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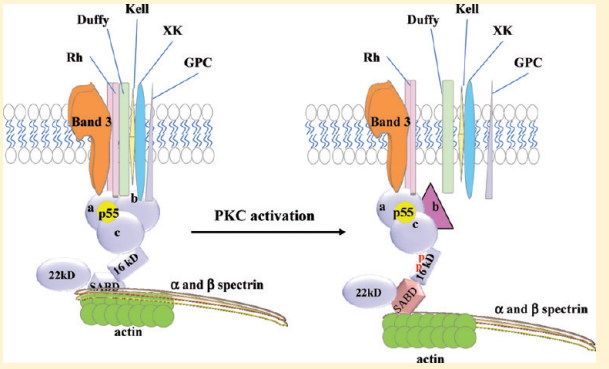
Phosphorylation-Dependent Perturbations of the 4.1R-Associated Multiprotein Complex of the Erythrocyte Membrane

Emilie Gauthier,[†] Xinhua Guo,[†] Narla Mohandas,[†] and Xiuli An*[,†,‡]

[†]Red Cell Physiology Laboratory and [‡]Laboratory of Membrane Biology, New York Blood Center, New York, New York 10065, United States

ABSTRACT: The bulk of the red blood cell membrane proteins are partitioned between two multiprotein complexes, one associated with ankyrin R and 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, phosphorlyation 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 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 for the 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, respectively), the rhesus proteins, Rh and RhAG, CD47, LW, Duffy, XK, and Kell.[1] Recent work has shown that several of these proteins are assembled into two multiprotein complexes. The first, commonly termed 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, facilitating coordinated CO_2 uptake and O_2 release.[2] The second multiprotein complex, which we have termed the "4.1R complex",[3] comprises the three principal components of the skeletal network junctions (spectin, 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 domain.[4] The crystal structure of the 30 kDa domain reveals a cloverleaf disposition of three globular lobes.[5] 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 Ca^{2+} and calmodulin, by PIP_2, and by phosphorylation. Binding of band 3, GPC, and p55 to 4.1R is modified by Ca^{2+} and calmodulin.[10,11] PIP_2, which binds in the cleft between lobes A and C, promotes binding of GPC but inhibits that of band 3.[12] 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 nonconserved 16 kDa domain.[13] Phosphorylation of the 10 kDa spectrin−actin domain by tyrosine kinase reduces the strength of the 4.1R−spectrin−actin complex,[14] while phosphorylation of serine 312 by PKC in situ weakens the binding of 4.1R to GPC[15] and the stability of the ternary junction complex, with accompanying reduction of the shear resistance of the membrane.[15]

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

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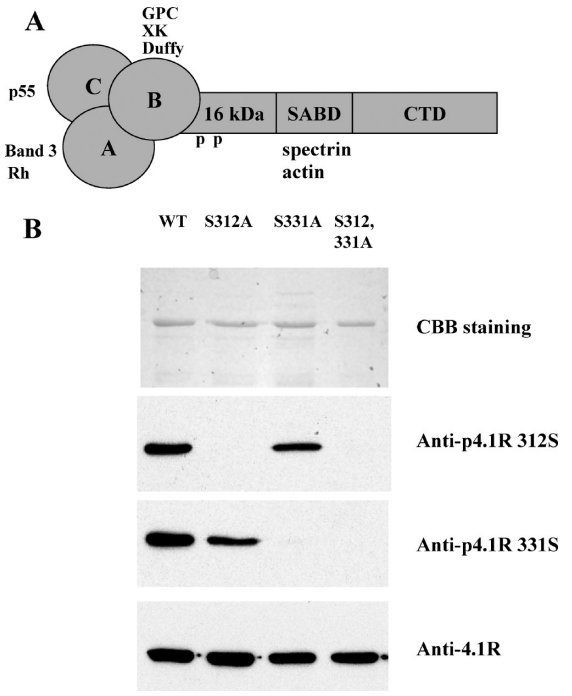

Figure 1. (A) Schematic representation of the 4.1R structure. The 80 kDa red cell 4.1R contains four functional domains: the N-terminal membrane-binding domain (also called the 30 kDa domain), consisting of lobe A, lobe B, and lobe C; the 16 kDa domain; the spectrin−actin binding domain (SABD); and the C-terminal domain (CTD). The binding sites for other membrane proteins and the phosphorylation sites are indicated. (B) Specificity of the anti-phosphoserine 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 or detected by Western blotting with anti-phospho-312S, anti-phospho-331S, and anti-4.1R antibodies.

