# Phosphatidylinositol-4,5-Biphosphate ($PIP_2$) Differentially Regulates the Interaction of Human Erythrocyte Protein 4.1 (4.1R) with Membrane Proteins†

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*Received January 4, 2006; Revised Manuscript Received March 20, 2006*

ABSTRACT: Human erythrocyte protein 4.1 (4.1R) participates in organizing the plasma membrane by linking several surface-exposed transmembrane proteins to the internal cytoskeleton. In the present study, we characterized the interaction of 4.1R with phosphatidylinositol-4,5-bisphosphate ($PIP_2$) and assessed the effect of $PIP_2$ on the interaction of 4.1R with membrane proteins. We found that 4.1R bound to $PIP_2$-containing liposomes through its N-terminal 30 kDa membrane-binding domain and $PIP_2$ binding induced a conformational change in this domain. Phosphatidylinositol-4-phosphate (PIP) was a less effective inducer of this conformational change, and phosphatidylinositol (PI) and inositol-1,4,5-phosphate ($IP_3$) induced no change. Replacement of amino acids K63,64 and K265,266 by alanine abolished the interaction of the membrane-binding domain with $PIP_2$. Importantly, binding of $PIP_2$ to 4.1R selectively modulated the ability of 4.1R to interact with its different binding partners. While $PIP_2$ significantly enhanced the binding of 4.1R to glycophorin C (GPC), it inhibited the binding of 4.1R to band 3 in vitro. $PIP_2$ had no effect on 4.1R binding to p55. Furthermore, GPC was more readily extracted by Titon X-100 from adenosine triphosphate (ATP)-depleted erythrocytes, implying that the GPC−4.1R interaction may be regulated by $PIP_2$ in situ. These findings define an important role for $PIP_2$ in regulating the function of 4.1R. Because 4.1R and its family members (4.1R, 4.1B, 4.1G, and 4.1N) are widely expressed and the $PIP_2$-binding motifs are highly conserved, it is likely that the functions of other 4.1 proteins are similarly regulated by $PIP_2$ in many different cell types.

Human erythrocyte protein 4.1 (4.1R)[1] is a major component of the red cell membrane skeleton. It plays an important role in maintaining and modulating erythrocyte morphology, membrane mechanical properties, and the complexity of the cell surface. It does so by its multiple interactions with skeletal proteins such as spectrin, actin, and p55, as well as with transmembrane proteins such as band 3 and glycophorin C (GPC) (for reviews, see refs *1* and *2*). Hereditary defects in 4.1R result in abnormally shaped erythrocytes with decreased membrane mechanical stability (*3*). Reconstitution of 4.1R-deficient membranes with exogenous 4.1R restores normal membrane mechanical stability (*4*, *5*). 4.1R is also required for retention of GPC in the membrane in humans with 4.1R deficiency, and in 4.1R null mice, GPC is lost from the red cell membrane (*6*, *7*).

4.1R contains four functional domains (*8*). At the N terminus, a 30 kDa N-terminal domain can link to the cytoplasmic tails of the transmembrane proteins GPC (*9*), the anion exchanger band 3 (*10*), and CD44 (*11*), as well as the membrane lipid phosphatidylserine (PS) (*12*). A 10 kDa domain promotes the binding of spectrin to actin (*13*, *14*). Between these two lies a 16 kDa domain, and at the C terminus is a 22/24 kDa domain, which binds to NuMA (nuclear mitotic apparatus) in nonerythroid cells (*15*).

The activities of the 30 kDa membrane-binding domain are tightly regulated. $Ca^{2+}$/calmodulin binds to the domain and decreases the affinity of 4.1R for GPC, band 3, and p55 (*16*−*18*). Phosphorylation of Ser312 in the 16 kDa domain by protein kinase C (PKC) decreases erythrocyte membrane mechanical stability by reducing the ability of phosphorylated 4.1R to form a ternary complex with spectrin and actin (*19*). Furthermore, the ability of phosphorylated 4.1R to interact with GPC is also markedly decreased (*19*).

The membrane-binding domain is closely related in both sequence and folding to N-terminal domains of ezrin, radixin, and moesin (the ERM proteins). For this reason, it is sometimes referred to as a FERM domain (4.1, ERM, *20*). The ERM proteins, like 4.1R, bind various transmembrane proteins via this domain. The fold of this domain is likened to a cloverleaf. It has three globular lobes that are tightly linked to each other (*21*, *22*). Importantly, the membrane-binding activities of the ERM proteins are modulated by the phospholipid phosphatidylinositol-4,5-bisphosphate ($PIP_2$). $PIP_2$ unmasks some of the activities of the ERM proteins;

† This work was supported in part by NIH Grants DK 26263, DK 32094, and HL31579.

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[1] Abbreviations: 4.1R, human erythrocyte protein 4.1; $PIP_2$, phosphatidylinositol-4,5-bisphosphate; PIP, phosphatidylinositol-4-phosphate; PI, phosphatidylinositol; $IP_3$, inositol-1,4,5-phosphate; PS, phosphatidylserine; PC, phosphatidylcholine; GPC, glycophorin C; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; ATP, adenosine triphosphate; FRET, fluorescence resonance energy transfer; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

10.1021/bi060015v CCC: $33.50 
Published on Web 04/19/2006

for example, the transmembrane protein CD44 only binds to ERM proteins in the presence of $PIP_2$ (*23*). A parallel with 4.1R is suggested by the observation that $PIP_2$ regulates 4.1R−membrane interactions (*24*).

