecholamine regulation of avian NKCC has been shown to be modulated by oxygen tension and osmolarity (21, 68) suggests that regulation of NKCC in erythrocytes probably occurred early in erythrocyte evolution and that participation of oxygen in this regulation was also an early event. It would be interesting to determine whether the catecholamine-mediated control of NKCC activity might also involve band 3-WNK1 interactions.

Finally, with >20 proteins thought to bind band 3 in red blood cells (AQP1, GAPDH, LDH, PFK, PK, aldolase, Syk, casein kinase 1, SHP2, adducin, ankyrin, protein 4.1, protein 4.2, carbonic anhydrase 2, RhAG, Rh, CD47, glycophorin A, Lyn, Hck, Fgr, and WNK1), the question naturally arises of whether sufficient copies of band 3 exist to accommodate them all. Rough estimates of the number of copies of band 3's most prominent protein ligands suggest that the answer is affirmative. Thus, there are 1.2×10^6 copies of band 3 per RBC (69, 70). If one assumes that there are also 120,000 ankyrins (71), 30,000 adducin dimers (72), 350,000 glyceraldehyde-3-phosphate dehydrogenases (73), 100,000 aldolases, 30,000 phosphofructokinases (74), 200,000 proteins 4.1 (75), and 200,000 proteins 4.2 (76), ~300,000 copies of band 3 should still remain to accommodate all of the above signaling enzymes, which far exceeds their numbers in RBCs. Thus, even if all known band 3 peripheral protein ligands were to compete for the same deoxyHb-binding site, there should still be sufficient deoxyHb sites available to accommodate them all. As a consequence, the potential for regulation of still other erythrocyte pathways by O_2 via competition for the deoxyHb-binding site on band 3 is still not exhausted.

Experimental procedures

Generation of transgenic mice and blood analysis

All mouse studies were approved by the National Human Genome Research Institute Animal Care and Use Committee (protocol number (G-04-2)). The National Institutes of Health Intramural Research Program is accredited by Association for Assessment and Accreditation of Laboratory Animal Care International. Transgenic mice expressing a "humanized" band 3 with the human deoxyHb-binding site, a band 3 lacking deoxyHb-binding site, or a band 3 with high-affinity deoxyHb-

binding site were generated through standard homologous recombination techniques in embryonic stem cells as described previously (34). Detailed protocols are included in the supporting Materials and methods. Blood was collected by retro-orbital bleeding from 6–10-week-old WT mice or transgenic mice with homozygous band 3 mutations. Complete blood counts were performed on an automatic hematology analyzer (Siemens ADVIA 120 hematology system) following the manufacturer's instructions. Reticulocyte counts were determined using Syto RNASelect green fluorescent cell stain (Invitrogen). Osmotic fragility was determined by measuring hemolysis of erythrocytes placed in sodium chloride solutions of varying concentrations as described previously (77).

Tonometry

Blood was collected by retro-orbital bleeding and shipped on ice overnight prior to analysis. Red blood cells were washed and resuspended at 20% hematocrit in equilibration buffer (10 mM MOPS, 150 mM $NaNO_3$, 10 mM glucose, pH 7.4, 330 ± 5 mOsm). The samples were placed in tonometers (Eschweiler) and equilibrated with fully humidified air or N_2 for 20 min at 37 °C.

Analysis of O_2-dependent K^+ influx for NKCC1

NKCC1 activity was measured as bumetanide (NKCC specific inhibitor)-sensitive K^+ influx using $^{86}Rb^+$ as a tracer for K^+ as described previously (78). Oxygenated or deoxygenated RBCs from tonometers were diluted to a hematocrit of ~2% into equilibration buffer containing 7.5 mM of K^+ and 1 mM ouabain (pre-equilibrated at the same O_2 tension), with or without the presence of bumetanide (10 μM). $^{86}Rb^+$ was added into each sample to a final concentration of 1 μCi/ml. Influx was measured for 10 min at 37 °C with continuous equilibration of air or N_2. Influx was terminated by washing the cells with ice-cold isotonic $MgCl_2$ solution. The cells were then lysed in 0.1% Triton X-100, and hemoglobin was removed by 5% TCA precipitation. Cell $^{86}Rb^+$ was determined with a scintillation counter (PerkinElmer). NKCC1 activity was expressed as bumetanide-sensitive K^+ influx in mmol/liter of cells/h.