MATERIALS AND METHODS

Materials. Human venous blood was drawn from healthy volunteers with informed consent. pMAL vector and MBP resin were obtained from New England Biolabs (Beverly, MA). pET31b-(+) and nickel columns were from Novagen (Madison, WI). BL21(DE3) bacteria and the Quick-Change site-directed mutagenesis kit were from Stratagene (La Jolla, CA). 4α-Phorbol 12, 13-didecanoate (PMA), the reduced form of glutathione, and isopropyl β-D-thiogalactopyranoside (IPTG) were from Sigma (St. Louis, MO). Glutathione Sepharose 4B was from Amersham Pharmacia Biotech (Piscataway, NJ). Millipore Centriprep YM-30 was from Fisher Scientific (Pittsburgh, PA). Calyculin A and the 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 the XK cytoplasmic domain, and the 4.1R binding region of GPC were from Genemed Synthesis Inc. (San Antonio, TX). The sequences are as follows: Duffy, HQATRTLLPSLPLPEGWFSHLDTLGSKS; Rh, NLKIWKAPHEAKYFDDQVFWKFPHLAVGF; XK, HPCK-KLFSSSVSEGFQRWLRCFCWACRQQKPCEPIG; GPC, RY-MYRHKGTYHTNEAKG. Non-muscle actin was from Cytoskeleton, Inc. (Denver, CO).

Methods. *Preparation of Proteins.* Spectrin was prepared from erythrocytes according to the method of Tyler et al.[17] The recombinant fragment of the N-terminal region of β-spectrin, comprising residues 1−301, 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 previously.[19] 4.1R-S312A, 4.1R-S331A, and 4.1R-S312,331A mutants were generated by in vitro mutagenesis using the Quick-Change site-directed mutagenesis kit, according to the supplier's instructions. The cDNA encoding p55 protein, cloned into the pMAL vector, or the cDNA encoding 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 dialyzed against phosphate-buffered saline [10 mM phosphate (pH 7.4) and 150 mM NaCl] for pull-down assays and against hypotonic buffer [5 mM Tris and 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 mutants mentioned above at a concentration of 6.25 μM were mixed with the catalytic subunit of PKC (25 ng/mL) in reaction buffer (60 mM MES, 1.5 mM EGTA, 15 mM $MgCl_2$, 125 μM ATP, and 0.05 μM calyculin A) for 15 min 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 three times with ice-cold phosphate-buffered saline. Packed red cells were incubated at 37 °C for 10 min in the presence of 0.02 μM calyculin A and then treated either without or with 2 μM PMA for 5 or 90 min.

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 and 5 mM Tris (pH 7.4)], followed by three washes in 35 volumes of the same buffer; 50 μL of the packed ghosts was resuspended in 150 μL of PBS, containing Triton X-100 and 0.02 μM calyculin A, for 30 min 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 21000*g* in the cold for 20 min, and the pellet was resuspended in PAGE sample buffer and boiled for 5 min.

Incorporation of the 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 and 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 the β-spectrin N-terminal peptide (residues 1−301) as described previously.[20] Calyculin A (0.02 μM) 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.

