Modulation of Erythrocyte Membrane Mechanical Function by Protein 4.1 Phosphorylation*

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Erythrocyte membrane mechanical function is regulated by the spectrin-based membrane skeleton composed of α- and β-spectrin, actin, protein 4.1R (4.1R), and adducin. Post-translational modifications of these proteins have been suggested to modulate membrane mechanical function. Indeed, β-spectrin phosphorylation by casein kinase I has been shown to decrease membrane mechanical stability. However, the effects of the phosphorylation of skeletal proteins by protein kinase C (PKC), a serine/threonine kinase, have not been elucidated. In the present study, we explored the functional consequences of the phosphorylation of 4.1R and adducin by PKC. We identified Ser-312 in 4.1R as the PKC phosphorylation site. Using antibodies raised against phosphopeptides of 4.1R and adducin, we documented significant differences in the time course of phosphorylation of adducin and 4.1R by PKC. Although adducin was phosphorylated rapidly by the activation of membrane-bound atypical PKC by phorbol 12-myristate 13-acetate stimulation, there was a significant delay in the phosphorylation of 4.1R because of delayed recruitment of conventional PKC from cytosol to the membrane. This differential time course in the phosphorylation of 4.1R and adducin in conjunction with membrane mechanical stability measurements enabled us to document that, although phosphorylation of adducin by PKC has little effect on membrane mechanical stability, additional phosphorylation of 4.1R results in a marked decrease in membrane mechanical stability. We further showed that the phosphorylation of 4.1R by PKC results in its decreased ability to form a ternary complex with spectrin and actin as well as dissociation of glycoporphin C from the membrane skeleton. These findings have enabled us to define a regulatory role for 4.1R phosphorylation in dynamic regulation of red cell membrane properties.

The maintenance of normal membrane deformability and mechanical stability is critical for human red blood cells to undergo extensive deformations in the microvasculature, which is necessary to perform their function of oxygen delivery during their 120-day life span. The well-characterized spectrin-based membrane skeleton, composed of α- and β-spectrin, actin, protein 4.1R (4.1R), adducin, dematin, tropomyosin, and tropomodulin, plays a critical role in regulating membrane mechanical function (1). Qualitative and quantitative defects in α- and β-spectrin and 4.1R that lead either to an impaired spectrin-spectrin self-association or to a weakened spectrin-actin-4.1R ternary complex have been shown to result in decreased membrane mechanical stability (2–5).

A number of studies over the years have documented that skeletal proteins β-spectrin, 4.1R, adducin, and dematin can be phosphorylated by a number of different kinases and are dephosphorylated by various phosphatases (6, 7). In vitro studies using purified proteins have shown that phosphorylation can alter skeletal protein function (6, 7). However, our understanding of how the phosphorylation of various red cell membrane skeletal proteins regulates the mechanical function of intact red cell membranes has not been well defined. In a previous study, we documented that phosphorylation of β-spectrin by casein kinase I decreases the mechanical stability of intact membranes (8).

4.1R and adducin form a ternary protein complex with spectrin and actin, and both of them promote the association of spectrin with actin filaments (9–13). 4.1R also binds to the integral membrane proteins glycophorin C (GPC)† and band 3. Although an important role for 4.1R in regulating membrane mechanical stability has been documented (5), the role of adducin in regulating membrane mechanical function has yet to be delineated.

Limited chymotryptic digestion of 4.1R generates four distinct polypeptides, the 30-, 16-, 10-, and 22/24-kDa fragments (14). The 30-kDa N-terminal domain, referred to as the membrane binding or FERM domain (15), is responsible for binding 4.1R to transmembrane proteins GPC (16–19) and band 3 (20–22) and to p55 (23) and calmodulin (24). The function of the 16-kDa domain has yet to be defined. The 10-kDa domain, referred to as the spectrin-actin binding (SAB) domain, harbors the binding sites for spectrin and actin (25, 26). The C-terminal 22/24-kDa domains can interact with immunophilin FK506-binding protein (27), nuclear mitotic apparatus protein (NuMA) (28), and zonula occluden-2 (ZO-2) (29). 4.1R can be phosphorylated both in vitro and in vivo by protein kinase A (PKA) and PKC (21, 30–33). Previous studies (30) have documented that in solution, PKA stimulates the incorporation of 32P into Ser-331 and Ser-467 of purified 4.1R but that the phosphorylation of these residues has no effect on the ability of 4.1R to interact with its red cell membrane binding partners. However, phosphorylation of 4.1R by PKC, at a site yet to be defined, decreases the ability of 4.1R to promote spectrin-actin

