Global transformation of erythrocyte properties via engagement of an SH2-like sequence in band 3

Estela Puchulu-Campanella\textsuperscript{a,b}, Francesco M. Turrini, Yen-Hsing Li\textsuperscript{a,b}, and Philip S. Low\textsuperscript{a,b,1}

\textsuperscript{a}Purdue Institute for Drug Discovery, Purdue University, West Lafayette, IN 47907; \textsuperscript{b}Department of Chemistry, Purdue University, West Lafayette, IN 47907; and \textsuperscript{1}Department of Oncology, University of Turin, 10126 Turin, Italy

Edited by Vincent T. Marchesi, Yale University School of Medicine, New Haven, CT, and approved October 12, 2016 (received for review July 20, 2016)

Src homology 2 (SH2) domains are composed of weakly conserved sequences of \( \sim 100 \) aa that bind phosphotyrosines in signaling proteins and thereby mediate intra- and intermolecular protein-protein interactions. In exploring the mechanism whereby tyrosine phosphorylation of the erythrocyte anion transporter, band 3, triggers membrane destabilization, vesiculation, and fragmentation, we discovered a SH2 signature motif positioned between membrane-spanning helices 4 and 5. Evidence that this exposed cytoplasmic sequence contributes to a functional SH2-like domain is provided by observations that: (i) it contains the most conserved sequence of SH2 domains, GSFLEVLR; (ii) it binds the tyrosine phosphorylated cytosolic domain of band 3 (cdb3-PO4) with \( K_{D} = 14 \) nM; (iii) binding of cdb3-PO4 to erythrocyte membranes is inhibited both by antibodies against the SH2 signature sequence and dephosphorylation of cdb3-PO4; (iv) label transfer experiments demonstrate the covalent transfer of photoactivatable biotin from isolated cdb3-PO4 (but not cdb3) to band 3 in erythrocyte membranes; and (v) phosphorylation-induced binding of cdb3-PO4 to the membrane-spanning domain of band 3 in intact cells causes global changes in membrane properties, including (i) displacement of a glycolytic enzyme complex from the membrane, (ii) inhibition of anion transport, and (iii) rupture of the band 3–ankyrin bridge connecting the spectrin-based cytoskeleton to the membrane. Because SH2-like motifs are not retrieved by normal homology searches for SH2 domain signature sequence in band 3, positioned in a cytoplasmic loop between two membrane-spanning helices. The experiments described below document the functionality of this remarkable SH2-like domain, summarizing its properties and describing the structural consequences of its docking with the tyrosine phosphorylated residues in the cytoplasmic domain of band 3.

Results

Tyrosine Phosphorylation of Intact Band 3 but Not the Isolated Cytoplasmic Domain of Band 3 Reduces Its Binding Affinity for Ankyrin.

Our motivation to search for a possible SH2-like domain in band 3 arose from two apparently contradictory observations. First, ankyrin

P

rotein tyrosine phosphorylation is involved in the regulation of most cellular process, including proliferation, survival, differentiation, responses to stress, and control of cell shape/motility (1). Whereas phosphotyrosine binding (PTB) domains may mediate association with tyrosine-containing sequences regardless of their phosphorylation state, Src homology 2 (SH2) domains promote protein association only when critical tyrosine residues become phosphorylated. This strict dependence on tyrosine phosphorylation creates a molecular switch that can be sensibly controlled by upstream tyrosine kinases (1). Whereas some SH2 domains facilitate association between heterologous proteins in a signaling pathway, others mediate intrapolyptide interactions that change protein conformation and thereby alter signaling function (2). In all cases, association between the SH2 domain and the interacting phosphotyrosine involves formation of weak interactions between a highly conserved G(S/T)FLVLR sequence of the SH2 domain and the phosphate present on the phosphorylated tyrosine. To date, all 120 known SH2 domains in the human genome are composed of moderately conserved sequences of \( \sim 100 \) contiguous aa (3), and no functional SH2 domain has been reported that contains only the critically conserved residues interrupted by unrelated sequences.

Band 3 (the erythrocyte anion exchanger, AE1, or SLC4A1) constitutes the most abundant polypeptide in the erythrocyte membrane \((\sim 1.2 \times 10^{6} \text{ copies per cell})\) where it serves as a major center of membrane organization (4). The cytoplasmic domain of band 3 (cdb3) binds and organizes at least 13 different peripheral proteins on the membrane, whereas the membrane-spanning domain catalyzes anion transport and binds a different constellation of membrane proteins. Proteins believed to interact directly with band 3 include most glycolytic enzymes, multiple kinases, two phosphatases, protein 41, protein 42, adducin, ankyrin, carbonic anhydrase, hemoglobin, peroxiredoxin, RH polyepitope, CD47, and glycophorins A and B (5–14). As anticipated, physiological modulations of band 3 regulate processes involving these proteins, including glucose metabolism, membrane mechanical stability, cell morphology, intracellular redox balance, cytoplasmic pH, and anion and cation transport across the membrane (15–20). Included among the stimuli that regulate band 3 functions is the phosphorylation of band 3 on tyrosines 8, 21, 359, and 904 (21, 22). These phosphorylation events control the partitioning of glucose metabolism between glycolysis and the pentose phosphate pathway (15), regulate the linkage between band 3 and the spectrin-based cytoskeleton, and modulate cell morphology/stability (22). In our search for a mechanism to explain these multifarious effects, we discovered a highly conserved SH2 domain signature sequence in band 3, positioned in a cytoplasmic loop between two membrane-spanning helices. The experiments described below document the functionality of this remarkable SH2-like domain, summarizing its properties and describing the structural consequences of its docking with the tyrosine phosphorylated residues in the cytoplasmic domain of band 3.