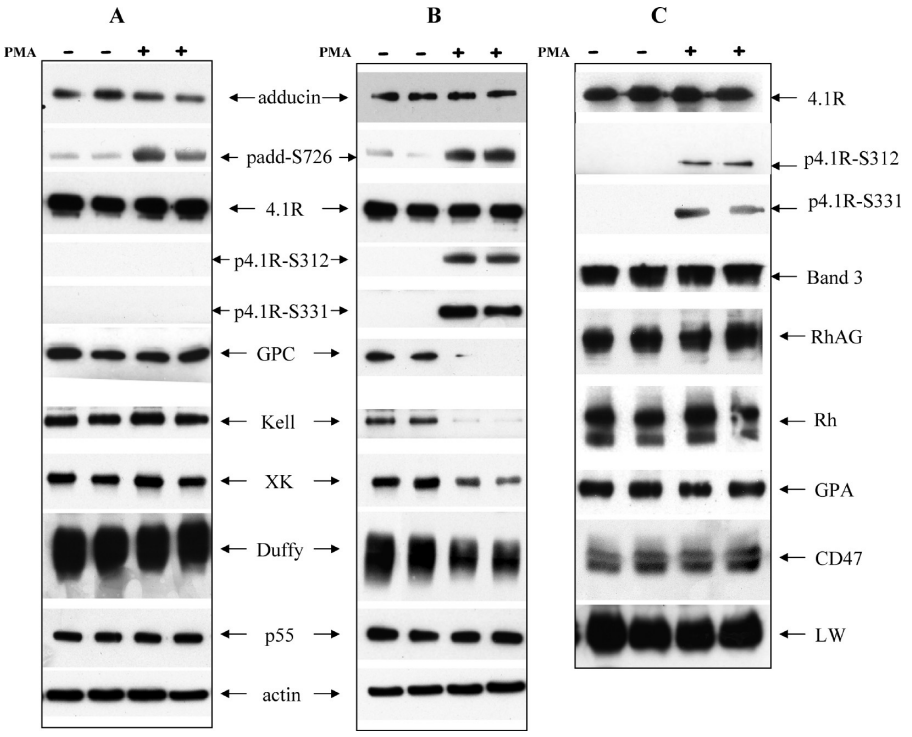

Figure 2. Detergent extractability of transmembrane proteins. (A and B) Detergent extractability of components of the 4.1R-based multiprotein complex. Red cells were not treated or were treated with 2 μM PMA for 5 (A) or 90 min (B) at 37 °C. Membrane proteins were extracted with Triton X-100, and proteins 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 level of 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 μM PMA for 90 min. The experiments were performed as described above. Note that no difference is observed between untreated and PMA-treated membranes.

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 resuspended in 20 volumes of extraction buffer [0.625 M NaCl, 6.25 mM sodium phosphate, 0.625 mM EGTA, 0.625 mM DTT, and 2% Triton (pH 7.4)] and incubated for 30 min at 4 °C, followed by centrifugation at 21000*g* in the cold for 20 min. The pellet was resuspended 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 h at room temperature. Streptavidin beads were added and recovered by centrifugation at 21000*g* for 5 min. 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, the GST-tagged cytoplasmic domain of band 3, or the GST-tagged βI spectrin fragment, using amylose beads or glutathione beads.

Actin Pelleting Assay. F-Actin (7 μM with respect to monomer) was incubated with unphosphorylated or phosphorylated 4.1R or its mutants (1.7 μM) for 60 min at room temperature in binding buffer [130 mM KCl, 20 mM NaCl, 10 mM Tris, 0.1 mM EGTA, 10 mM β_2-mercaptoethanol, 0.2 mM ATP, 2 mM $MgCl_2$, and 30 μM PMSF (pH 7.4)] and then centrifuged at 4 °C for 30 min at 90000 rpm (313000*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 the anti-4.1R antibody.

SDS−PAGE and Western Blotting. The samples were separated by SDS−PAGE in 10% gels. The proteins were transferred to a nitrocellulose membrane, blocked for 2 h in blocking buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 0.01% Tween, 4% nonfat dried milk powder, and 1% BSA], incubated for 1 h with the desired primary antibody, and then washed and incubated with anti-rabbit, anti-mouse, or anti-rat IgG coupled to HRP. The 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. Figure 1A shows the four structural domains of 4.1R and the subdomains 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