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‡ The abbreviations used are: GPC, glycophorin C; SAB, spectrin-actin binding; PK, protein kinase; IOV, inside-out vesicle; PMA, phorbol 12-myristate 13-acetate; aPKC, atypical PKC; cPKC, conventional PKC; P-4.1R, phosphorylated protein 4.1R; PVDF, polyvinylidene difluoride; TBS, Tris-buffered saline; DI, deformability index; FERM, 4.1-ezrin-radixin-moesin.
interaction and its ability to bind to the cytoplasmic domain of band 3 in inside-out vesicles (IOV) but has no effect on 4.1R binding to GPC (21). In a subsequent study (31), phorbol 12-myristate 13-acetate (PMA) treatment of red blood cells has been shown to result in a decreased association of GPC with the membrane skeleton, suggesting that PKC phosphorylation releases GPC from its skeletal protein-binding partner. The effect of the phosphorylation of 4.1R on the mechanical function of an intact membrane has yet to be defined.

Adducin is present as a heterotetramer of d/β subunits in a 1:1 ratio in the red cell (35). Adducin subunits are related in sequence, and all of them contain an N-terminal globular head domain, a neck domain, and a C-terminal protease-sensitive tail domain (36). The tail domain of all adducin subunits ends with a highly conserved 22-residue myristoylated alanine-rich protein kinase C substrate (MARCKS)-related domain and contains the PKC phosphorylation site. In fact, Ser-726 of protein kinase C substrate (MARCKS)-related domain and contain-
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rate of decline in DI is a measure of membrane mechanical stability, and the time required for the DI to reach half-maximum value, designated T_{50}, is a quantitative measure of decreased stability. T_{50} values for membranes with various extents of 4.1R and adducin phosphorylation were determined.

Extractability of P-4.1R from Ghosts of PMA-treated Erythrocytes—
4.1R was extracted from ghosts of erythrocytes treated with or without PMA under the low ionic condition (0.1 mM EDTA, pH 8.5). Briefly, erythrocytes pretreated with 0.02 mM calyculin A were subsequently treated with or without PMA at 37 °C for 90 min. Ghosts prepared from these red cells were suspended in 0.1 mM EDTA (pH 8.5) and incubated at 37 °C for 30 min. The cell suspensions were centrifuged for 20 min at 40,000 g, and the 4.1R associated with the membranes and the extract 4.1R in the supernatant were analyzed by immunoblotting with both an anti-4.1R antibody that specifically recognizes phosphorylated 4.1R and an anti-4.1R antibody that recognizes both phosphorylated and non-phosphorylated 4.1R.

Measurement of 4.1, P-4.1R, and GPC Content of Triton Shells Prepared from PMA-treated Erythrocytes—Erythrocytes were treated with 2 μM PMA for 0, 5, or 90 min at 37 °C. 200 μl of cell suspension was added to 1 ml of phosphate-buffered saline containing 1% Triton X-100 followed by incubation for 30 min at 0 °C. The suspension was subsequently centrifuged for 30 min at 20,000 g, and the pellet was washed using cold phosphate-buffered saline. The Triton shells were analyzed by immunoblotting using antibodies against 4.1R and reductin 4.1R, and GPC.

Measurement of the Ability of 4.1R and P-4.1R to Form a Ternary Complex with Spectrin and Actin—The ability of 4.1R and P-4.1R to form a ternary complex with spectrin and actin was measured using a co-sedimentation assay with F-actin and purified spectrin from erythrocytes as described previously (25). F-actin (12 μM) and spectrin (1.6 μM) were incubated with phosphorylated or unphosphorylated 4.1R (1.7 μM) in binding buffer (130 mM KCl, 20 mM NaCl, 10 mM Tris, 0.1 mM EGTA, 10 mM β-mercaptoethanol, 0.2 mM ATP, 2 mM MgCl₂, 30 μM phenylmethylsulfonyl fluoride, pH 7.4) for 60 min on ice, prior to sedimentation. Samples were applied to a 0.2-ml cushion of 10% sucrose in binding buffer and centrifuged at 0 °C for 150 min at 30,000 × g. Equivalent portions of the supernatant and pellet of the three proteins were analyzed for their contents by SDS-PAGE (7.5% acrylamide).

Other Analytical Techniques—The protein concentration during the purification of 4.1R, P-4.1R, and spectrin was assayed using the Bradford method (43).

