Structural and Functional Characterization of Protein 4.1R-Phosphatidylserine Interaction

POTENTIAL ROLE IN 4.1R SORTING WITHIN CELLS*

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Xiu-Li An‡‡, Yuichi Takakuwa‡, Sumie Manno‡, Bong-Gyoon Han‡, Philippe Gascard‡
and Narla Mohandas‡‡

From the ‡Department of Biochemistry, School of Medicine, Tokyo Women’s Medical University, Shinjuku, Tokyo 162-8666 and the ¶Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720

Erythrocyte protein 4.1R is a multifunctional protein that binds to various membrane proteins and to phosphatidylserine. In the present study, we report two important observations concerning 4.1R-phosphatidylserine interaction. Biochemically, a major finding of the present study is that 4.1R binding to phosphatidylserine appears to be a two-step process in which 4.1R first interacts with serine head group of phosphatidylserine through the positively charged amino acids YKRS and subsequently forms a tight hydrophobic interaction with fatty acid moieties. 4.1R failed to dissociate from phosphatidylserine liposomes under high ionic strength but could be released specifically by phospholipase A2, but not by phospholipase C or D. Biochemical analyses showed that acyl chains were associated with 4.1R released by phospholipase A2. Importantly, the association of acyl chains with 4.1R impaired its ability to interact with calmodulin, band 3, and glycophorin C. Removal of acyl chains restored 4.1R binding. These data indicate that acyl chains of phosphatidylserine play an important role in its interaction with 4.1R and on 4.1R function. In terms of biological significance, we have obtained evidence that 4.1R-phosphatidylserine interaction may play an important role in cellular sorting of 4.1R.

Protein 4.1R (4.1R)1 in erythrocytes has multiple binding sites for transmembrane and skeletal proteins and plays a critical role in maintaining cell morphology and membrane mechanical properties (1, 2). The mechanical stability of the erythrocyte membrane is regulated by 4.1R interaction with the spectrin-actin network (5, 4) and with membrane proteins, such as band 3 (5). Hereditary defects in 4.1R result in abnormally shaped erythrocytes with decreased membrane mechanical stability, manifested clinically as hemolytic anemia (6). Four main structural/functional domains with apparent molecular masses of 30, 16, 10, and 22–24 kDa have been identified in 4.1R (7). A 30-kDa N-terminal membrane binding domain possesses binding sites for the cytoplasmic tails of integral membrane proteins, such as band 3 (8), glycophorin C (GPC) (9), and CD44 (10). This domain also binds to p55 (9, 11) and calmodulin (CaM) (12, 13). A 10-kDa internal domain contains the critical spectrin-actin-binding activity required for membrane mechanical stability (4, 14–18), whereas the C-terminal 22–24-kDa domain binds the immunophilin FKBP13 (19), NuMA (20), and ZO-2 (21). The function of the 16-kDa domain remains to be defined.

Protein 4.1R has also been shown to bind phosphatidylserine (PS) (22–24), which is exclusively localized in the inner leaflet of the erythrocyte membrane. However, in contrast to extensive structural and functional characterization of the various protein-protein interactions involving 4.1R, little is known about the nature and the function of 4.1R interaction with PS. Recent studies are beginning to shed light on the potential function of PS interaction with cytoskeletal proteins. For example, PS interaction with the actin-binding protein talin has been shown to mediate anchorage of actin filaments to the phospholipid bilayer (25, 26), whereas PS interaction with microtubule-protein tau or with microtubule-associated GTPase dynamin has been shown to regulate protein function and/or phosphorylation state (27, 28).