Significance

Src homology 2 (SH2) domains regulate signaling by binding phosphotyrosines. While all known SH2 domains consist of contiguous sequences of \( \sim 100 \) aa, we describe a highly conserved SH2-like sequence motif located in the second cytoplasmic loop of membrane-spanning domain of erythrocyte membrane band 3. Upon tyrosine phosphorylation of the cytoplasmic domain of band 3, the domain rotates to bind the SH2 signature sequence within the membrane-spanning domain of band 3, promoting significant changes in erythrocyte properties. Because sequence homology searches do not recognize SH2-like sequence motifs, the possibility arises that many other SH2-like structures remain undetected in membrane-spanning proteins where they perform critical steps in kinase-facilitated signaling pathways.

Author contributions: E.P.-C., F.M.T., and P.S.L. designed research; E.P.-C. and Y.-H.L. performed research; E.P.-C., F.M.T., and P.S.L. analyzed data; and E.P.-C. and P.S.L. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

1To whom correspondence should be addressed. Email: plow@purdue.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1611904113/-/DCSupplemental.
binding to the cytoplasmic domain of band 3 (cdb3) in potassium iodide (KI)-striped inside-out erythrocyte membrane vesicles (KI-IOVs) was found to be partially inhibited by tyrosine phosphorylation of band 3, whereas binding of ankyrin to the isolated dimeric cdb3 was not. Thus, as shown in Fig. 1, titration of the D3D4 domain of ankyrin into a suspension of KI-IOVs derived from unmodified human erythrocytes exhibits cooperative binding yielding a binding constant of ∼70 nM, whereas the same binding isotherm for D3D4–ankyrin association with KI-IOVs derived from o-vanadate-treated RBCs results in a K_d of ∼60 nM (Fig. 1B); i.e., demonstrating that o-vanadate induced tyrosine phosphorylation of band 3 reduces ankyrin affinity for KI-IOVs. Evidence that this decrease in ankyrin affinity indeed derives from tyrosine phosphorylation of band 3 is provided both by blots showing that the loss of ankyrin affinity correlates with the phosphorylation of band 3 (Fig. 1A) and that the phosphorylation-induced decrease in ankyrin affinity is reversed upon removal of the tyrosine phosphates by phosphatases (K_d ∼85 nM; Fig. 1A and B). Importantly, these data are congruent with previously published observations demonstrating that phosphorylation of Y8 and Y21 of band 3 by spleen tyrosine kinase (SYK) blocks ankyrin binding to band 3, both in intact RBCs and in KI-IOVs (22).

The aforementioned conflicting observation arose when the phosphorylation-induced inhibition of ankyrin binding to band 3 was examined using the purified cdb3 (i.e., the domain to which ankyrin binds) (6) rather than intact band 3 in KI-IOVs. Thus, as shown in Fig. 1C, tyrosine phosphorylation of purified cdb3 by recombinant SYK kinase (i.e., the major tyrosine kinase that phosphorylates band 3 in vivo) (14) has no impact on the binding of D3D4–ankyrin (23) to cdb3, as demonstrated by the similar ability of phosphorylated and nonphosphorylated cdb3 to copellet D3D4–ankyrin in standard pull-down assays.

**Discovery of a Functional SH2-Like Sequence in Band 3.** To determine why tyrosine phosphorylation might inhibit band 3–ankyrin interactions in both whole RBCs and KI-IOVs, but not when purified phospho-cdb3 (cdb3-PO4) was used as the binding partner, we searched for an SH2 domain on the erythrocyte membrane that might bind cdb3-PO4 in situ but be absent in a binding assay containing only purified cdb3. The signature sequence for which we screened was GSFLVR, because this sequence is the most highly conserved among all 120 known human SH2 domains (24) and is shown to be essential for phosphorytosine binding (24, 25). Importantly, the only KI-IOV protein retrieved in the homology search was band 3 (Fig. 2A and Fig. S1), and the highly conserved 509–GSFLVR–514 peptide was surprisingly found in an exposed region of the cytoplasmic loop connecting membrane-spanning helices 4 and 5 (Fig. S2) (26, 27). Moreover, the weakly conserved sequences flanking GSFLVR in classical SH2 domains were absent from the sequences flanking 509–GSFLVR–514 in band 3. Although the probability that the GSFLVR sequence could have evolved by chance is only 1 in 20^6 (i.e., there are six residues and each has a random chance of 1/20 of being the conserved amino acid), the absence of the expected flanking sequences in a classical SH2 domain mandated that we demonstrate the functionality of this hypothesized SH2-like domain by standard methods.

