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Phosphorylation-dependent perturbations of the 4.1R-associated multiprotein complex of the erythrocyte membrane

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Abstract

The bulk of the red blood cell membrane proteins are partitioned between two multiprotein complexes, one associated with ankyrin R, the other with protein 4.1R. Here we examine the effect of phosphorylation of 4.1R on its interactions with its partners in the membrane. We show that activation of protein kinase C in the intact cell leads to phosphorylation of 4.1R at two sites, serine-312 and serine-331. This renders the 4.1R-associated transmembrane proteins GPC, Duffy, XK and Kell readily extractable by nonionic detergent with no effect on the retention of band 3 and Rh, both of which also interact with 4.1R. In solution, phosphorylation at either serine suppresses the capacity of 4.1R to bind to the cytoplasmic domains of GPC, Duffy and XK. Phosphorylation also exerts an effect on the stability *in situ* of the ternary spectrin-actin-4.1R complex, which characterizes the junctions of the membrane skeletal network, as measured by the enhanced competitive entry of a β -spectrin peptide possessing both actin- and 4.1R-binding sites. Thus phosphorylation weakens the affinity of the 4.1R for β -spectrin. The two 4.1R phosphorylation sites lie in a domain flanked in the sequence by the spectrin- and actin-binding domain and a domain containing the binding sites for transmembrane proteins. It thus appears that phosphorylation of a regulatory domain in 4.1R results in structural changes transmitted to the functional interaction centers of the protein. We consider possible implications of our findings to altered membrane function of normal reticulocytes and sickle red cells.

The red cell membrane is a composite structure, comprising a membrane skeletal lattice, attached to the lipid bilayer, mainly through interactions with transmembrane proteins. The major skeletal proteins are α - and β -spectrin, F-actin, ankyrin R, protein 4.1R, adducin, dematin, tropomyosin, tropomodulin, protein 4.2 and p55, whereas the principal transmembrane proteins are band 3, glycophorins A and C (GPA and GPC), the rhesus proteins, Rh and RhAG, CD47, LW, Duffy, XK and Kell¹. Recent work has shown that several of these proteins are assembled into two multiprotein complexes. The first, commonly referred to as the ankyrin R-based complex, contains ankyrin R, band 3, GPA, protein 4.2, RhAG, Rh, GPB, CD47 and LW. This ankyrin R-based complex is thought to function as a metabolon, engaged in chloride–bicarbonate exchange, and facilitating

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coordinated CO₂ uptake and O₂ release². The second multiprotein complex, which we have termed the '4.1R complex'³, comprises the three principal components of the skeletal network junctions (spectrin, actin and 4.1R), together with tropomyosin, tropomodulin, adducin, dematin, p55, and the transmembrane proteins, GPC, XK, Kell, Duffy, band 3 and Rh. Both ankyrin R and 4.1R based complexes participate in linking the membrane skeleton to the lipid bilayer.

The binding sites in 4.1R for integral membrane proteins are located in the N-terminal 30 kDa membrane-binding domain, while spectrin and actin bind to the 10 kDa domain⁴. The crystal structure of the 30 kDa domain reveals a cloverleaf disposition of three globular lobes⁵. Lobe A contains the binding sites for band 3 and Rh, Lobe B contains the binding sites for GPC, XK and Duffy, while the p55 binding site is in Lobe C^{3,6-9}.

Protein interactions involving 4.1R can be regulated by Ca²⁺ and calmodulin, by PIP₂ and by phosphorylation. Binding of band 3, GPC and p55 to 4.1R is modified by Ca²⁺ and calmodulin^{10,11}. PIP₂, which binds in the cleft between lobes A and C, promotes binding of GPC but inhibits that of band 3¹². 4.1R is a substrate for the cAMP-dependent protein kinase (PKA), for tyrosine kinase and for protein kinase C (PKC). In solution PKA phosphorylates serine-331 in the non-conserved 16kD domain¹³. Phosphorylation of the 10 kDa spectrin-actin domain by tyrosine kinase reduces the strength of 4.1R-spectrin-actin complex¹⁴, while phosphorylation of serine-312 by PKC *in situ* weakens the binding of 4.1R to GPC¹⁵ and stability of the ternary junction complex, with accompanying reduction of the shear-resistance of the membrane¹⁵.