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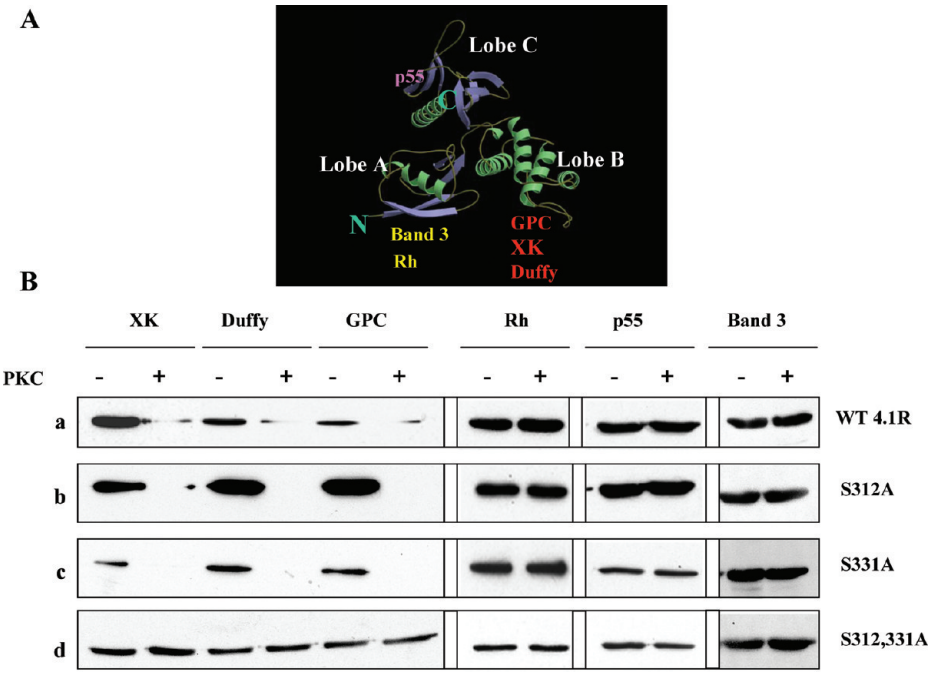

Figure 3. Effect of 4.1R phosphorylation on binding to XK, Duffy, GPC, Rh, band 3, and p55. His-tagged wild-type 4.1R and mutants S312A, S331A, and S312,331A were phosphorylated by the catalytic subunit of PKC in solution. The binding of unphosphorylated or phosphorylated 4.1R proteins to the cytoplasmic domain of XK, Duffy, GPC, Rh, band 3, and p55 was assessed by pull-down assays. Binding was detected by Western blotting with the anti-4.1R antibody. Note that phosphorylation of serine 312, serine 331, or both almost abolished binding to XK, Duffy, and GPC but not to Rh, band 3, and p55.

wild-type 4.1R and the mutants S312A, S331A, and S312,331A and exposed them in solution to the catalytic subunit of PKC. Figure 1B shows that the anti-p4.1R 312S antibody recognized wild-type and S331A 4.1R, but not the S312A mutant or the S312,331A double mutant. Similarly, the anti-p4.1R 331S antibody recognized wild-type 4.1R and the S312A mutant but not the S331A mutant or 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 with nonionic detergent before and following exposure of the cell to PMA for periods of 5 or 90 min. After a 5 min exposure, only adducin but not 4.1R had been significantly phosphorylated (Figure 2A), in agreement with the results of Manno et al.[15] Phosphorylation of adducin did not measurably alter the detergent extractability of GPC, Kell, XK, or Duffy, implying that their interaction with the membrane skeleton remained unaffected. In marked contrast, after a 90 min 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 (Figure 2B). By striking contrast, the retention of band 3, RhAG, Rh, GPA, CD47, and LW, all components of the ankyrin R complex, was unaffected (Figure 2C). We conclude that phosphorylation of 4.1R selectively governs the attachment to the membrane skeleton of those transmembrane proteins that 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 improve our understanding of the basis of the effects described above 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 is depicted in Figure 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 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 (row a of Figure 3B) but not with Rh, p55, and band 3. Mutation of either of the two phosphorylation sites in 4.1R was sufficient to inhibit binding to XK, Duffy, and GPC (rows b and c of Figure 3B). With both phosphorylation sites eliminated, the mutant protein bound normally (row d of Figure 3B), 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 constitute the nodes of the membrane skeletal lattice. The red cells of individuals with a hereditary deficiency with respect to 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 membranes.[20] The incorporation of the same spectrin fragment is modestly but significantly enhanced following phosphorylation of 4.1R (Figure 4A,B), reflecting a reduction in the level of cohesion of the junction complex in situ.