To gain insight into the feature of 4.1R-PS interaction, we performed a detailed characterization of this interaction using PS liposomes and PS-loaded inside-out vesicles (IOVs) prepared from erythrocytes. We obtained evidence that suggests that 4.1R interaction with PS liposomes is a two-step process in which 4.1R first intercalates into PS liposomes through interaction with the serine head group of PS and subsequently forms a tight hydrophobic interaction with the fatty acid moieties. The positively charged cluster of amino acids YKRS, in the 30-kDa membrane binding domain of 4.1R, was documented to play an important role in the initial interaction of 4.1R with PS. Because of the subsequent tight hydrophobic interaction, 4.1R bound to PS could be released only by treatment with phospholipase A2 (PLA2), resulting in the release of 4.1R associated with fatty acids (FAs). 4.1R-associated FAs significantly impaired the ability of 4.1R to interact with CaM, band 3, and GPC. Removal of FAs restored 4.1R binding. Finally, whereas Ca2+/CaM regulates the interactions of 4.1R with various membrane proteins (10, 12, 29–31), it has no effect on 4.1R binding to PS liposomes. Moreover, CaM promptly dissociated from 4.1R upon PS binding. Taken together, these data indicate that YKRS residues in 4.1R membrane binding domain are responsible for intercalation of 4.1R into PS liposomes, whereas the acyl chains of PS play an

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‡ To whom correspondence should be addressed: Lawrence Berkeley National Laboratory, University of California, Mail Stop 74h/157, 1 Cyclotron Rd., Berkeley, CA 94720. Tel.: 510-486-7029; Fax: 510-486-6746; E-mail: mnaarl@lbl.gov.

1 The abbreviations used are: 4.1R, protein 4.1R; GPC, glycophorin C; PLA2, phospholipase A2; PLC, phospholipase C; PLD, phospholipase D; PS, phosphatidylserine; PC, phosphatidylcholine; FA, fatty acid; ER, endoplasmic reticulum; GFP, green fluorescent protein; CaM, calmodulin; IOV, inside-out vesicle; PAGE, polyacrylamide gel electrophoresis.

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important role in subsequent tight hydrophobic association of PS with 4.1R.

To investigate further the biological significance of 4.1R-PS interaction, we transfected wild type 4.1R and YRKS-AAAA mutant 4.1R, which fails to bind PS into COS-7 cells. We confirmed previous observation that wild type 4.1R is broadly distributed in the nucleus, cytoplasm, and plasma membrane (40). In marked contrast, mutant 4.1R mostly accumulated in the endoplasmic reticulum (ER). This evidence strongly implies that 4.1R-PS interaction may play an important role in 4.1R sorting and localization within cells.

**EXPERIMENTAL PROCEDURES**

**Materials**

After obtaining informed consent, blood was obtained from healthy human volunteers. Purified PS, phosphatidylcholine (PC), CaM from bovine brain, synthetic phosphatidic acid, lyso-PS, PLAs, from bee venom, phospholipase C (PLC) from Bacillus cereus, phospholipase D (PLD) from cabbages, and paraformaldehyde, were purchased from Sigma. Glutathione-Sepharose 4B, calmodulin-Sepharose CL-4B, and PreScission proteinase were purchased from Amersham Pharmacia Biotech. Precoated Silica Gel 60 plates were obtained from Merck. pET31b(+) vector and nickel resin were purchased from Novagen (Madison, WI). QuickChange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). 4.1R-GFP-C3 vector was from CLONTECH (Palo Alto, CA). Monoclonal anti-GFP antibody was purchased from Roche Molecular Biochemicals. Anti-mouse site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). one-Sepharose 4B, calmodulin-Sepharose CL-4B, and PreScission proteinase cleavage site were introduced from Roche Molecular Biochemicals. Anti-mouse IgG coupled to horseradish peroxidase was from Jackson ImmunoResearch Laboratories (West Grove, PA). Cell culture medium DME-H21, IgG coupled to horseradish peroxidase was from Jackson ImmunoResearch Laboratories (West Grove, PA). Cell culture medium DME-H21, IgG coupled to horseradish peroxidase was from Jackson ImmunoResearch Laboratories (West Grove, PA). Cell culture medium DME-H21, IgG coupled to horseradish peroxidase was from Jackson ImmunoResearch Laboratories (West Grove, PA). Cell culture medium DME-H21, IgG coupled to horseradish peroxidase was from Jackson ImmunoResearch Laboratories (West Grove, PA).