To explore the functional properties of the putative SH2 domain signature sequence, we prepared stripped and trypsin-digested inside-out erythrocyte membrane vesicles (SD-IOVs) that lack all peripheral proteins as well as the cdb3, but still contain a functional membrane-spanning domain of band 3. As shown in Fig. 2B, addition of increasing concentrations of unmodified cdb3 to these SD-IOVs showed a low affinity interaction with an apparent K_d of ∼70 nM. In contrast, titration of SYK-phosphorylated cdb3 (cdb3-PO4) into the same SD-IOVs produced an analogous binding isotherm with an apparent K_d of ∼14 nM. Because dephosphorylation of cdb3-PO4 by treatment with the tyrosine phosphatase PTP1B reduced the high-affinity interaction to the same level seen with nonphosphorylated cdb3 (i.e., K_d ∼155 nM), we conclude that the high-affinity association of cdb3-PO4 requires tyrosine phosphorylation of band 3. Importantly, similar results were also obtained using a pull-down assay (Fig. 2C and D), confirming that the high-affinity binding of cdb3 to SD-IOVs requires the SYK-mediated tyrosine phosphorylation of band 3.

To further establish the presence of a functional SH2-like sequence in band 3, we next prepared an antibody against the cytoplasmic loop in the membrane-spanning domain of band 3 containing the GSFLVR signature sequence (507–EGFSFLVR–FISRYTQE–522) and examined its ability to block association of cdb3-PO4 with SD-IOVs. As seen in Fig. 3A, the prominent polypeptide recognized by this anti-SH2 peptide antiserum in erythrocyte membranes is band 3 (lane 2), as demonstrated by the staining of the same polypeptide with a monoclonal antibody to band 3 (lane 3). However, because two additional proteins were also recognized by the anti-SH2 peptide antiserum, it was essential that we include the preimmune serum that recognizes the same two additional proteins as a control in the studies using the anti-SH2 signature sequence antiserum. Importantly, preincubation of the anti-SH2 signature sequence antiserum with SD-IOVs was found to reduce cdb3-PO4 binding to a level similar to nonphosphorylated cdb3 (Fig. 3B), whereas preincubation with the preimmune serum from the same rabbit caused no decrease in cdb3-PO4 binding. These data also support the hypothesis that the high-affinity cdb3-PO4 binding site on SD-IOVs resides in the discontinuous SH2 domain of band 3.
SBED connecting cdb3-PO4 to its unknown binding partner on the mitaminating to catalyze photocross-linking, the disulfide bridge in sulfo-cdb3-PO4 to the membrane-spanning domain of band 3. After the presence of SD-IOVs might mediate phototransfer of biotin from scientific Pierce) and examined whether its photoactivation in the biotinylated photocross-linking reagent, sulfo-SBED (Thermo Scientific) was blocked with either anti-SH2 signature sequence antibody (Fig. 3C, lane 2) or an ~10-fold excess of unlabeled cdb3-PO4 (Fig. 3C, lane 3) before incubation with sulfo-SBED-labeled cdb3-PO4. As shown in lanes 2 and 3, both the anti-SH2 signature sequence antisera and excess cdb3-PO4 1 successfully competed for cdb3-PO4 cross-linking to SD-IOVs, i.e., confirming that cdb3-PO4 is indeed selectively binding to the SH2 domain signature motif in band 3 and not some other erythrocyte membrane protein. Based on all of these results, we conclude that tyrosine phosphorylation of cdb3 promotes its association with an SH2-like domain within the membrane-spanning domain of band 3.

Impact of cdb3-PO4 Binding to the SH2 Domain Signature Motif of Band 3 on Erythrocyte Properties. To explore whether engagement of the SH2-like motif by phosphorylated cdb3 might impact important properties of the human erythrocyte, we induced tyrosine phosphorylation in intact erythrocytes and examined its impact on erythrocyte biology. As shown in Fig. 4, lane 1, addition of pervanadate to whole erythrocytes results in prominent tyrosine phosphorylation of band 3. Because this phosphorylation can be quantitatively prevented by pretreatment with SYK kinase inhibitors (SYK inh.II, PRT06607, R406, and imatinib; Fig. 4, lanes 2–5), we conclude that the major tyrosine kinase responsible for the pervanadate-induced phosphorylation of band 3 is likely SYK, although the fact that each of the above inhibitors undoubtedly has off-target activities against other tyrosine kinases precludes any conclusion that SYK must be the only kinase involved. Examination of the morphology of the

To further confirm the interaction of cdb3-PO4, with the SH2 domain signature motif of band 3, we labeled cdb3-PO4 with the biotinylated photocross-linking reagent, sulfo-SBED (Thermo Scientific Pierce) and examined whether its photoactivation in the presence of SD-IOVs might mediate phototransfer of biotin from cdb3-PO4 to the membrane-spanning domain of band 3. After washing the SD-IOVs to remove unbound cdb3-PO4 and then illuminating to catalyze photocross-linking, the disulfide bridge in sulfo-SBED connecting cdb3-PO4 to its unknown binding partner on the membrane was reduced and the identity of this biotinylated partner on the membrane was determined by Western blotting. As seen in Fig. 3C, the predominant biotinylated partner (lane 1 in streptavidin-HRP) was band 3, as confirmed by subsequent staining with anti-band 3 monoclonal antibody. To more conclusively establish these results, parallel experiments were performed in which the SH2 domain was blocked with either anti-SH2 signature sequence antibody (Fig. 3C, lane 2) or an ~10-fold excess of unlabeled cdb3-PO4 (Fig. 3C, lane 3) before incubation with sulfo-SBED-labeled cdb3-PO4. As shown in lanes 2 and 3, both the anti-SH2 signature sequence antisera and excess cdb3-PO4 1 successfully competed for cdb3-PO4 cross-linking to SD-IOVs, i.e., confirming that cdb3-PO4 is indeed selectively binding to the SH2 domain signature motif in band 3 and not some other erythrocyte membrane protein. Based on all of these results, we conclude that tyrosine phosphorylation of cdb3 promotes its association with an SH2-like domain within the membrane-spanning domain of band 3.
glycolytic enzymes (Fig. S3) from the membranes of only tyrosine phosphorylated cells, the SH2-like motif-mediated conformational change in band 3 can be concluded to also impact the phosphorylation of band 3 (28) might be regulated by tyrosine phosphorylation of SYK-mediated tyrosine phosphorylation of band 3 is linked to the observed membrane destabilization.