We present here the results of an investigation into the nature of phosphorylation effects on the interactions of 4.1R with its partners in the red cell membrane, and consider the physiological and pathological implications.

MATERIALS AND METHODS

MATERIALS

Human venous blood was drawn from healthy volunteers with informed consent. pMAL vector, MBP resin were obtained from New England Biolabs (Beverly, MA). pET31b(+), nickel columns from Novagen (Madison, WI), BL21 (DE3) bacteria and Quick-Change site-directed mutagenesis kit from Stratagene (LaJolla, CA), 4 α -phorbol 12,13-didecanoate (PMA), reduced form of glutathione and isopropyl β -D-thiogalactopyranoside (IPTG) from Sigma (St Louis, MO), glutathione Sepharose 4B from Amersham Pharmacia Biotech (Piscataway, NJ), Millipore Centriprep YM-30 from Fisher Scientific (Pittsburgh, PA). Calyculin A and catalytic subunit of Protein Kinase C were from Calbiochem (San Diego, CA), SDS/PAGE and electrophoresis reagents from Bio-Rad (Hercules, CA), and SuperSignal West Pico chemiluminescence detection kit reagents and GelCode blue reagent from Pierce (Rockford, IL). Antibodies were generated and characterized in our laboratory^{3,16}. Horseradish peroxidase-conjugated anti-rabbit IgG, HRP-conjugated anti-mouse IgG and anti-rat IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Biotin-labeled synthetic peptides corresponding to the cytoplasmic tail of Duffy, Rh, the membrane proximal part of XK cytoplasmic domain, the 4.1R binding region

of GPC were from Genemed Synthesis Inc. (San Antonio, TX). The sequences are following: Duffy: HQATRTLPSLPLPEGWFSHLDTLGSKS; Rh: NLKIWKAPHEAKYF DDQVFWKFPHLAVGF; XK: HPCKKLFSSSVSEGFQRWLRCFCWACRQQKPCEPIG; GPC: RYMYRHKGTYHTNEAKG. Non-muscle actin from Cytoskeleton, Inc (Denver, CO).

METHODS

Preparation of proteins—Spectrin was prepared from erythrocytes according to Tyler *et al.*,¹⁷. The recombinant fragment of the N-terminal region of β -spectrin, comprising of residues 1 to 301, was prepared by cloning and expression in *Escherichia coli* BL21, as described by An *et al*¹⁸. The full-length His-tagged-4.1R (80 kDa) was produced and purified as described previously¹⁹. 4.1R-S312A, 4.1R-S331A and 4.1R-S312,331A mutants were generated by *in-vitro* mutagenesis using the QuickChange site-directed-Mutagenesis kit, according to the supplier's instructions. The cDNA coding for p55 protein, cloned into the pMAL vector, or the cDNA coding the cytoplasmic domain of band 3, cloned into pGEX-4T-2, was transformed into *E. coli* BL21 (DE3) for protein expression. The GST-tagged recombinant proteins were purified using glutathione-Sepharose 4B beads. The MBP-tagged recombinant protein was purified on an amylose resin affinity column. Proteins were dialysed against phosphate-buffered saline (10 mM phosphate, pH 7.4, 150 mM NaCl) for pull-down assays and against hypotonic buffer (5 mM Tris, 5 mM KCl, pH 7.4) for resealing experiments.