**Methods**

**Preparation of 4.1R—4.1R from erythrocytes was prepared according to the method described by Tyler et al. (32) with minor modifications. For some experiments, 4.1R was radiolabeled using Bolton-Hunter reagent (2000 Bq/mmol, PerkinElmer Life Sciences) and dialyzed against binding buffer (10 mM Tris, pH 7.4, 130 mM KCl, 20 mM NaCl, 1 mM Na$_2$HPO$_4$) at room temperature. Liposome-bound 4.1R was separated and analyzed as described above. The gels were stained with Coomassie Brilliant Blue to visualize the proteins in various fractions.

**Measurement of Protein-Liposome Interaction**—Gel filtration chromatography was used to assay 4.1R-liposome interaction, whereas sedimentation assay was used to quantify 4.1R-PS interaction. To perform gel filtration chromatography, 210 µg of proteins was incubated with liposomes (250 nm) in a total volume of 1.2 ml of binding buffer for 1 h at room temperature. To separate the liposome-bound protein from the unbound protein, 1 ml of the incubated mixture was incubated with a 1 × 30 cm Sepharose 4B column at a flow rate of 0.4 ml/min. 2-ml fractions of flow-through were collected and analyzed by 9% SDS-PAGE. The gels were stained with Coomassie Brilliant Blue and analyzed by densitometry.

**Analysis of 4.1R Binding to PS Liposomes in the Presence of CaM**—To assay 4.1R binding to PS liposomes in the presence of CaM, 4.1R (25 µg/ml) was preincubated with increasing concentrations of CaM (0–14.4 µM) in a total volume of 0.4 ml in the presence of 100 µM Ca$^{2+}$ or EGTA at room temperature for 30 min. 250 nM of PS liposomes were subsequently added to the mixture, and the incubation was continued for an additional 1 h at room temperature. Liposome-bound 4.1R was separated by centrifugation at 230,000 × g for 1 h at 4 °C. Under these conditions, liposome-bound 4.1R sediments with liposomes, whereas unbound 4.1R remained in the supernatant. Pellets were washed three times with binding buffer, and equivalent amounts of pellets were analyzed by 9% SDS-PAGE. Gels were stained with Coomassie Brilliant Blue and analyzed by densitometry.

**Effects of Ionic Strength and Detergents on 4.1R-PS Complex**—To study the effects of ionic strength on 4.1R-PS interaction, the incubation was carried out in binding buffer containing 0.5% Tween 20 or Triton X-100. Following incubation, liposome-bound 4.1R and unbound 4.1R were separated and analyzed as described above.

**Effects of PLAs, PLC, and PLD on 4.1R-PS Complex**—To study the effects of phospholipase treatment on 4.1R-PS liposome complex. PS liposomes with bound 4.1R were treated with either 10 units of PLA$_2$, or 60 units of PLD for 1 h at room temperature. The gels were stained with Coomassie Brilliant Blue to visualize the proteins in various fractions. Hydrolysis of PS by phospholipases was confirmed by TLC.

**Characterization of Protein 4.1R-PS Interaction**

Measurement of 4.1R Binding to IOVs and Loading of IOVs with Phospholipids—Inside-out vesicles depleted of all peripheral proteins (ph11 IOVs) were prepared as previously described (35) with minor modifications. Phospholipids (PS or PC) were loaded into pH 11 IOVs according to the method described by Brockman and Anderson (36) with minor modifications. Briefly, phospholipids dissolved in an equal volume mixture of chloroform and methanol at a concentration of 20 µg/ml were rapidly injected into 1 ml of IOV suspension (2.4 µg of protein). The final concentration of chloroform/methanol by volume in the mixture was 2%. The mixture was incubated overnight on ice with constant gentle stirring followed by three washes of the phospholipid-loaded IOVs with binding buffer. The loading of pH 11 IOVs with either PS or PC was confirmed by thin layer chromatography (TLC).