In this context, we have asked whether the essential activities of 4.1R in the red cell are modulated by $PIP_2$. We report here that 4.1R binds the phospholipids $PIP_2$ and phosphatidylinositol-4-phosphate (PIP). These lipids but not phosphatidylinositol (PI) or inositol-1,4,5-phosphate ($IP_3$), the headgroup of $PIP_2$, caused a conformational change paralleled by an increase in GPC binding and a decrease in binding to band 3. Metabolic depletion of red cells (which reduces cellular $PIP_2$ content) resulted in a significant decrease of GPC retention in cytoskeletal extracts but no measurable increase in the retention of band 3. It appears that in situ $PIP_2$ selectively regulates the linkage of GPC to the red cell cytoskeleton. We conclude that $PIP_2$ is a major, selective regulatory cofactor in red cell membrane−cytoskeletal interactions.

## EXPERIMENTAL PROCEDURES

*Materials.* pGEX-4T-2 vector was purchased from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). pET28-c(+) vector and nickel column were from Novagen (Madison, WI). BL21 (DE3) bacteria and QuickChange site-directed mutagenesis kit were from Stratagene (La Jolla, CA). Vectors (pECFP-N1 and pEYFP-N1) used as templates to amply ECFP and EYFP sequences were from BD Biosciences Clontech (Palo Alto, CA). Restriction enzymes were from New England BioLabs (Beverly, MA). Reduced forms of glutathione and isopropyl-$\beta$-D-thiogalactopyranoside (IPTG) were purchased from Sigma (St. Louis, MO). Cholesterol, phosphatidylcholine (PC), PS, PI, PIP, $PIP_2$, and $IP_3$ were all purchased from Avanti (Alabaster, AL). Proteinase inhibitor cocktail set II was from Calbiochem (San Diego, CA). Rabbit anti-human 4.1R, band 3, and GPC antibodies were prepared in our laboratory. The glutathione-*S*-transferase (GST)-p55 construct was kindly provided by Dr. Wataru Nunomura (Tokyo Women's Medical University, Japan). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG was from Jackson ImmunoResearch Laboratory (West Grove, PA). Renaissance chemiluminescence detection kit and Gelcode staining reagent were from Pierce Biotechnology, Inc. (Rockford, IL).

*Methods: Preparation of Recombinant 4.1R Proteins and Liposomes.* Subcloning, expression, and purification of human 4.1R 80 kDa was described previously (*25*). The four 4.1R domains (30, 16, 10, and 22/24 kDa) and the cytoplasmic domains of band 3 and GPC were subcloned into pGEX-4T-2 vector using *Eco*RI and *Sal*I cloning sites up- and downstream, respectively. The cDNA was transformed into *Escherichia coli* BL21 (DE3) for protein expression. The GST-tagged recombinant proteins were purified by glutathione sepharose-4B affinity column. Replacement of lysines 63, 64, 265, and 266 by alanines in the 30 kDa domain was accomplished using the QuickChange site-directed mutagenesis. The fidelity of all subcloned fragments was confirmed by DNA sequencing. Liposomes were prepared using the sonication method (*12*).

*Engineering, Expression, and Purification of Fluorescent 30 kDa Constructs.* The cyan fluorescent protein (CFP) sequence was first subcloned into the pGEX-4T-2 vector using *Bam*HI and *Eco*RI cloning sites up- and downstream, respectively. The resulting vector is designated pGEX-4T-2-CFP. The yellow fluorescent protein (YFP) sequence was subcloned into the pGEX-4T-2-CFP vector using *Sal*I and *Not*I cloning sites. The resulting vector is designated pGEX-4T-2-CFP−YFP. Then, CFP−YFP was cut from the pGEX-4T-2-CFP−YFP vector using *Bam*I and *Not*I and pasted into the pET28-c(+) vector. Finally, wild-type or mutant 30 kDa was cut from the pGEX-4T-2 vector and inserted between CFP and YFP using *Eco*RI and *Sal*I cloning sites. The resulting His-tagged CFP−30 kDa−YFP was expressed in *E. coli* BL21 (DE3) and purified by using nickel column. The fidelity of all subcloned fragments was confirmed by DNA sequencing at each step.

*Measurement of the Protein−Liposome Interaction.* The sedimentation assay was employed to measure the interaction between proteins and liposomes (*26*). To perform the sedimentation assay, following incubation of the protein with liposomes, liposome-bound protein was separated by centrifugation at 230000*g* for 30 min at 4 °C. Under these conditions, the bound proteins sediment with liposomes, while unbound proteins remain in the supernatant. Pellets were analyzed using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Gels were stained with GelCode staining reagent and analyzed by densitometry.

*Fluorescence Resonance Energy Transfer (FRET).* Fluorescence energy transfer was measured using a Perkin−Elmer LS-50 fluorescence spectrophotometer. The excitation wavelength was set to 433 nm; emission spectra from 450 to 600 nm were recorded during excitation; and the ratio of emission intensities at 527 and 476 nm was quantitated to determine the FRET efficiency.