In-vitro phosphorylation of 4.1R by PKC—The recombinant His-tagged wild-type 4.1R and the above mutants at a concentration of 6.25 μ M were mixed with the catalytic subunit of PKC (25 ng/mL) in the reaction buffer (60 mM MES, 1.5 mM EGTA, 15 mM MgCl₂, 125 μ M ATP, 0.05 μ M calyculin A) for 15 minutes at 30°C. The resultant samples served to check the specificity of the phospho-specific antibodies (anti-p4.1R S312 or anti-p4.1R S331) by Western blotting and were used for pull-down assays.

PMA treatment of erythrocytes to activate PKC—Red cells from fresh blood from human volunteers or from anesthetized wild-type mice were washed 3 times with ice-cold phosphate-buffered-saline. Packed red cells were incubated at 37°C for 10 minutes in the presence of 0.02 μ M of calyculin A and then treated either without or with 2 μ M of PMA for 5 or 90 minutes.

Detergent extraction of transmembrane proteins from erythrocyte ghosts—Ghosts from control and PMA-treated erythrocytes were prepared by lysing the cells in ice-cold hypotonic buffer (5 mM KCl, 5 mM Tris, pH 7.4), followed by three washes in 35 volumes of the same buffer. 50 μ l of the packed ghosts were re-suspended in 150 μ l of PBS, containing Triton X-100 and 0.02 μ M calyculin A, for 30 minutes at 4°C. The concentrations of Triton X-100 required for extraction of the proteins in question differed from 1% for Kell, XK, Band 3 and GPA to 0.05% for Rh, RhAG, CD47 and Duffy and 0.01% for LW. Following incubation, the cell suspension was centrifuged at 21,000 g in the cold for 20 minutes and the pellet was re-suspended in PAGE sample buffer and boiled for 5 minutes.

Incorporation of β -spectrin peptide into erythrocyte ghosts—The freshly drawn human red blood cells, before and after treatment with PMA and calyculin A were washed with ice-cold PBS, lysed with hypotonic buffer (5 mM KCl, 5 mM Tris, pH 7.4) and washed three times with 35 volumes of the same buffer. The ghosts were resealed in the absence or presence of varying concentrations of β -spectrin N-terminal peptide (1-301) as described previously²⁰. Calyculin A (0.02 μ M) was present at all stages of cell processing for samples treated with PMA. As a control, BSA (bovine serum albumin) was also introduced into the ghosts as described above.

Preparation of membrane skeletons from resealed ghosts—To measure the incorporation of the β -spectrin peptide into the ghost membrane skeleton, the resealed ghosts were re-suspended in 20 volumes of extraction buffer (0.625 M NaCl, 6.25 mM sodium phosphate, 0.625 mM EGTA, 0.625 mM DTT, 2% Triton, pH 7.4) and incubated for 30 minutes at 4°C, followed by centrifugation at 21,000g in the cold for 20 minutes. The pellet was re-suspended in electrophoresis sample buffer and analyzed by 10% SDS/PAGE. The gels were stained with Coomassie Blue and the bands were quantified by densitometry.

Pull-down assays—Unphosphorylated and in vitro phosphorylated His-tagged wild-type 4.1R or its mutants, were mixed with biotin-labeled cytoplasmic domains of XK, Duffy, Rh, or GPC and incubated for 1 hour at room temperature. Streptavidin beads were added and recovered by centrifugation at 21,000g for 5 minutes. The beads were washed three times with PBS and eluted with 10% SDS. The elute was analyzed by SDS/PAGE. Binding of 4.1R was detected by western blotting with an anti-4.1R antibody. Similar experiments were performed to detect the binding of 4.1R and its mutants to MBP-tagged-p55, GST-tagged cytoplasmic domain of band 3 or GST-tagged β I spectrin fragment, using amylose beads or glutathione beads respectively.