RESULTS

Identification of Phosphorylated Amino Acid of 4.1R in Intact Membranes—To initially identify the domain of 4.1R phosphorylated in intact membranes, erythrocytes were incubated with PMA, an activator of PKC, in the presence of calyculin A, a phosphatase inhibitor. The P-4.1R was separated by SDS-PAGE and proteolyzed by α-chymotrypsin. Cleavage products separated by SDS-PAGE were electrophoretically transferred to a PVDF membrane, and phosphorylated peptides were identified by anti-phosphoserine antibody. As shown in Fig. 1A, only the 16-kDa chymotryptic fragment of 4.1R was phosphorylated by PKC, whereas the chymotryptic fragments corresponding to the other three domains, 30-kDa FERM domain, 10-kDa SAB domain, and the C-terminal 22/24-kDa domains, were not phosphorylated. The consensus sequences identified previously (34) for phosphorylation by cPKC (and/or novel PKC) were (K/R)₁₋₃(X)₀₋₂(S/T) > (S/T)X₀₋₂(R/K)₁₋₃ > (K/R)₁₋₃X₀₋₂(S/T). There are nine serine residues in the 16-kDa domain, and based on the identified consensus sequences, all nine of the serine residues in the 16-kDa domain potentially can be phosphorylated. To determine which of the serine residues were phosphorylated in intact membranes, we used amino acid sequence analysis. Arg-299 was the first amino acid residue in the 16-kDa domain. As shown in Fig. 1B, the recovery of serine residues from the 16-kDa domain of unphosphorylated 4.1R decreased linearly with increasing cycles. Analysis performed on P-4.1R showed a similar linear decrease in the recovery of various serines except for Ser-312. The recovery of Ser-312 at cycle 14 of P-4.1R was markedly decreased (Fig. 1B), implying that Ser-312 was phosphorylated.

For further biochemical characterization, P-4.1R was purified from ghosts prepared from PMA-treated erythrocytes. Although 4.1R cannot be extracted from ghosts prepared from normal erythrocytes under hypotonic condition (0.1 mM EDTA, pH 8.5), surprisingly, almost all of the P-4.1R was extracted from ghosts of PMA-treated erythrocytes under this condition. Isolated P-4.1R was further purified from 4.1R by Q-Sepharose column chromatography with a linear gradient of 100–500 mM KCl in Buffer A. Although 4.1R eluted at 240 mM KCl, the elution of P-4.1R was shifted to 340 mM KCl (data not shown). This finding suggests that P-4.1R is more negatively charged than 4.1R and that phosphorylation may induce a conformational change in 4.1R. Biochemical analysis showed that the phosphate content of P-4.1R isolated under these conditions was 1.3 mol of phosphorous/mol of 4.1R. Thus, it appears that the intrinsic PKC phosphorylates only one site in membrane-associated 4.1R in erythrocytes.

To confirm that the phosphorylation site of 4.1R in intact
membranes was indeed Ser-312, anti-P-4.1R antibody was made using synthetic peptide (CAAAQ\textsuperscript{307}TRAAS(phospho)-ALID). Although the antibody detected a strong band corresponding to 4.1R in membranes prepared from erythrocytes treated with PMA, it did not detect 4.1R in membranes treated with 4\alpha-phorbol 12,13-didecanoate, the inactive phorbol ester (Fig. 2A). This finding strongly supports our conclusion that Ser-312 is the residue in 4.1R that is phosphorylated in vivo by PKC. We also generated an antibody against P-adducin using the corresponding phosphopeptide. As with 4.1R, anti-P-adducin antibody specifically recognized phosphorylated \(\alpha\)-adducin and \(\beta\)-adducin but not unphosphorylated adducin (Fig. 2A).

**Involvement of P-4.1R and P-adducin in Membrane Stability**—The time-dependent phosphorylation of 4.1R and adducin in intact membranes monitored using the two phosphorylation-specific antibodies is shown in Fig. 2A. Both 4.1R and adducin exhibited a time-dependent increase in the extent of phosphorylation. Interestingly, although phosphorylation of adducin occurred very soon after the addition of PMA, there was a lag phase before the phosphorylation of 4.1R could be documented (Fig. 2B).