*GST Pull-Down Assays.* The GST-tagged recombinant cytoplasmic domain of band 3, GPC, or full-length p55 was coupled to glutathione sepharose 4B beads at room temperature for 30 min. Beads were pelleted and washed. 4.1R (at a concentration of 1 $\mu$M), preincubated without or with various concentrations of phospholipid vesicles for 30 min at room temperature, was added to the beads with protein coupled at a concentration of 1 $\mu$M in a final volume of 80 $\mu$L. The mixture was incubated for another 1 h at room temperature, pelleted, washed, and eluted with 10% SDS. The supernatant was analyzed by SDS−PAGE, followed by transfer to nitrocellulose membrane, and probed with anti-4.1R antibody. GST was used as a negative control in all experiments, and correction was made for the vestigial binding of 4.1R to GST. The extent of binding was analyzed by densitometry of Western blots.

*Adenosine Triphosphate (ATP) Depletion and Triton Extraction of Erythrocytes.* ATP depletion was performed as described previously (*27*). Briefly, erythrocytes were washed 3 times in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) at pH 7.4, 150 mM NaCl, 6 mM iodoacetamide, 10 mM inosine, and 1 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). The washed cells were suspended in the same medium at a hematocrit of 30% and incubated at 37 °C for different periods of time. After ATP depletion, the cells were extracted with 0.5% Triton X-100 in phosphate-buffered saline. The resulting Triton shells were analyzed by immunoblotting using antibodies against GPC and band 3.

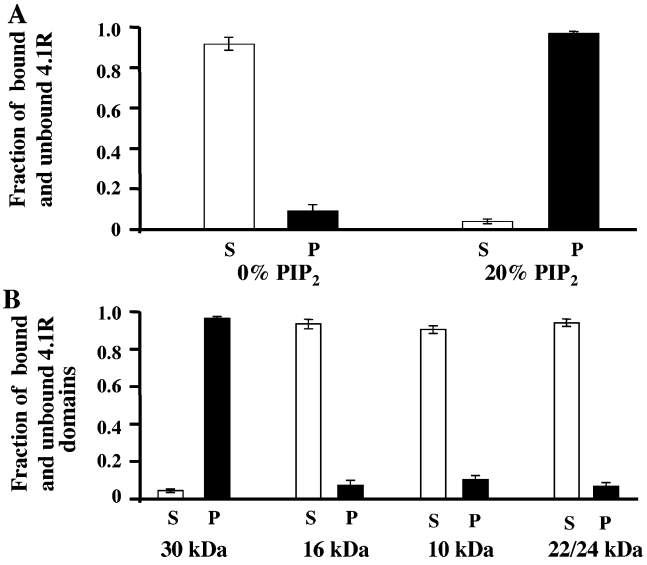


FIGURE 1: Binding of 4.1R and its structural domains to $PIP_2$-containing liposomes. (A) 4.1R was incubated with PC or 20% $PIP_2$-containing liposomes, and the bound and unbound proteins were separated by ultracentrifugation. Proteins in the supernatant (S) and pellet (P) were resolved by 10% SDS−PAGE and evaluated by densitometry. 4.1R was found in the $PIP_2$-containing lipid pellet but not in the PC lipid pellet. (B) Various domains of 4.1R were incubated with 20% $PIP_2$-containing liposomes, and binding was assessed as described above. Only the 30 kDa fragment was found in the pellet.

*SDS−PAGE and Western Blot.* SDS−PAGE of samples was performed using 10% acrylamide gels. Proteins were transferred onto a nitrocellulose membrane. All steps were performed at room temperature. After the blot was blocked for 1 h in blocking buffer (10 mM Tris at pH 7.4, 150 mM NaCl, 0.5% Tween-20, and 5% nonfat dry milk), it was probed for 1 h with the primary antibody. After several washes, the blot was incubated with anti-rabbit IgG coupled to HRP. After several washes, the blot was developed using the Renaissance chemiluminescence detection kit.

## RESULTS

*Binding of 4.1R and Its Structural Domains to Liposomes Containing $PIP_2$.* A sedimentation assay was used to study the interaction between proteins and liposomes. Our criterion for phospholipid binding capacity was recovery of the protein in the liposome pellet after high-speed centrifugation. We first examined the binding of full-length 4.1R to PC liposomes (20% cholesterol and 80% PC) and PC liposomes containing $PIP_2$ (20% $PIP_2$, 20% cholesterol, and 60% PC). As shown in Figure 1A, under the experimental conditions employed, more than 95% of 4.1R cosedimented with $PIP_2$-containing liposomes but none cosedimented with PC liposomes. To define the functional domain in 4.1R that binds $PIP_2$, the ability of four recombinant fragments of 4.1R (the 30 kDa membrane-binding domain, the 16 kDa regulatory domain, the 10 kDa spectrin−actin binding domain, and the 22/24 kDa C-terminal domain) to bind $PIP_2$-containing liposomes was evaluated. As shown in Figure 1B, while more than 95% of the 30 kDa fragment cosedimented with $PIP_2$-containing liposomes, the 16, 10, and 22/24 kDa fragments failed to bind and remained in the supernatant. Thus, we have localized a $PIP_2$-binding site to the 30 kDa membrane-binding domain of 4.1R. The cytoplasmic domains of GPC and band 3 and full-length p55 did not bind to $PIP_2$-containing liposomes in our assay (data not shown).