Actin pelleting assay—F-actin (7 μ M with respect to monomer) was incubated with unphosphorylated or phosphorylated 4.1R or its mutants (1.7 μ M) for 60 minutes at room temperature in binding buffer (130 mM KCl, 20 mM NaCl, 10 mM Tris, 0.1 mM EGTA, 10 mM β_2 mercapto-ethanol, 0.2 mM ATP, 2 mM $MgCl_2$, 30 μ M PMSF, pH7.4) and then centrifuged at 4°C for 30 min at 90,000 rpm (313,000 $\times$ g) in a Beckman Optima TL ultracentrifuge with a TLA-100 rotor. The pellet was dispersed in the original sample volume and analyzed by 10% SDS-PAGE, followed by western blotting with anti-4.1R antibody.

SDS/PAGE electrophoresis and western blotting—The samples were separated by SDS/PAGE electrophoresis in 10% gels. The proteins were transferred to a nitrocellulose membrane, blocked for 2 hours in blocking buffer (10mM Tris, pH7.4, 150mM NaCl, 0.01% Tween, 4% nonfat dried milk powder, 1% BSA), incubated for 1 hour with the desired primary antibody, then washed and incubated with anti-rabbit, anti-mouse or anti-rat IgG coupled to HRP. SuperSignal West Pico chemiluminescence detection kit was used to identify the polypeptides. All steps were performed at room temperature.

RESULTS

Specificity of anti-phospho-4.1R antibodies

Fig 1A shows the four structural domains of 4.1R and the sub-domains of the N-terminal 30 kDa membrane binding domain. The binding sites for its various binding partners are indicated. The phosphorylation sites are in the 16 kDa domain. To examine the effects of phosphorylation of 4.1R on its interactions with other membrane proteins we first generated antibodies directed against the phosphoserine residues at positions 312 and 331 in the sequence, using the oligopeptides, AAAQTRQAS(p)ALID and TASKRAS(p)RSLDGAAA as antigens. To assess the specificity of these antibodies, we prepared His-tagged recombinant wild-type 4.1R and the mutants S312A, S331A and S312,331A and exposed them in solution to the catalytic subunit of PKC. Fig 1B shows that the anti-p4.1R 312S antibody recognized wild-type and the S331A 4.1R, but not the S312A or the S312,331A double mutant. Similarly, the anti-p4.1R 331S antibody recognized wild-type 4.1R and the S312A mutant but neither the S331A nor the double mutant. Thus both antibodies are sequence-specific.

Weakening of the linkage of transmembrane proteins to the cytoskeleton following phosphorylation of 4.1R by PKC

To examine the effect of 4.1R phosphorylation on the linkage of transmembrane proteins to the membrane skeleton we measured their susceptibility to extraction from the membranes by nonionic detergent before and following exposure of the cell to PMA for periods of 5 or 90 minutes. After 5 minutes exposure, only adducin but not 4.1R had been significantly phosphorylated (Fig 2A), in agreement with Manno et al ¹⁵. Phosphorylation of adducin did not measurably alter the detergent extractability of GPC, Kell, XK and Duffy, implying that their interaction with the membrane skeleton remained unaffected. In marked contrast, after 90 minutes exposure to PMA, in addition to phosphorylation of adducin, 4.1R was also phosphorylated at both serine sites which led to substantial dissociation of all four of these proteins by the dilute detergent medium (Fig 2B). By striking contrast, the retention of band 3, RhAG, Rh, GPA, CD47 and LW, all components of the ankyrin R complex was unaffected (Fig 2C). We conclude that phosphorylation of 4.1R selectively governs the attachment to the membrane skeleton of those transmembrane proteins which form a part of the 4.1R-based multiprotein complex.