The observed difference between the kinetics of 4.1R and adducin phosphorylation by PKC suggests that different PKC isoforms may be responsible for the phosphorylation of these two skeletal proteins. PKC is a group of 11 distinct isoforms, and not all isoforms are PMA-inducible. The various isoforms differ in their cofactor requirement, substrate specificity, and subcellular localization. To determine whether the observed differences in the time course of phosphorylation was caused by different PKC isoforms, we probed for the membrane association of PMA-inducible cPKC (such as PKC\(\beta\)) and aPKC (such as PKC\(\zeta\)). As shown in Fig. 3, aPKC (such as PKC\(\zeta\)) appears to be membrane bound, although there is a significant delay in the recruitment of cPKC (such as PKC\(\beta\)) to the membrane. Thus, the observed differences between the kinetics of phosphorylation of 4.1R and adducin in erythrocyte membranes could be accounted for by the different PKC isoforms responsible for the phosphorylation of these two skeletal proteins, aPKC (such as PKC\(\zeta\)) for adducin and cPKC (such as PKC\(\beta\)) for 4.1R.

To determine the consequences of phosphorylation of 4.1R and/or adducin on membrane mechanical cohesion, we quantified the membrane mechanical stability of resealed ghosts prepared from erythrocytes treated with PMA for different times. In erythrocytes treated for 5 min in which only adducin was phosphorylated (Fig. 4C), no significant changes in membrane mechanical stability could be documented (Fig. 4A). In erythrocytes treated for 90 min, both 4.1R and adducin were phosphorylated (Fig. 4D), and the membrane mechanical stability was markedly decreased (Fig. 4B). These findings imply that although phosphorylation of adducin by PKC has no effect on membrane mechanical stability, additional phosphorylation of 4.1R decreases the mechanical function of the membrane.

PMA dose-dependent increases in the magnitude of 4.1R and adducin phosphorylation and consequent changes in membrane mechanical stability were explored (Fig. 5). Immunoblot analysis with anti P-4.1R and P-adducin antibodies showed that the phosphorylation of adducin reached maximal levels at 0.1 \(\mu\)M, whereas that of 4.1R increased with increasing PMA concentrations, reaching maximal levels at approximately 1.5 \(\mu\)M (Fig. 5A). \(T_{50}\), a measure of membrane mechanical stability, showed a PMA dose-dependent decrease, a 30% decrease in membrane mechanical stability, at 1.5 \(\mu\)M PMA (Fig. 5B). The membrane mechanical stability did not show significant further decreases at higher concentrations of PMA. Importantly, these findings showed an inverse correlation between the PKC phosphorylation of 4.1R and membrane mechanical stability (Fig. 5B).

**Regulation of 4.1R Binding to Membrane Proteins by Phosphorylation**—The effect of phosphorylation on the ability of 4.1R to bind membrane proteins was evaluated by comparing the extractability of 4.1R and P-4.1R from erythrocyte membranes. 4.1R was tightly associated with IOVs prepared from untreated red cells (Fig. 6, lane 2'), and no 4.1R could be detected in the low ionic strength extraction buffer (lane 3'). In marked contrast, P-4.1R was extracted readily from PMA-treated erythrocytes. Little or no 4.1R was associated with IOVs prepared from untreated red cells (Fig. 6, lane 2'), and no 4.1R could be detected in the low ionic strength extraction buffer (lane 3'). Western blotting with antibody specific for phosphorylated 4.1R confirmed that although 4.1R was unphosphorylated in untreated red cells (Fig. 6, lanes 1'), it was phosphorylated in PMA-treated cells (Fig. 6, lane 4'). Interestingly the 4.1R released from PMA-treated erythrocytes was
the phosphorylated form (Fig. 6, lane 6'). These findings imply that the phosphorylation of 4.1R significantly reduces the affinity of its interaction with membrane proteins, GPC, and band 3.

To obtain further evidence of the effect of 4.1R phosphorylation on its ability to interact with GPC, the amount of GPC retained in Triton shells prepared from erythrocytes treated
with 2 μM PMA after 0, 5, and 90 min was quantitated (Fig. 7). Triton shells containing the equivalent amounts of spectrin/actin (Fig. 7, left panel) were examined by immunoblot analysis using antibodies against 4.1R, P-4.1R, and GPC (right panel). Immunoblotting with anti 4.1R antibody that recognizes both unphosphorylated and phosphorylated 4.1R showed that an equivalent amount of 4.1R was associated with spectrin and actin in all three Triton shell preparations. GPC remained associated with Triton shells prepared from untreated membranes and membranes treated for 5 min with PMA in which 4.1R was unphosphorylated. In marked contrast, very little GPC was associated with Triton shells when 4.1R was maximally phosphorylated following a 90-min incubation with PMA. These results imply that the interaction of 4.1R with GPC is reduced dramatically following phosphorylation of 4.1R.