To pursue the relevance of SH2-like motif engagement to still other erythrocyte properties, we next examined whether the organization of the well-characterized glycolytic enzyme complex on band 3 (28) might be regulated by tyrosine phosphorylation of band 3. As seen by displacement of GAPDH (Fig. 4C, pervanadate column), we conclude that SYK-mediated tyrosine phosphorylation of band 3 is linked to the observed membrane destabilization.

Because another major function of band 3 involves its catalysis for HCO₃⁻ exchange across the membrane (i.e., a process required for transport of CO₂ as HCO₃⁻ from the tissues to the lungs), we next examined the effect of tyrosine phosphorylation on anion exchange through band 3. As shown in Fig. 4D, tyrosine phosphorylation of band 3 (promoted by resealing of SYK kinase plus ATP into lysed erythrocytes) causes complete inhibition of anion transport (i.e., the level of anion exchange across the resealed erythrocyte membrane is reduced to a level seen in DIDS-inhibited samples) (29). Moreover, prevention of the tyrosine phosphorylation by preaddition of SYK kinase inhibitors prevents this blockade of anion transport. Furthermore, resealing of the anti-SH2 domain antibody (but not the preimmune serum) into the lysed red cells eliminates the kinase-mediated inhibition of anion transport. Taken together, these data suggest that the phosphorylation-induced engagement of the SH2 domain signature sequence in band 3 with cdb3-PO₄ also induces changes in anion transport.

Based on all of the above studies, we wish to propose a mechanism to explain the phosphorylation-stimulated changes in erythrocyte membrane properties. Upon administration of pervanadate (a potent tyrosine phosphatase inhibitor), the constitutive equilibrium in tyrosine phosphorylation of band 3 shifts strongly toward the phosphorylated state; i.e., tyrosines 8, 21, 359, and 904 in band 3 all become phosphorylated (30). As cartooned in Fig. 4E, this phosphorylation promotes the interaction of cdb3-PO₄ (and perhaps phosphotyrosine 904 at the cytoplasmically exposed COOH terminus in the membrane-spanning domain of band 3) with the SH2-like motif in the membrane-spanning domain of band 3, inducing a global conformational change in the anion transporter that causes release of glycolytic enzymes (21), displacement of ankyrin from cdb3 (22), and inhibition of anion transport. Whereas dissociation of both the glycolytic enzymes and ankyrin from cdb3 can be explained as logical consequences of the induced reorientation/conformation of cdb3, the inhibition of anion transport may be more complex. Thus, the amino acid sequence that encompasses the SH2 signature motif specifically forms the bridge that precisely

---

**Fig. 4.** Tyrosine phosphorylation of band 3 induces shape changes in erythrocytes, dissociation of glycolytic enzymes from the membrane, and inhibition of anion exchange across the membrane. (A) Western blot showing induction of band 3 tyrosine phosphorylation by 5 μM pervanadate (lane 1) and its prevention by preincubation with 10 μM SYK inhibitor II (lane 2), 10 μM R406 (lane 3), 10 μM PRT062607 (lane 4), or 20 μM imatinib (lane 5). (B) Erythrocyte shape changes induced by 5 μM pervanadate include echinocytes and spherocytes. (C) Erythrocyte shape changes (Top row) and displacement of glyceroldehyde-3-phosphate dehydrogenase from the membrane (Bottom row) only occur in erythrocytes in which pervanadate has induced tyrosine phosphorylation of the membrane (Middle row). Analogous findings with lactate dehydrogenase are shown in Fig. S3. Tracings of anion exchange in lysed erythrocytes following resealing in the presence of SYK kinase + ATP, SYK kinase + SYK kinase inhibitor II, and/or SYK kinase + anti-SH2 antisera, as indicated. Preimmune serum is used as a control for treatment with anti-SH2 domain antisera. DIDS blocks all 3-mediated anion transport. (E) Proposed mechanism of regulation of erythrocyte properties by tyrosine phosphorylation of band 3 and engagement of an SH2-like motif in msdb3.
spans between the two halves of the membrane-spanning domain of band 3. Because these two halves of band 3 must pivot back and forth to catalyze anion transport (26, 27), binding of cdb3-PO4 solely to one of the two conformations would be expected to lock the anion transporter in that conformation and thereby inhibit anion transport.

Based on analysis of the crystal structure of band 3 (26, 27), the GSFLVR bridging peptide can be seen to exist in an α-helical conformation when the transport site faces outwards, but must extend to an elongated conformation when the transport site opens up to the cytoplasm. Given that the GSFLVR sequence in SH2 domains exists in an elongated conformation, one would predict that cdb3-PO4 (or phosphorylated Y904) might only bind to the inward facing conformation of band 3.