Effect of phosphorylation of 4.1R on its interactions with membrane proteins in solution

To better understand the basis of the above effects in the cell we investigated the consequences of phosphorylation on pairwise interactions between 4.1R and its binding partners in solution. The disposition of binding sites for these proteins on the N-terminal 30 kDa domain of 4.1R are depicted in Fig 3A. Soluble fragments (either synthetic peptides or recombinant) corresponding to the cytosolic domains of the transmembrane proteins, XK, Duffy, GPC, Band 3 and Rh, as well as full-length p55, were prepared and their interaction with phosphorylated and unphosphorylated 4.1R, and its mutants lacking of either or both of the phosphorylation sites was examined by pull-down assays. Phosphorylation of 4.1R inhibited its interactions with only three of its binding partners, namely XK, Duffy and GPC (Fig 3B-a) but not with Rh, p55 and band 3. Mutation of either one of the two

phosphorylation sites in 4.1R was sufficient to inhibit binding to XK, Duffy and GPC (Fig 3B-b and 3B-c). With both phosphorylation sites eliminated, the mutant protein bound normally (Fig 3B-d) showing that the mutations themselves did not perturb the function of the protein.

Destabilization of the spectrin-actin-4.1R ternary complex by 4.1R phosphorylation

The first recognized function of 4.1R was in the formation of a high-affinity ternary complex with spectrin and F-actin, which constitutes the nodes of the membrane skeletal lattice. The red cells of individuals with hereditary deficiency of 4.1R have highly unstable membranes, due to the weak nature of the binary spectrin-actin interaction. We have previously found that even in normal cells the junctions are appreciably labile as evidenced by the incorporation of the N-terminal fragment of β -spectrin, containing the actin and 4.1R binding sites, into the junctions of intact membranes²⁰. The incorporation of the same spectrin fragment is modestly but significantly enhanced following phosphorylation of 4.1R (Fig 4A and 4B), reflecting a reduction in cohesion of the junction complex *in situ*. As a control, bovine serum albumin was not incorporated into the skeleton (Fig 4C).

Phosphorylation of 4.1R perturbs its interaction with spectrin but not with actin

To determine whether it is the interaction of 4.1R with spectrin or with actin (or both) that is affected by phosphorylation, we examined its effect on the formation of the binary complexes. The interaction with spectrin was assessed by pull-down assay with the GST-tagged β -spectrin N-terminal fragment, which contains the 4.1R-binding site. The interaction with F-actin was measured by an actin pelleting assay. Quantitative analysis reveals that phosphorylation of 4.1R and of its mutants, S312A and S331A by PKC reduced their binding to the spectrin fragment by ~ 30%, while that of the double mutant, lacking both phosphorylation sites, was unaffected (Fig 5A and B). Binding to F-actin was unaffected by phosphorylation in all cases (Fig 5C and D). We conclude that there is a small but significant effect of phosphorylation of 4.1R on its interaction with spectrin and thus on the stability of the junctional complex.

DISCUSSION

Protein 4.1R is a multifarious protein with binding capacity for a wide range of integral^{3,8,21,22} and skeletal proteins^{23,24} in the red cell and other membrane constituents such as phospholipids^{19,25}. These interactions permit the formation of the large 4.1R-based protein complexes in the membrane, which, as we have shown, are dissociated, wholly or partly, on phosphorylation of the 4.1R by PKC. The spectrin-actin-4.1R ternary complex, and thus the skeletal network junctions, are concomitantly loosened, and since the multiprotein 4.1R-linked complexes are necessarily confined to the junctions, some reciprocal effects may prevail.

It was previously shown that residue Ser-331 of 4.1R can be phosphorylated by PKA in solution¹³, and that Ser-312 is phosphorylated by PKC *in situ*¹⁵. We have now established that both serines are phosphorylated by PKC both in solution and in the cell. Moreover, our specific antibodies have now allowed us to discriminate between effects at these two sites,

and have revealed that phosphorylation at either one is sufficient to eliminate or grossly weaken the affinity of the protein for the known 4.1R-binding transmembrane proteins. By contrast, phosphorylation of 4.1R has no discernible effect on its interactions with the transmembrane proteins, Rh and band 3, a population of which appears also to be associated with the 4.1R-based complex in the membrane.