*Polyphosphoinositide-Induced a Conformational Change in the 30 kDa Domain of 4.1R.* It is possible that $PIP_2$ binding induces a conformational change in the 30 kDa membrane-binding domain of 4.1R. To address this, we designed a reporter fusion protein in which CFP and YFP were fused to the N and C termini of the domain, respectively. We then measured FRET between the two fluorophores prior to and following the binding of polyphosphoinositides or related compounds. A schematic representation of the CFP−30 kDa−YFP reporter is shown in Figure 2A. Efficient energy transfer in the absence of lipids was demonstrated by a substantial emission at 527 nm (YFP emission peak at 527 nm) upon excitation of CFP at 433 nm (Figure 2B). In contrast, no emission at 527 nm could be seen when 30 kDa with YFP alone was excited at 433 nm (Figure 2C), demonstrating that the observed emission noted with CFP−30kDa−YFP is the result of energy transfer. In the presence of 10 $\mu$M $PIP_2$, a reduction in the intensity of emission at 527 nm with a corresponding increase in the intensity of cyan emission was noted, consistent with a structural change leading to a decreased efficiency of energy transfer (Figure 2B), and the decrease in FRET efficiency induced by $PIP_2$ was dose-dependent (Figure 2D). PIP also reduced efficiency of energy transfer but to a lesser extent than $PIP_2$. PIP and $PIP_2$ had no effect on emission of CFP−30 kDa alone or 30 kDa−YFP alone (data not shown), implying that the observed changes in energy transfer are due to reduced energy transfer but not the result of the direct effect of the lipids on fluorescence emission. In contrast to $PIP_2$ and PIP, PI and $IP_3$ had no effect on the efficiency of energy transfer. Furthermore, cholesterol, PC, and PS had no effect on energy transfer (data not shown).

*Identification of the $PIP_2$-Binding Motif in the 30 kDa Domain.* In ezrin, pairs of basic residues are required for interactions with $PIP_2$ (*28*). The sequence of human 4.1R contains several such dibasic motifs of which one, residues K265 and K266 in lobe C of the membrane-binding domain, aligns with a KR motif in the ERM proteins that is required for $PIP_2$ binding. In lobe A, there is a dibasic motif at K63,64. These motifs are conserved as K or R among all human and mouse 4.1 proteins, although K63,64 does not align with a similar motif in the ERM proteins (Figure 3A). Furthermore, the crystal structure of the 4.1R 30 kDa domain revealed that these two pairs of basic residues are located on the surface and contribute to a positively charged cleft between lobes A and C (parts B and C of Figure 3). To test the significance of these residues, we mutated each pair to alanine. The interaction of the mutant 30 kDa domain with $PIP_2$-containing liposomes was examined by the FRET assay. As shown in Figure 3D, while $PIP_2$-containing liposomes induced a significant decrease in energy transfer of wild-type 30 kDa domain, the extent of decrease was reduced in the two mutant proteins K63,64A and K265,266A. No decrease in energy transfer was noted in the protein in which all four lysines were mutated to alanines. These results indicate that K63,64 and K265,266 are integral to the $PIP_2$-binding sites and that the presence of both K63,64 and K265,266 are required for optimal binding.

*Effect of $PIP_2$ on the Function of the Membrane-Binding Domain of 4.1R.* Having documented that $PIP_2$ can induce a