Discussion

We report the existence of a functional SH2-like motif that resides in a cytoplasmic loop between consecutive helices of a membrane-spanning protein. We further show that docking of this SH2 signature sequence with the tyrosine phosphorylated cdb3 induces major changes in the structure and function of the whole membrane, including dissociation of a glycolytic enzyme complex from the membrane, displacement of ankyrin (i.e., the major bridge connecting the membrane to the spectrin-based cytoskeleton) from band 3, and inhibition of anion transport through band 3. Release of the glycolytic enzyme complex from the membrane has been previously shown to shift the catabolism of glucose from the pentose phosphate pathway to glycolysis (15), whereas disjunction of ankyrin from band 3 has been found to significantly destabilize the membrane (22).

Inhibition of anion transport not only suppresses transport of CO2 as HCO3− from the tissues to the lungs, but also affects intracellular pH and the volume of the red cells (20). Whether other red cell properties are similarly modulated by tyrosine phosphorylation of band 3 will require further scrutiny, but the fact that the cdb3 is known to interact with at least 13 other proteins should render this conjecture highly likely.

Although the SH2 docking mechanism can account for the data presented here, it must also be recognized that the proposed mechanism is not unique in its ability to explain our observations. First, whereas we have focused on the interaction between cdb3-PO4 and the SH2 sequence motif, it is also plausible that phosphorylated Y904 at the COOH terminus of band 3 could similarly dock with the SH2 sequence motif. Thus, Y904 resides in a cytoplasmically exposed, unstructured peptide (i.e., the COOH-terminus of the major bridge connecting the membrane to the spectrin-based cytoskeleton) from band 3, and inhibition of anion transport through band 3. Release of the glycolytic enzyme complex from the membrane has been previously shown to shift the catabolism of glucose from the pentose phosphate pathway to glycolysis (15), whereas disjunction of ankyrin from band 3 has been found to significantly destabilize the membrane (22).

Inhibition of anion transport not only suppresses transport of CO2 as HCO3− from the tissues to the lungs, but also affects intracellular pH and the volume of the red cells (20). Whether other red cell properties are similarly modulated by tyrosine phosphorylation of band 3 will require further scrutiny, but the fact that the cdb3 is known to interact with at least 13 other proteins should render this conjecture highly likely.

Although the SH2 docking mechanism can account for the data presented here, it must also be recognized that the proposed mechanism is not unique in its ability to explain our observations. First, whereas we have focused on the interaction between cdb3-PO4 and the SH2 sequence motif, it is also plausible that phosphorylated Y904 at the COOH terminus of band 3 could similarly dock with the SH2 sequence motif. Thus, Y904 resides in a cytoplasmically exposed, unstructured peptide (i.e., the COOH-terminal residues 883–911 are not resolved in the crystal structure) whose sequence (DEYED) is similar to the peptide containing Y8 (DDYED) and Y21 (EEYED). Importantly, if phosphorylated Y904 were to bind the SH2 motif, the biological consequences would likely be limited to inhibition of anion transport, because the conformation/orientation of cdb3 would remain unaltered. In this scenario, a kinase that phosphorylated solely Y904 could regulate anion transport without altering glycolysis or membrane stability. Second, because band 3 exists in an equilibrium between this scenario, a kinase that phosphorylated solely Y904 could regulate anion transport without altering glycolysis or membrane stability. Therefore, because the GSFLVR sequence in SH2 domains exists in an elongated conformation, one would predict that cdb3-PO4 (or phosphorylated Y904) might only bind to the inward facing conformation of band 3.

Discussion

We report the existence of a functional SH2-like motif that resides in a cytoplasmic loop between consecutive helices of a membrane-spanning protein. We further show that docking of this SH2 signature sequence with the tyrosine phosphorylated cdb3 induces major changes in the structure and function of the whole membrane, including dissociation of a glycolytic enzyme complex from the membrane, displacement of ankyrin (i.e., the major bridge connecting the membrane to the spectrin-based cytoskeleton) from band 3, and inhibition of anion transport through band 3. Release of the glycolytic enzyme complex from the membrane has been previously shown to shift the catabolism of glucose from the pentose phosphate pathway to glycolysis (15), whereas disjunction of ankyrin from band 3 has been found to significantly destabilize the membrane (22).

Inhibition of anion transport not only suppresses transport of CO2 as HCO3− from the tissues to the lungs, but also affects intracellular pH and the volume of the red cells (20). Whether other red cell properties are similarly modulated by tyrosine phosphorylation of band 3 will require further scrutiny, but the fact that the cdb3 is known to interact with at least 13 other proteins should render this conjecture highly likely.