The smaller effect of phosphorylation of 4.1R on the stability of the ternary junction complex is in qualitative agreement with an earlier report ¹⁵. Functional characterization of the 4.1R spectrin-actin-binding (SAB) domain identified two spectrin-binding motifs: the N-terminal 21-amino acid cassette encoded by exon 16 and the region encompassing residues 27-43 within the C-terminal 59 residues of the SAB domain encoded by exon 17 ^{26,27}. An eight-amino acid actin-binding motif lies between the two spectrin-binding motifs ²⁸. Since the phosphorylation of 4.1R decreased its binding to spectrin by about 30%, only one of the two spectrin binding motifs may be affected by the phosphoryl group in the neighboring 16 kDa domain.

Another noteworthy aspect of our observations is that introduction of a single phosphoryl group in the 16 kDa domain of the protein engenders a large change in the affinities of binding sites in another domain, far removed in the amino acid sequence. The crystal structure of the protein, missing however the C-terminal 16kDa domain ⁵, comprises three globular elements, or lobes. The N-terminal lobe A contains the binding site(s) for band 3 and Rh, lobe B contains the site(s) for GPC, Kell, Duffy and XK, and lobe C contains p55 binding site. Since there is no disturbance by phosphorylation of the interaction with band 3, Rh and p55, it appears that the effects are confined to lobe B. This could in principle involve a structural change, which masks or otherwise inactivates the binding site(s), or a direct interaction between the two parts of the protein (the lobe B and 16 kDa). The nature of the conformational change transmitted between the domains remains to be explored.

Based on the available data from this and previous work, we propose the working model depicted in Fig 6 for the effect of 4.1R phosphorylation on the multiprotein complex. As shown in the left-hand panel, under normal condition, transmembrane proteins GPC, XK, Duffy, Rh, band 3, protein p55, as well as skeletal proteins spectrin and actin, all associate directly with 4.1R. Kell is also present in the complex through its interaction with XK. Upon PKC activation, residues ser-312 and ser-331 in the 16 kDa domain are phosphorylated, resulting in conformational changes in SABD and in lobe B of the 30 kDa domain. These diminish the inter-protein interactions, as indicated in the right-hand panel.

The physiological significance of 4.1R phosphorylation in the mature normal red cell is uncertain, since the protein is normally unphosphorylated. On the other hand, extensive phosphorylation of 4.1R occurs in reticulocytes ¹⁶, in malaria-infected red cells ^{29,30}, and also in sickle cells ³¹. There may thus be a role for 4.1R phosphorylation in early-stage erythrocytes and in pathological states.

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Abbreviations

4.1R	human erythrocyte protein 4.1
GPC	glycophorin C
GPA	glycophorin A
PKC	protein kinase C
PKA	protein kinase A
PMA	4 α -phorbol 12,13-didecanoate
IPTG	isopropyl β -D-thiogalactopyranoside
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Fig 1.

(A) Schematic representation of 4.1R structure. The 80 kDa red cell 4.1R contains four functional domains: The N-terminal membrane-binding domain (also called 30 kDa domain), consisting of lobe A, lobe B and lobe C), the 16 kDa domain, the spectrin-actin binding domain (SABD) and the C-terminal domain (CTD). The binding sites for other membrane proteins and the phosphorylation sites are indicated. **(B) Specificity of the anti-phosphoserines 312S and 331S antibodies.** The His-tagged Wild-Type, S312A, S331A and S312,331A recombinant 4.1R proteins were phosphorylated in the presence of the catalytic subunit of PKC in vitro and were stained after PAGE with Coomassie Blue staining

or detected by Western Blotting with anti-Phospho312S, - Phospho331S and anti-4.1R antibodies.