Analysis of O_2-dependent phosphorylation of erythrocyte NKCC1 and OSR1

Oxygenated or deoxygenated RBCs were lysed in 10 volumes of ice-cold 5 mM phosphate buffer containing 1 mM EDTA, pH 8.0, in the presence of phosphatase and protease inhibitors (Roche). The membrane fractions were pelleted at 25,000 × *g* for 30 min at 4 °C and separated by SDS-PAGE. NKCC1 was detected by immunoblotting using a monoclonal anti-NKCC1 antibody (Developmental Studies Hybridoma Bank). Phosphorylated NKCC1 was analyzed by using anti-phospho (Thr^{212}/Thr^{217})-NKCC1 antibody (Millipore). For analysis of OSR1, whole cell lysates in 1% Triton X-100 were separated by SDS-PAGE and immunoblotted with anti-OSR1 antibody (Developmental Studies Hybridoma Bank) to detect total OSR1 or with anti-phospho (Thr^{185})-OSR1 (Sigma) and anti-phospho (Ser^{325})-OSR1 (Millipore) to detect phosphorylated OSR1.

Immunoprecipitation and assay of WNK1

Preparation and assay of WNK1 were modified from the method published by Zagórska *et al.* (27). Briefly, oxygenated or deoxygenated RBCs were lysed in 10 volumes of lysis buffer (20 mM Tris-Cl, 150 mM NaCl, 1% NP-40, 1 mM EDTA, pH 7.5) containing protease and phosphatase inhibitors (Roche) and centrifuged at 16,000 × *g* for 10 min at 4 °C to remove the cell debris. Clear cell lysate was incubated with 4 μg of anti-WNK1 antibody (Abcam) and 30 μl of 50% slurry of protein A/G–agarose beads (Pierce) for 2 h at 4 °C with end-over-end rotation. The beads were then washed twice with lysis buffer containing 0.5 M NaCl and twice with kinase assay buffer (20 mM Tris-Cl, pH 7.4, 1 mM DTT). *In vitro* phosphorylation reactions were carried out in a final volume of 30 μl in kinase assay buffer containing 5 μM of recombinant kinase inactive GST-tagged OSR1 (D164A), 10 mM $MgCl_2$, and 0.1 mM ATP. After 30 min, reactions were terminated by adding SDS sample buffer. Phosphorylation of OSR1 was analyzed by immunoblotting with anti-phospho (Thr^{185})-OSR1 and anti-phospho (Ser^{325})-OSR1.

Immunofluorescent staining of WNK1 in intact erythrocytes

Immunofluorescent staining of WNK1 in oxygenated deoxygenated erythrocytes was performed as described previously (41). Briefly, deoxygenated erythrocytes were fixed in 1% formaldehyde under deoxygenated condition for 20 min and then further fixed in 0.5% acrolein (Sigma) for 5 min. Oxygenated erythrocytes were fixed in 0.5% acrolein for 5 min. Fixed erythrocytes were permeabilized in 0.1% Triton X-100 and blocked in 0.2% fish skin gelatin (Sigma) and then immunostained with anti-WNK1 (1:100 dilution; final concentration, 10 μg/ml), followed by Alexa Fluor 488–labeled secondary antibody (Jackson ImmunoResearch Laboratories).

GST-pulldown assay

GST-pulldown assays were performed as described previously (79). Briefly, clear lysates from HEK 293 cells expressing Myc-tagged WNK1 proteins were precleaned with GSH-agarose beads and GST protein and then incubated with 10 μg of cdb3–GST fusion proteins or GST and 50 μl of 50% slurry of GSH-agarose beads for 2 h at 4 °C with end-over-end mixing. After incubation, the beads were washed four times with the lysis buffer. Pelleted proteins were separated by SDS-PAGE and immunoblotted with anti-Myc antibody to detect WNK1 proteins.

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