Although the SH2 docking mechanism can account for the data presented here, it must also be recognized that the proposed mechanism is not unique in its ability to explain our observations. First, whereas we have focused on the interaction between cdb3-PO4 and the SH2 sequence motif, it is also plausible that phosphorylated Y904 at the COOH terminus of band 3 could similarly dock with the SH2 sequence motif. Thus, Y904 resides in a cytoplasmically exposed, unstructured peptide (i.e., the COOH-terminal residues 883–911 are not resolved in the crystal structure) whose sequence (DEYED) is similar to the peptide containing Y8 (DDYED) and Y21 (EEYED). Importantly, if phosphorylated Y904 were to bind the SH2 motif, the biological consequences would likely be limited to inhibition of anion transport, because the conformation/orientation of cdb3 would remain unaltered. In this scenario, a kinase that phosphorylated solely Y904 could regulate anion transport without altering glycolysis or membrane stability. Second, because band 3 exists in an equilibrium between this scenario, a kinase that phosphorylated solely Y904 could regulate anion transport without altering glycolysis or membrane stability. Therefore, because the GSFLVR sequence in SH2 domains exists in an elongated conformation, one would predict that cdb3-PO4 (or phosphorylated Y904) might only bind to the inward facing conformation of band 3.
Preparation of SD-IOVs. Membranes were prepared using established protocols (6). IOVs were then prepared as described in SI Materials and Methods and stripped by incubation in 1 M KI (6) or 0.1 M EDTA, pH 11 (19). When required, stripped IOVs were trypsinized following established protocols (19).

Determination of the Affinity of Ankyrin for cdb3 or p-cdb3. The affinity of cdb3-PO4, p-cdb3, and cdb3 for the SH2 domain contained in SD-IOVs was determined using an ELISA type of assay and it is described in SI Materials and Methods.

Administration of binding of cdb3-PO4 to (SD-IOV) by anti-SH2 signature sequence antisera and identification of the polypeptide in erythrocyte membranes that binds sulfo-SBED-cdb3-PO4 were both performed as described in SI Materials and Methods.

Methods. Sulfo-SBED derivatization of cdb3-PO4, untagged-cdb3-PO4 was derivatized with sulfo-SBED in the dark following a previously published protocol (S, 28).

Determination of the Rate Band 3 Catalyzed Chloride Exchange Across the Erythrocyte Membrane. The rate of chloride exchange was measured following the fluorescence dequenching of CL–6–methoxy-N–(3-sulfopropyl)quinolinium (SPQ; Setareh Biotech) according to the method of Illisly and Verkman (52) with a few modifications described in SI Materials and Methods.

Confocal Microscopy and Immunoblot Analysis. Erythrocytes were processed as described in SI Materials and Methods.

Acknowledgments. We thank Ann Liu and Karson Putt for assistance in editing this manuscript. This work was supported by NIH Grant GM024417-36.
Cdb3 containing a COOH-terminal His-Tag was di-
to SD-IOVs by Anti-SH2 Signature 1o f5 strain BL21 (DE3)/pLysS using a GST-Trap-HP BioRad sample

1.5 mM KH-phosphate buffer (50 mM Hepes pH 7.4, 2 mM EDTA, 3 mM DTT, and 1.5 mM sodium azide, pH 8.0). Equal concentrations of control, phosphorylated, or dephosphorylated KI-IOV, as determined by Bradford assay, were mixed with increasing concentrations of GST-D3D4-ankyrin in binding buffer containing 2 mg/mL BSA and incubated overnight with rotation at 4 °C. Next, samples were pelleted, the supernatants discarded, and pellets were washed twice in binding buffer with BSA and once in the same buffer without BSA before quanti-
tation using a GST Colorimetric Activity Assay Kit (Novagen) and a Beckman Coulter 640 spectrophotometer.

Cdb3 Phosphorylation and Dephosphorylation Assays. Cdb3 was phosphorylated by incubation at 30 °C for 45 min with GST-tagged SYK tyrosine kinase (Promega) in 50 mM Tris buffer, pH 7.5, containing 20 mM MgCl₂, 1.5 mM ATP, 5 mM MnCl₂, and 1 mM DTT. The reaction was stopped by adding 10 μM SYK inhibitor II. For dephosphorylation, aliquots of phosphorylated cdb3 (cdB3-Po4) were dialyzed into tyrosine phosphatase assay buffer (50 mM Hepes pH 7.4, 2 mM EDTA, 3 mM DTT, and 100 mM NaCl) and incubated at 37 °C for 60 min with PTP1B (Sigma) in a 1:4 ratio of phosphatase-to-phosphorylated cdb3.

Analysis of the Affinity of Ankyrin for cdb3 or p-cdb3. Phosphory-
lated or unmodified His-tagged-cdb3 (2 μM) was mixed with 2 μM purified GST-D3D4-ankyrin and incubated at 4 °C overnight in 7.5 mM phosphate buffer, pH 7, containing 10% (wt/vol) sucrose, 90 mM KCl, 10 mM imidazole, 0.4 mM phenyl-
methylsulfonyl fluoride, and 1 mg/mL BSA. Preequilibrated Ni-
NTA beads were incubated with the mixture for 30 min at 4 °C and washed twice in the above buffer, followed by two washes in the same buffer lacking BSA. The bound complexes of His-tagged-
cdb3-ankyrin were eluted with 200 mM imidazole and mixed with BioRad sample buffer (1:1) before electrophoresis in a 10% (wt/vol) polyacrylamide gel. After SDS/PAGE separation and transfer to a nitrocellulose membrane, the membrane was stained and the desired protein bands were visualized using polyclonal antiankyrin and anti-
tiphosphotyrosine. Analysis of blots was performed with a ChemiDoc Imaging System using Image Lab software (Bio-Rad).