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Fig 2. Detergent extractability of transmembrane proteins

(A) and (B) Detergent extractability of components of the 4.1R-based multiprotein complex. Red cells were treated either without or with 2 μ M of PMA for 5 (A) or 90 minutes (B) at 37°C. Membrane proteins were extracted with Triton X-100 and proteins retained in the membrane skeletons were analyzed by SDS-PAGE and probed with antibodies as indicated. Note that only adducin is phosphorylated after 5 min and both adducin and 4.1R are phosphorylated after 90 min. Note also the decreased retention of GPC, XK, Kell and Duffy in membranes treated for 90 min but not when treated for 5 min.

(C) Detergent extractability of components of the ankyrin R-based macromolecular complex. Red cells were treated with 2 μ M PMA for 90 minutes. The experiments were performed as described above. Note that no difference is observed between untreated and PMA-treated membranes.

Fig 3. Effect of 4.1R phosphorylation on binding to XK, Duffy, GPC, Rh, band 3 and p55
 The His-tagged Wild-type 4.1R and its mutants S312A, S331A S312,331A were phosphorylated by the catalytic subunit of PKC in solution. The binding of unphosphorylated or phosphorylated 4.1R proteins to the cytoplasmic domain of XK, Duffy, GPC, Rh, band 3 and p55 was measured by pulldown assays. Binding was detected by western blotting with anti-4.1R antibody. Note that phosphorylation of either or both Ser-312 and Ser-331 almost abolished binding to XK, Duffy and GPC but not to Rh, band 3 and p55.

Fig 4. Effect of the 4.1R phosphorylation by PKC on the incorporation of β -spectrin polypeptide into membrane skeletal junctions

GST-tagged polypeptide 1-301 was introduced into ghosts before and after treatment with PMA for 90 minutes at 37°C. Triton shells from the resealed ghosts were analyzed by electrophoresis in 10% SDS and Coomassie Blue staining (A). The incorporated peptide was quantified by Image J software. Quantitative analysis revealed significantly higher incorporation of the peptide into the cytoskeletons of PMA-treated than untreated cells (B). GST-tagged polypeptide β I 1-301 or BSA was introduced into ghosts and their retention in the triton shell was examined as described above. Lane 1, ghosts; lane 2, ghosts resealed with GST- β I 1-301; lane 3, triton shell of ghosts resealed with GST- β I 1-301; lane 4, GST- β I 1-301; lane 5, ghosts; lane 6, ghosts resealed with BSA; lane 7, triton shell of ghosts resealed with BSA; lane 8, BSA. Note that in contrast to β I 1-301, BSA is not retained in the triton shell (C).

Fig 5. Effects of 4.1R phosphorylation on its interactions with spectrin and actin in solution

The His-tagged wild-type 4.1R and its mutants, S312A, S331A and S312,331A, were phosphorylated by PKC in solution. Binding of the unphosphorylated and phosphorylated 4.1R proteins to GST-tagged β -spectrin polypeptide and to actin was observed by GST pulldown assay (A) and the actin pelleting assay (C), respectively. The binding of 4.1R was detected by western blotting with anti-4.1R antibody. Quantitative analysis from three independent experiments for binding to spectrin or actin is shown in Fig 5B and 5D respectively. Note that phosphorylation of either or both Ser-312 and Ser-331 suppressed the binding to β -spectrin polypeptide, but had no effect on binding to actin.

Fig 6. Working model for the effect of 4.1R phosphorylation on the 4.1R multiprotein complex
Left-hand panel: 4.1R-based complex contains transmembrane proteins GPC, XK, Kell, Duffy, Rh, band 3, and protein p55, as well as skeletal proteins spectrin and actin. While GPC, XK and Duffy bind to lobe B, Rh and band 3 bind to lobe A, and p55 to lobe C of the N-terminal membrane binding region of 4.1R. SABD binds to spectrin and actin. **Right-hand panel:** residues ser-312 and ser-331 in the 16 kDa domain are phosphorylated upon PKC activation (red p). This results in conformational changes in SABD and lobe B in the 30 kDa domain as indicated, and causes in turn a weakening of the 4.1R-spectrin association, and liberation of GPC, XK, Kell, and Duffy.