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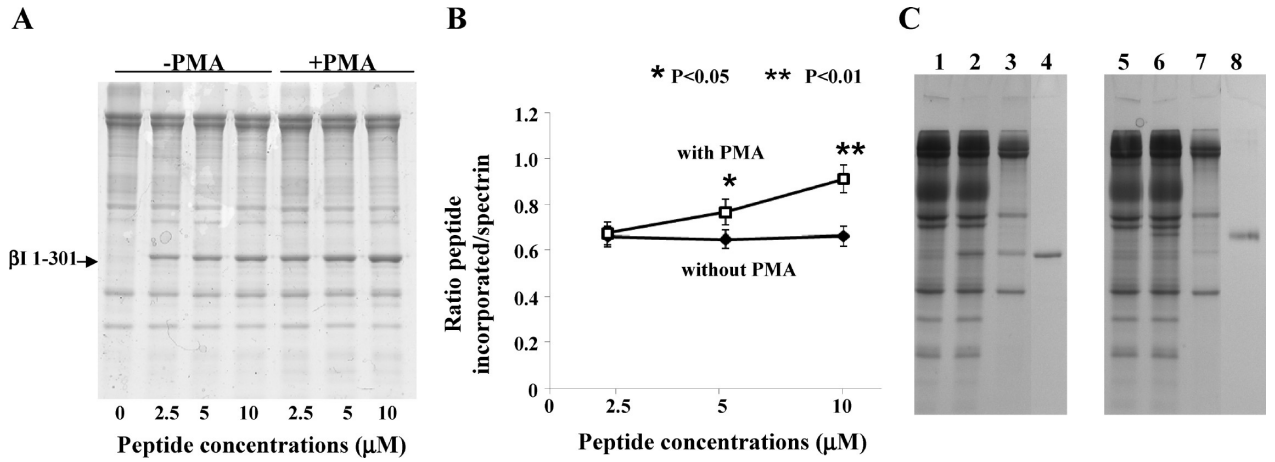

Figure 4. (A) Effect of the 4.1R phosphorylation by PKC on the incorporation of the β-spectrin polypeptide into membrane skeletal junctions. GST-tagged polypeptide 1−301 was introduced into ghosts before and after treatment with PMA for 90 min at 37 °C. Triton shells from the resealed ghosts were analyzed by electrophoresis in 10% SDS and Coomassie Blue staining. (B) The incorporated peptide was quantified with Image J. Quantitative analysis revealed significantly higher levels of incorporation of the peptide into the cytoskeletons of PMA-treated vs untreated cells. (C) GST-tagged polypeptide βI 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-βI 1−301; lane 3, Triton shell of ghosts resealed with GST-βI 1−301; lane 4, GST-βI 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 βI 1−301, BSA is not retained in the Triton shell.