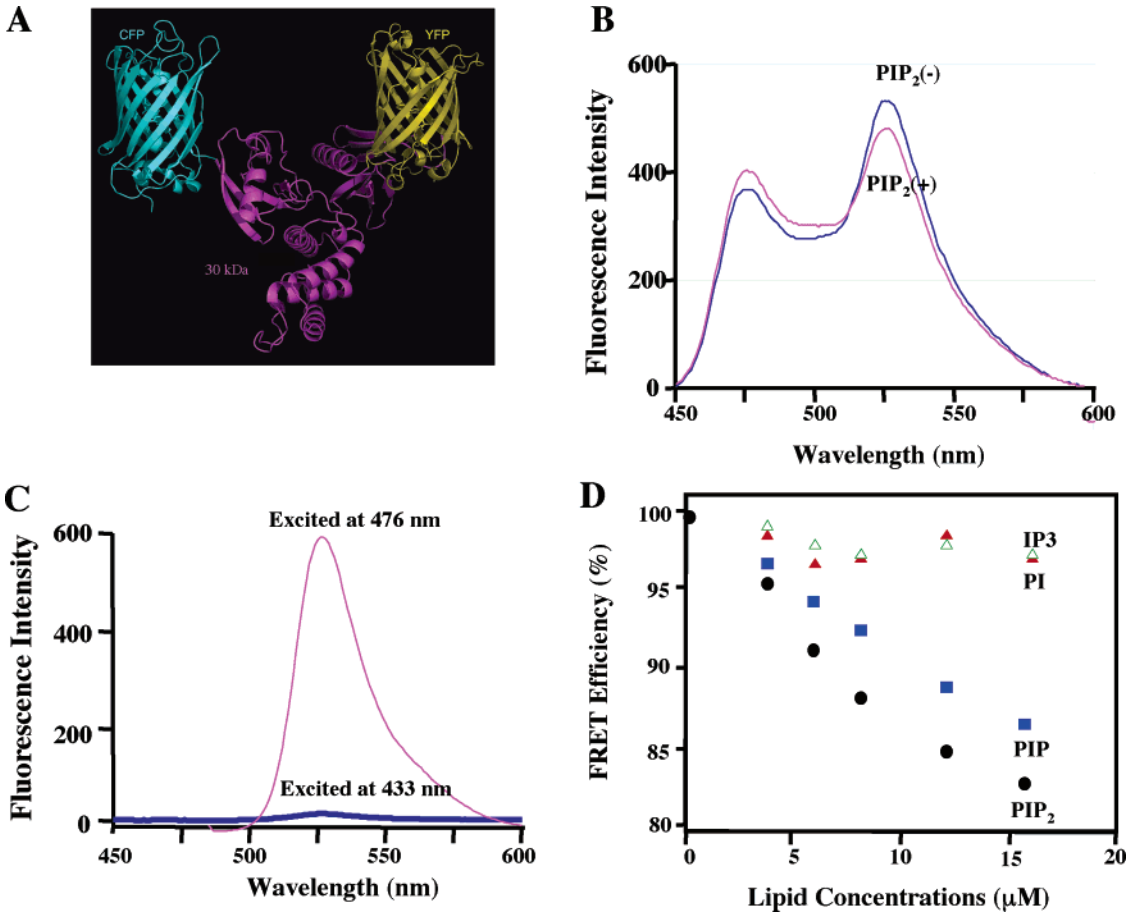


FIGURE 2: Conformational change of the 30 kDa domain induced by phosphoinositides. (A) Diagram of CFP−30 kDa−YFP reporter construct. The individual parts of the reporter (CFP, 30 kDa domain of 4.1R, and YFP) are colored cyan, magenta, and yellow, respectively. The estimated distance between the two fluorophores is 3−6 nm. The figure is based on structures in the PDB 1CV7, 1GG3, and 1YFP. (B) Spectra of CFP−30 kDa−YFP. CFP−30 kDa−YFP was excited at 433 nm, and an emission spectrum was recorded as described in the Experimental Procedures. CFP−30 kDa−YFP displayed a characteristic two-peak spectrum with emission peaks at 476 and 527 nm. In the presence of 16 $\mu$M $PIP_2$, the emission at 527 nm was decreased and emission at 476 nm was increased. (C) Spectra of 30 kDa−YFP excited at 433 and 476 nm and emission spectrum recorded as described in the Experimental Procedures. Note that no fluorescence emission is seen when 30 kDa−YFP was excited at 433 nm. (D) Effect of phosphoinositides on FRET efficiency of CFP−30 kDa−YFP. CFP−30 kDa−YFP spectra in the presence of various concentrations of phosphoinositides were recorded as described above. FRET efficiency was determined by the ratio of YFP emission (527 nm) over CFP emission (476 nm) and normalized, taking the FRET efficiency as 100% in the absence of lipid. FRET efficiency was plotted against the concentration of lipid.

conformational change in the 30 kDa domain, we explored the effect of $PIP_2$ on the ability of this domain to interact with its binding partners, GPC, band 3, and p55. $PIP_2$-containing liposomes enhanced the binding of 4.1R to the cytoplasmic domain of GPC (Figure 4A). In contrast, $PIP_2$-containing liposomes markedly decreased the binding of 4.1R to the cytoplasmic domain of band 3 (Figure 4B). The effect of $PIP_2$ on both the 4.1R−GPC and 4.1R−band 3 interactions follows a dose-dependent manner, with a maximal effect observed at 10 $\mu$M $PIP_2$ (Figure 4C). $PIP_2$ had no effect on the binding of 4.1R to p55 (data not shown). Consistent with our FRET data, PIP had slight effect on 4.1R binding to GPC and band 3, while PI and $IP_3$ had no effect (parts A and B of Figure 4). The specificity of the effect of $PIP_2$ on regulating the interaction of 4.1R with GPC and band 3 was documented by the findings that $PIP_2$ did not affect the interaction of the K63,64,265,266A mutant 30 kDa domain (that fails to bind $PIP_2$) with either GPC or band 3 (parts D and E of Figure 4).

*Extractability of GPC and Band 3 from ATP-Depleted Erythrocytes.* To explore whether the interactions of GPC and band 3 with 4.1R are also regulated by $PIP_2$ in situ, we studied the extent of alteration in the interaction of GPC with the membrane skeleton in ATP-depleted erythrocytes. We treated erythrocytes with iodoacetamide to inhibit glycolysis and incubated the cells for various lengths of time to deplete cells of ATP. It is well-established that an early event in the metabolic depletion of erythrocytes is dephosphorylation of $PIP_2$ (*27*).