Preparation of SD-IOVs. Fresh blood from multiple healthy donors was washed and erythrocyte membranes (ghosts) were prepared as described in the main text (6). IOVs were then prepared by washing the ghosts in 0.1 mM EDTA pH 8.0 containing 0.5 mM DTT and incubating them in 40 volumes of the same buffer on ice for 30 min. The membranes were then incubated at 37 °C for 45 min with shaking, and the resulting vesicles were collected by centrifuging at 45,000 × g for 60 min at 4 °C. Vesicles were stripped by either incubation in 1 M KI or 0.1 mM EDTA, pH 11. Stripped vesicles were washed in 5 mM sodium phosphate, pH 8.0, 1 mM EDTA, 0.4 mM phenylmethylsulfonyl fluoride, and used immediately or supplemented with 10 mg/mL sucrose and frozen in liquid nitrogen for later use.

Determination of the Affinity of cdb3-Po4, d-cdb3, and cdb3 for Band 3 in SD-IOVs. The affinity of cdb3-Po4, d-cdb3, and cdb3 for the SH2 domain contained in SD-IOVs was determined using 96-well plates (Costar) coated with SD-IOVs. Plates were coated by incubation of 20 μg/mL (protein content) SD-IOVs in the wells overnight at 4 °C. Coated plates were washed twice in Hepes buffer (25 mM Hepes, pH 7.5, containing 100 mM NaCl, 10 mM MgCl₂, 0.5 mM o-vanadate, 0.4 mM phenylmethylsulfonyl fluo-
ride, and 7 mM sodium azide), blocked with 2 mg/mL BSA, and incubated with soluble untagged cdb3-Po4, d-cdb3, or cdb3 in 1 mg/mL BSA overnight. Plates were again washed four times with 1 mg/mL BSA at 4 °C before incubation with anti-cdb3 in 1 mg/mL BSA for 60 min at 20 °C. After three additional washes without PMSF and azide, plates were incubated with anti-mouse HRP for 20 min at 20 °C and then washed three times and visualized with 3, 3′, 5, 5′ tetramethyl benzidine (TMB) peroxidase substrate from Biolegend. Reaction was stopped by addition of stop solution (Biolegend) and the amount of bound cdb3, d-cdb3, or cdb3-Po4 was finally determined by measuring the intensity of peroxidase product formation at 450 nm using a VersaMax ELISA microplate reader with SoftMax Pro Software (Molecular Devices).

Blocking of Binding of cdb3-Po4 to SD-IOVs by Anti-SH2 Signature Sequence Antiserum. SD-IOVs were incubated overnight on ice with different concentrations of anti-SH2 antiserum in the above Hepes buffer. Aliquots were then mixed on ice for 15 min before incubation overnight on ice with agitation. Aliquots were next rinsed three times in the same buffer before addition of 4× BioRad sample buffer and separation by SD/PAGE. Separated samples were then transferred to nitrocellulose and probed with either anti-phospho-
ryosine antibody or anti-cdb3 antibody before incubation with anti-
mouse–HRP. Analysis of the blots was performed with a ChemiDoc Imaging System using Image Lab software (Bio-Rad).

Sulfo-SBED Derivatization of cdb3-Po4. Briefly, cdb3-Po4 was di-
yzed into the above Hepes buffer and incubated at 20 °C in the dark with sulfo-SBED (Thermo Fisher) at a 1:1 molar ratio with rotation for 30 min. Upon completion of derivatization, cdb3-
Po4-sulfo-SBED was washed three times in the dark at 4 °C with Hepes buffer using an Amicon Ultra 0.5 mL 10-kDa filter.

Identification of the Polypeptide in Erythrocyte Membranes That Binds Sulfo-SEBD-cdb3-Po4. Sodium hydroxide stripped IOVs were incubated overnight in the dark on ice in Hepes buffer...
containing 2 mg/mL BSA. When desired, some IOV aliquots were subsequently blocked with anti-SH2 antisera for 4–6 h before addition of the sulfo-SBED-labeled cdb3. In other cases, the stripped IOVs were mixed simultaneously with the sulfo-SBED cdb3-PO4 in the presence of a 10-fold excess of unlabeled cdb3-PO4. Next, all samples were incubated on ice with agitation for 30 min before a subsequent overnight incubation on ice. Samples were then spun at 22,000 × g at 4 °C to pellet the IOVs, and pellets were washed twice in Hepes buffer containing 1 mg/mL BSA and twice in Hepes lacking BSA before incubating with 300 nm UV light on ice for 15 min. All samples were resuspended and incubated for 40 min at 37 °C in 1 mL of 1 M KI containing protease inhibitor mixture (Roche), and 100 mM DTT. Samples were then washed once in 5 mM sodium phosphate buffer, pH 8.0, containing 1 mM EDTA, protease inhibitors and phosphatase inhibitors before resuspending in BioRad sample buffer containing 100 mM DTT. Finally, samples were separated electrophoretically in a 10% (wt/vol) SDS/PAGE gel, transferred to nitrocellulose, and probed with streptavidin-HRP or anti-band 3 (gift from Michael Jennings, University of Arkansas, Little Rock, AR).