Regulation of 4.1R Interaction with Spectrin and Actin by Phosphorylation—The effect of phosphorylation on the ability of 4.1R to form a ternary complex with spectrin and actin was assayed using a pelleting assay. As shown in Fig. 8A, although some spectrin could be pelleted in association with F-actin (lane 4) under the experimental conditions used, the addition of unphosphorylated 4.1R to a mixture of spectrin and actin significantly increased the amount of spectrin pelleted (compare lanes 4 and 5). Interestingly, the ability of P-4.1R to promote spectrin-actin interaction was decreased significantly compared with 4.1R (Fig. 8B). These findings imply that the phosphorylation of 4.1R significantly reduces its ability to form a ternary complex with spectrin and actin.

### DISCUSSION

Biochemical and functional studies have documented an important role for the spectrin-based membrane skeleton in regulating erythrocyte membrane mechanical function (1). Specifically, decreased self-association of spectrin dimers resulting from mutations in either α- or β-spectrin and the weakening of spectrin-actin-4.1R interactions caused by mutations in β-spectrin and 4.1R have been shown to decrease membrane mechanical stability (1, 5). Skeletal proteins, β-spectrin, 4.1R, adducin, and dematin can be phosphorylated by a number of different kinases and dephosphorylated by various phosphatases (7). In vitro studies using purified proteins have shown that phosphorylation can alter skeletal protein function (6, 7). However, our understanding of how phosphorylation of various red cell membrane skeletal proteins can regulate mechanical function of intact red cell membranes has not been well defined. A major finding of the present study is the documentation that phosphorylation of 4.1R, but not adducin, by PKC modulates membrane mechanical function.

A distinctive feature of the present study design warrants mention. In contrast to earlier studies that relied on the use of [32P]ATP to monitor phosphorylation, we used specific antibodies that recognized only the phosphorylated state of the proteins. The use of these antibodies enabled us to document significant differences in time course of phosphorylation of adducin and 4.1R in intact membranes. The rapid phosphorylation of adducin is the result of activation of membrane-associated aPKC (such as PKCζ) by PMA stimulation, whereas the gradual phosphorylation of 4.1R is caused by the delayed recruitment of cPKC (such as PKCβ) to the membrane. This differential time course between the phosphorylation of 4.1R and adducin in conjunction with membrane mechanical stability measurements enabled us to ascertain that although the phosphorylation of adducin alone by PKC has little effect on...
membrane mechanical stability, the additional phosphorylation of 4.1R results in a marked decrease in membrane mechanical stability.

Spectrin-actin interaction is essential for maintaining membrane mechanical stability. In solution, both 4.1R and adducin accentuate spectrin-actin interaction. However, phosphorylation of either 4.1R or adducin by PKC decreases its ability to promote spectrin-actin interaction in vitro (30). It is interesting to note that in intact membranes PKC phosphorylation of only adducin had little effect on membrane mechanical function, whereas phosphorylation of both 4.1R and adducin resulted in decreased membrane mechanical cohesion. This finding suggests that 4.1R phosphorylation in intact membranes destabilizes spectrin-actin interaction, leading to decreased membrane mechanical stability. It should be noted, however, that we could not exclude the possibility that 4.1R and adducin could act cooperatively in regulating membrane mechanical function.

Although a number of different serine residues in purified 4.1R are phosphorylated by PKC in solution, only Ser-312 in the 16-kDa domain of 4.1R is phosphorylated in intact membranes. Our biochemical studies showed that 4.1R phosphorylated at Ser-312 exhibits a decreased ability to promote spectrin-actin interaction. Our finding that the interaction between 4.1R and the N-terminal domain of β-spectrin and actin filaments is mediated by the 10-kDa SAB domain implies that the phosphorylation of Ser-312 induces a conformational change in the SAB domain of 4.1R. Interestingly, phosphorylated 4.1R also exhibits decreased affinity for interaction with band 3 and GPC, mediated by the 10-kDa SAB domain. Phosphorylation of Ser-312 in the 16-kDa domain decreases membrane mechanical stability. This finding suggests that 4.1R phosphorylation in intact membranes destabilizes spectrin-actin interaction, leading to decreased membrane mechanical stability. It should be noted, however, that we could not exclude the possibility that 4.1R and adducin could act cooperatively in regulating membrane mechanical function.

In contrast to our findings, a previous study (42) failed to demonstrate alterations in membrane mechanical stability following phosphorylation of 4.1R by PKC. The reason for the discrepancy is most likely the result of dephosphorylation of 4.1R during the lysing and rescaling of ghosts in the earlier study (42). In fact, we confirmed that 4.1R is indeed dephosphorylated during processing of the ghosts in the absence of the phosphatase inhibitor calyculin A.