Figure 5A shows that GPC is associated with the membrane skeleton of normal (metabolically active) erythrocytes as a result of its interaction with 4.1R. With an increasing time of incubation in glucose-free buffer, GPC was progressively diminished from the cytoskeletal fraction (Figure 5A). This indicates that as $PIP_2$ is increasingly dephosphorylated, the 4.1R−GPC link is broken. In contrast, band 3 remained in the cytoskeletal fraction over the same period of incubation (Figure 5B). These data point to a potential role for $PIP_2$ in promoting the GPC−cytoskeleton interaction in situ.

## DISCUSSION

In the present study, we performed detailed structural and functional characterization of the 4.1R interaction with $PIP_2$.

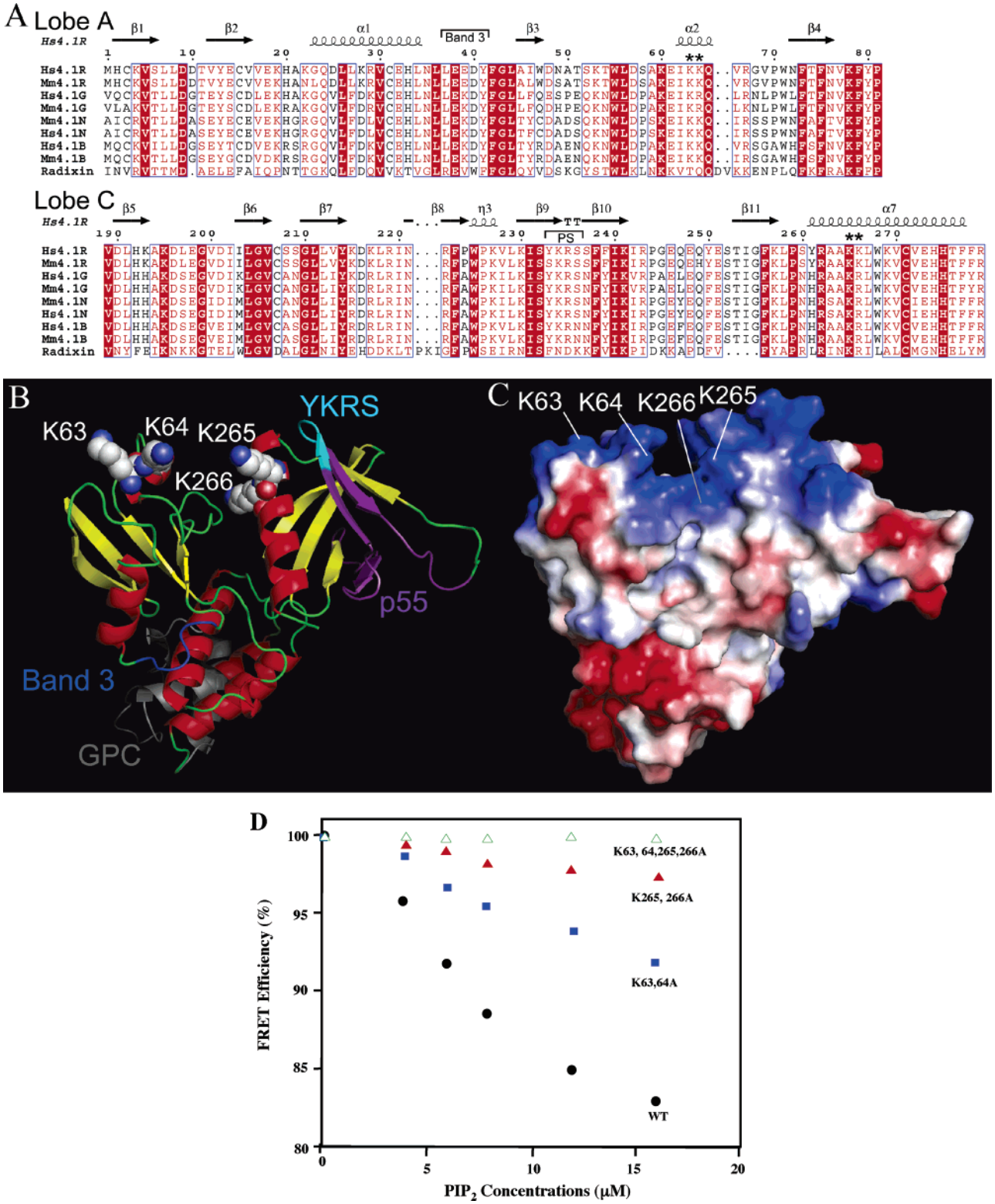


FIGURE 3: Identification of $PIP_2$-binding motif in the 30 kDa domain. (A) Potential $PIP_2$-binding motif in the protein 4.1 family. Human (Hs) and mouse (Mm) 4.1 sequences were obtained from the Uniprot database, and accessions numbers are Hs4.1B, Q9Y2J2; Hs4.1G, O43491; Hs4.1N, Q9H4G0; Hs4.1R, P11171; Mm4.1B, Q9WV92; Mm4.1G, Q80UE5; Mm4.1N, Q9Z2H5; Mm4.1R, P48193; and radixin, P35241. (B) Ribbon diagram of the 30 kDa domain. The crystal structure was taken from PDB 1GG3 residues K63,64 and K265,266 are displayed in space-filling representation, whereas band 3, GPC, p55, and PS-binding regions are presented in blue, gray, purple, and cyan colors; (C) Electrostatic potential surface of the molecule in the same orientation as in B. Residues K63,64 and K265,266 form a positively charged cleft on the surface. (D) Effect of $PIP_2$ on FRET efficiency of wild-type and mutant 30 kDa fragments.