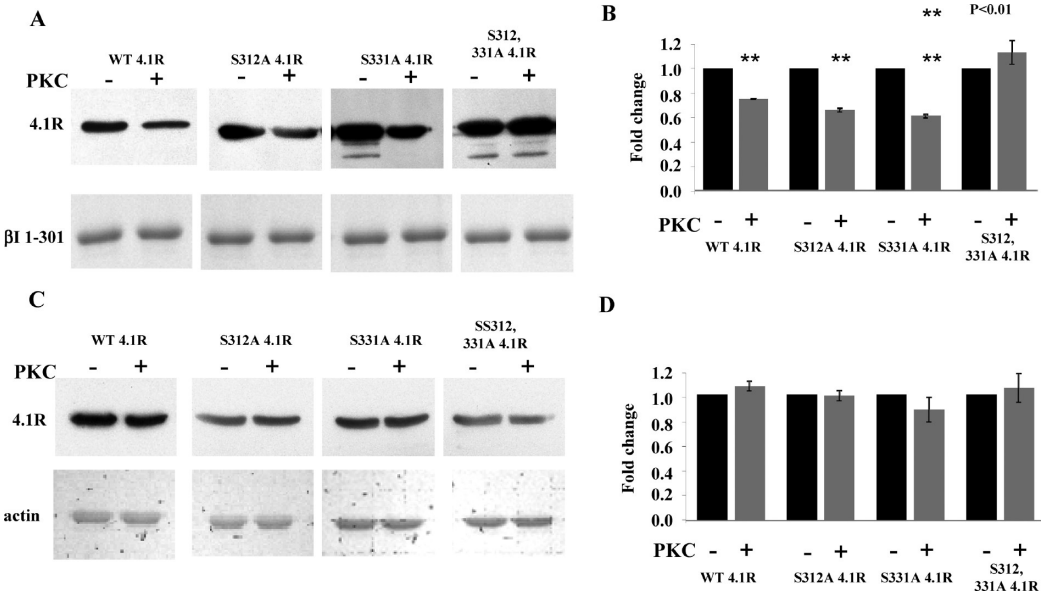

Figure 5. Effects of 4.1R phosphorylation on its interactions with spectrin and actin in solution. His-tagged wild-type 4.1R and its mutants, S312A, S331A, and S312,331A, were phosphorylated by PKC in solution. Binding of the unphosphorylated and phosphorylated 4.1R proteins to the GST-tagged β-spectrin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

As a control, bovine serum albumin was not incorporated into the skeleton (Figure 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 a 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 mutants S312A and S331A by PKC reduced their level of binding to the spectrin fragment by ~30%, while that of the double mutant, lacking both phosphorylation sites, was unaffected (Figure 5A,B). Binding to F-actin was unaffected by phosphorylation in all cases (Figure 5C,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.

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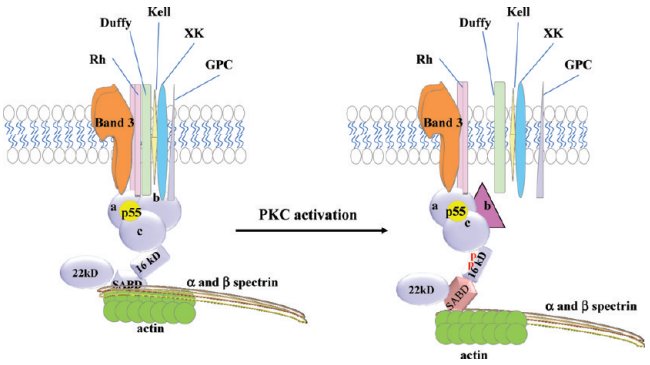

Figure 6. Working model for the effect of 4.1R phosphorylation on the 4.1R−multiprotein complex. In the left panel, the 4.1R-based complex contains transmembrane proteins GPC, XK, Kell, Duffy, Rh, band 3, and protein p55, as well as skeletal proteins spectrin and actin. While GPC, XK, and Duffy bind 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. In the right panel, residues serine 312 and serine 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.

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, upon phosphorylation of 4.1R by PKC. The spectrin−actin−4.1R ternary complex, and thus the skeletal network junctions, are concomitantly loosened, and because the multiprotein 4.1R-linked complexes are necessarily confined to the junctions, some reciprocal effects may prevail.

It was previously shown that residue serine 331 of 4.1R can be phosphorylated by PKA in solution[13] and that serine 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 weaker 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 59 C-terminal 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] Because the phosphorylation of 4.1R weakened its binding to spectrin by ∼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 the fact that introduction of a single phosphoryl group into 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 16 kDa 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 the site(s) for GPC, Kell, Duffy, and XK, and lobe C the p55-binding site. Because 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 (lobe B and the 16 kDa domain). The nature of the conformational change transmitted between the domains remains to be explored.

On the basis of the available data from this and previous work, we propose the working model depicted in Figure 6 for the effect of 4.1R phosphorylation on the multiprotein complex. As shown in the left panel, under normal conditions, transmembrane proteins GPC, XK, Duffy, Rh, band 3, and 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 serine 312 and serine 331 in the 16 kDa domain are phosphorylated, resulting in conformational changes in SABD and in lobe B of the 30 kDa domain. These weaken the interprotein interactions, as indicated in the right panel.

The physiological significance of 4.1R phosphorylation in the mature normal red cell is uncertain, because 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.

AUTHOR INFORMATION

Corresponding Author

*Laboratory of Membrane Biology, 310 E. 67th St., New York, NY 10065. Telephone: (212) 570-3247. Fax: (212) 570-3264. E-mail: xan@nybloodcenter.org.

Author Contributions

E.G. performed research, analyzed the data, and drafted the paper. X.G. performed experiments. X.A. and N.M. designed experiments, analyzed the data, and wrote the paper.

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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, 4α-phorbol 12,13-didecanoate; IPTG, isopropyl β-D-thiogalactopyranoside; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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