**Determination of the Rate Band 3 Catalyzed Chloride Exchange Across the Erythrocyte Membrane.** The rate of chloride exchange was measured following the fluorescence dequenching of CL−6-methoxy-N(-3-sulfopropyl)quinolinium (SPQ; Setareh Biotech) according to the method in ref. 52. Briefly, leaky membranes prepared as above, were treated on ice under low-light conditions with SYK tyrosine kinase and/or SYK kinase inhibitors and anti-SH2 antisera or preimmune serum. SPQ, 50 mM Hepes-Tris, pH 7.0, 5 mM Mg2+, 1.0 mM ATP, and chloride up to a final concentration of 100 mM KCl (similar isotonicity as buffer A) were added before rescaling for 45 min at 37 °C. Samples were washed three times in buffer A (50 mM Hepes-Tris pH 7.0 containing 100 mM sucrose and 100 mM KCl) and then an aliquot of 5 μL was added quickly to 200 μL cuvette fill with buffer B [50 mM Hepes-Tris, pH 7.0, containing 100 mM sucrose and 66 mM K2SO4 ± 0.3 mM H2DIDS (purchased from Setareh Biotech)] and the fluorescence dequenching of SPQ was monitored as Cl− (the quenching agent) exchanges for SO4−2 through band 3 using a Cary Eclipse fluorescence spectrophotometer (Agilent). The solubility of SPQ can change over time leading to poor assay performance. Therefore, SPQ was kept under N2 and stored at −20 °C.

**Confocal Microscopy and Immunoblot Analysis.** Erythrocytes were washed three times in PBS containing 5 mM glucose. Cells were resuspended at 30% Hct in the same buffer and aliquots incubated at 37 °C for 60 min with either PBS or the selected SYK inhibitors: 20 μM imatinib mesylate (SAS Alsachim-Bioparc), 10 μM PRT062607 (Selleckchem), or 10 μM SYK inhibitor II (EMD Millipore). To induce tyrosine phosphorylation, 5 μM pervanadate was added to each cell suspension and cells were incubated at 37 °C. All incubations, washing, and aliquot processing were performed in the dark to protect SYK inhibitor II from photoinactivation. Aliquots of 50 μL were removed and fixed for immunofluorescence microscopy in 1 mL fresh-made 0.5% acrolein in PBS for 5 min or plunged into ice-cold hemolysis buffer containing 0.5 mM o-vanadate and protease inhibitors for preparation of membranes and Western blot analysis. Fixed cells were washed three times in PBS containing 100 mM glycerine and 7 mM sodium azide. Next, cells were permeabilized in the same buffer containing 0.1% Triton X-100. Fixed and permeabilized cells were incubated in PBS containing 50 mM glycine, 7 mM sodium azide, and 0.2% cold fish gelatin for 60 min prior to staining with the antibodies. Antibodies used were a monoclonal anti-phosphotyrosine or a polyclonal anti-GAPDH or anti-LDH. Imaging was performed on an Olympus FluoViewFV1000.

Membranes for SDS/PAGE and Western blotting analyses were collected by centrifugation at 22,000 × g for 20 min at 4 °C and washed once in the same hemolysis buffer before resuspending in Bio-Rad sample buffer (1:1) and separation by SDS/PAGE on 10% (wt/vol) polyacrylamide gels. Separated proteins were transferred to nitrocellulose and probed with monoclonal anti-phosphotyrosine. Analysis of blots was performed as described above.
Fig. S1. Alignment of residues surrounding the GSFLVR sequence in band 3 with established SH2 domains present in enzymes, adaptors, scaffolds, and signal regulators in the human genome. Sequence alignment was performed with Geneious version 5.5.7 (www.geneious.com, using an identity matrix global alignment with free end gaps. Colors used to reveal aligned sequences constitute the default colors used by the ClustalX program, where amino acids labeled with the respective colors are orange (G, P, S, and T), red (H, K, and R), blue (F, W, and Y), and green (I, L, M, and V).
Fig. S2. Structure of the membrane-spanning domain of band 3 showing the location of the conserved SH2 domain sequence (GSFLVR; colored in magenta in A) that forms part of the cytoplasmic loop connecting membrane-spanning helices 4 and 5. This cytoplasmic loop also connects the “core” and “gate” domains of band 3 that must pivot to open and close the anion channel (double-pointed arrow). The most NH$_2$-terminal (G381) and COOH-terminal residues (D887) resolved in the crystal structure (26) are marked N and C, respectively. The highly specific covalent inhibitor of anion transport, DIDS, is shown with space-filling atoms. The figure was constructed using YASARA version 11.10.18, 1993–2015 (www.yasara.org). (B) Space filling model of the cytoplasmic view of A with the GSLVR residues shown in magenta. (C) Surface presentation of B with GSLVR labeled.
Fig. S3. Tyrosine phosphorylation of band 3 induces both cell shape changes and dissociation of glycolytic enzymes from the erythrocyte membrane. Displacement of the glycolytic enzyme, lactate dehydrogenase, from the membrane (Bottom row) only occurs in erythrocytes in which pervanadate has induced tyrosine phosphorylation of the membrane (Middle row). Moreover, comparison of pervanadate-treated cells in the absence (Middle column) and presence (Right column) of SYK inhibitor II demonstrates that the enzyme displacement is mediated by SYK tyrosine kinase.

Fig. S4. Alignment of SH2 domain signature sequences in tyrosine kinase-regulated membrane transporters. Alignment of individual proteins is centered on the most conserved SH2 domain motif, GSFLVR, using Geneious version 5.5.7 (www.geneious.com, and using identity matrix global alignment with free end gaps. Proteins are named as in Sprowl et al. (49).