We have provided direct evidence that (1) 4.1R binds $PIP_2$-containing liposomes through its N-terminal 30 kDa membrane-binding domain, (2) residues K63,64 and K265,266 in the 30 kDa domain are critical for the interaction, (3) the 30 kDa domain undergoes a conformational change(s) upon $PIP_2$ binding, and (4) $PIP_2$ differentially modulates the interaction of 4.1R with its different binding partners both in vitro and in situ.

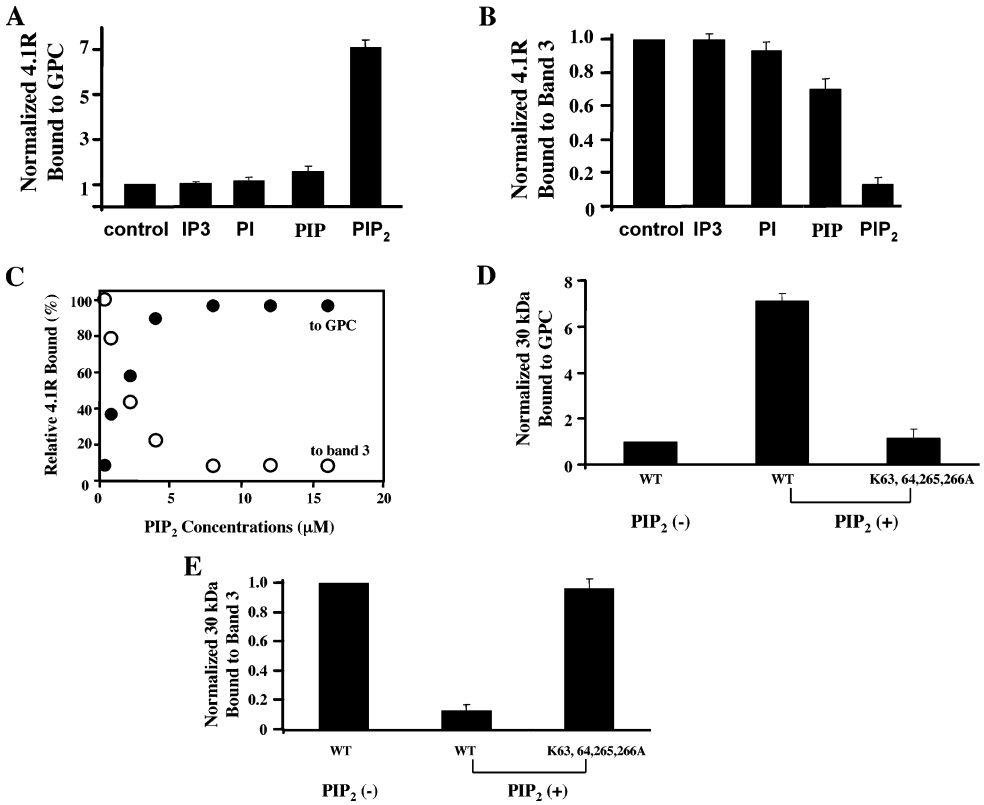


FIGURE 4: Effect of phosphoinositides on 4.1R and 30 kDa binding to the cytoplasmic domain of GPC and band 3. (A) Binding of 4.1R to the cytoplasmic domain of GPC was significantly enhanced by $PIP_2$, slightly by PIP, but not by PI and $IP_3$. (B) Binding of 4.1R to the cytoplasmic domain of band 3 was significantly inhibited by $PIP_2$, slightly by PIP, but not by PI and $IP_3$. (C) With increasing $PIP_2$ concentrations, binding of 4.1R to the cytoplasmic domain of GPC progressively increased and binding to cytoplasmic domain of band 3 progressively decreased, both reaching a plateau at 10 $\mu$M. (D) $PIP_2$ significantly increased the binding of wild-type 30 kDa to the cytoplasmic domain of GPC, while it had no effect on the K63,64,265,266A mutant. (E) $PIP_2$ significantly decreased the binding of wild-type 30 kDa to the cytoplasmic domain of band 3, while it had no effect on the K63,64,265,266A mutant.

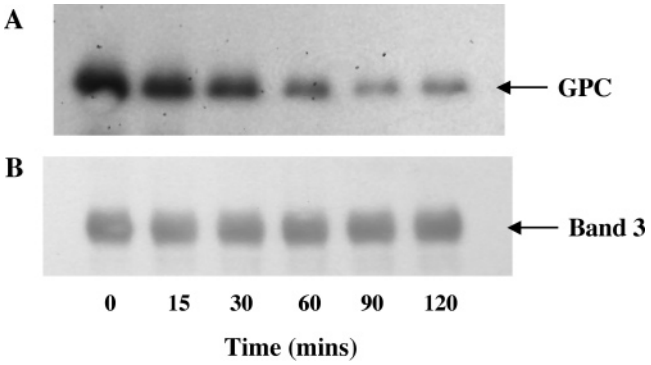


FIGURE 5: Immunoblot of GPC and band 3 in the Triton shells prepared from ATP-depleted red blood cells. The Triton shells were prepared from erythrocytes following ATP depletion as described in the Experimental Procedures. Proteins retained in the Triton shells were analyzed by SDS−PAGE and immunoblotting with anti-GPC and anti-band 3. Note the progressive decrease of GPC.

FRET methodology has been widely used to detect the conformational changes upon ligand binding (*29*, *30*). FRET occurs between a donor fluorophore and an acceptor molecule, with the emission spectrum of the donor overlapping with the absorption spectrum of the acceptor, and the distance between donor and acceptor is less than 10 nm. Because the N and C termini of the 30 kDa domain are in the A and C lobes of the folded structure, we anticipated that fusing CFP to the former and YFP to the latter will lead to the two fluorophores being localized within an estimated distance of 3−6 nm between them, leading to a significant FRET signal. This proved to be the case and gave us the opportunity to study conformational changes in the 30 kDa that occur upon ligand binding.

Several consensus $PIP_2$-binding motifs have been identified in various proteins including the protein 4.1 superfamily member ezrin (*28*), the calponin homology domains of the cytoskeletal proteins α-actinin and $\beta$-spectrin (*31*), the pleckstrin homology domain of phospholipase C (*32*), and other actin-interacting proteins such as gelsolin and profilin (*33*, *34*). The general feature of these motifs is that they contain a cluster of basic amino acids. In searching for the potential $PIP_2$-binding motif in 30 kDa of 4.1R, in addition to sequence alignment, we also took advantage of the availability of the crystal structure of the 30 kDa domain. The combination of these two approaches revealed two potential $PIP_2$-binding motifs K63,64 and K265,266. These residues are highly conserved in mammalian 4.1R, 4.1B, 4.1G, and 4.1N. Furthermore, these residues are all located on the surface of the molecule, and more importantly, they form a positively charged cleft, which could potentially serve as a docking site for the negatively charged $PIP_2$. The findings that the mutation of K63,64 or K265,266 only partially affects the interaction of 30 kDa with $PIP_2$ but that the double mutation of both K63,64 and K265,266 completely abolishes the binding of 30 kDa to $PIP_2$ strongly suggest that the cleft between the A and C lobes represents the $PIP_2$-binding site and that the two pairs of basic residues are integral to that site.

In the structure of the radixin FERM domain (*35*), $IP_3$ has been observed to bind between the A and C lobes. This binding causes substantial shifts in the structure (up to 6 Å). These observations have been used to propose a model for unmasking transmembrane-protein-binding sites upon ligand binding. It is interesting to note in this context that our FRET reporter did not reveal any conformational changes upon $IP_3$ binding nor does $IP_3$ modulate binding to GPC or band 3.

We have previously identified the PS-binding motif YRKS (residues 233−236) in the 30 kDa domain of 4.1R and demonstrated that the interaction of 4.1R to PS is critical for 4.1R assembly onto the membrane (*12*). It is important to note that the YRKS mutation had no effect on the ability of 30 kDa domain binding to $PIP_2$ (data not shown). The involvement of distinct motifs for PS and $PIP_2$ binding highlights the specificity of the interactions and indicates the distinct functions for 4.1R−PS and 4.1R−$PIP_2$ interactions. It also should be noted that the YRKS motif is not present in the ERM family and that ERM proteins do not bind to PS (*23*).

The GPC interaction with 4.1R was significantly enhanced by $PIP_2$ in vitro, and ATP depletion led to a decreased association of GPC with the membrane skeleton in situ. These finding are consistent with previous studies by Gascard et al. (*27*) who demonstrated ATP depletion or activation of erythrocyte phosphoinositol phospholipase C led to a reduction in membrane $PIP_2$ and a subsequent decrease of 4.1R binding to trypsin-resistant binding sites (probably GPC). The present findings are also consistent with findings by Anderson and Marchesi (*24*) that $PIP_2$ increases the binding of 4.1R to a population of glycophorin(s). However, it should be noted that in this study the identity of the specific glycophorin involved in the interaction was not defined.

Erythrocyte $PIP_2$ turns over slowly in healthy human erythrocytes (the half-time is several hours). There is presently little evidence to indicate a dynamic regulation of the $PIP_2$ content in response to signaling in mature red cells. In contrast, in complex cells such as neurons, $PIP_2$ is turned over rapidly in response to many signaling events. Because all mammalian 4.1 proteins (4.1R, 4.1B, 4.1N, and 4.1G) contain the $PIP_2$-binding motifs, our data strongly suggest that, in cells where $PIP_2$ functions within signaling pathways, analogous membrane−cytoskeleton interactions will be dynamically regulated. Members of the cell-adhesion molecule family neurexin/CASPR/paranodin bind to 4.1 proteins at the same site as GPC (*36*, *37*). It will be important to establish if cytoskeletal assemblies at cell junctions are regulated via cell signaling, operating via the nexus of $PIP_2$ and 4.1.

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BI060015V