**Preparation of Phospholipid Liposomes**—Small unilamellar phospholipid liposomes were prepared as described by Brockman and Anderson (36). Phospholipid liposomes were stored under nitrogen at 4 °C and used within 1 week of their preparation. The phospholipid concentration of liposomes was determined by phosphorus analysis using the method described by Bartlett (33). Measurement of 4.1R Binding to IOVs and Loading of IOVs with Phospholipids—Inside-out vesicles depleted of all peripheral proteins (pH11 IOVs) were prepared as previously described (35) with minor modifications. Phospholipids (PS or PC) were loaded into pH 11 IOVs according to the method described by Brockman and Anderson (36) with minor modifications. Briefly, phospholipids dissolved in an equal volume mixture of chloroform and methanol at a concentration of 20 µg/ml were rapidly injected into 1 ml of IOV suspension (2.4 µg of protein). The final concentration of chloroform/methanol by volume in the mixture was 2%. The mixture was incubated overnight on ice with constant gentle stirring followed by three washes of the phospholipid-loaded IOVs with binding buffer. The loading of pH 11 IOVs with either PS or PC was confirmed by thin layer chromatography (TLC).

**Preparation of IOVs and Loading of IOVs with Phospholipids**—IOVs were prepared as previously described (35) with minor modifications. Phospholipid liposomes were stored under nitrogen at 4 °C and used within 1 week of their preparation. The phospholipid concentration of liposomes was determined by phosphorus analysis using the method described by Bartlett (33).
Characterization of Protein 4.1R-PS Interaction

RESULTS

4.1R Binding to PS Liposomes—Gel filtration column chromatography was used to separate liposomes from unbound protein. PS liposomes incubated with 4.1R were applied onto a Sepharose 4B column. As shown in Fig. 1A, a large fraction of 4.1R eluted in the void volume in association with PS liposomes (fractions 5–8), whereas a small quantity of unbound 4.1R was found in later fractions (fractions 12–13). PS liposomes without 4.1R remained associated with the liposomes (Fig. 1B), implying that 4.1R is tightly bound to the liposomes. In marked contrast, there was no binding of 4.1R to PC liposomes (data not shown). However, 4.1R could bind to phosphatidic acid liposomes in a manner analogous to its binding to PS liposomes (data not shown). These data imply that 4.1R can bind only to liposomes in which the hydrophilic head groups of phospholipids are negatively charged.

To determine the dependence of 4.1R binding on liposome PS content, binding studies were performed using PS/PC liposomes with varying PS contents (Fig. 1C). Little 4.1R binding was seen when PS content was less than 30%. Binding progressively increased at higher PS contents, and maximal binding was seen when PS content was 40% or higher. This finding suggests that a threshold amount of the serine head group of PS is required for 4.1R binding to liposomes.

In order to identify the PS binding motif in 4.1R, the ability of various 4.1R recombinant proteins encoding different domains of the protein to bind PS liposomes was evaluated. The recombinant protein encoding the N-terminal 30-kDa domain of 4.1R bound PS liposomes, whereas the proteins encoding the 16- and 22/24-kDa domains did not (data not shown). Furthermore, a recombinant protein representing sequences encoded by exons 9–11 in the 30-kDa domain bound PS liposomes, whereas sequences encoded by exons 4–9 failed to do so (data not shown), implying that PS binding motif of 4.1R lies in sequences encoded by exons 10 and/or 11. A closer examination of the sequences encoded by these two exons revealed the presence in exon 10 of the sequence motif YKRS, similar to the PS binding motif FKKKS previously reported in MARCKS protein (37). Mutation of the YKRS sequence to AAAA abolished the ability of 4.1R to bind PS liposomes (Fig. 2). Thus, the positively charged cluster of amino acids YKRS, in the 30-kDa membrane binding domain of 4.1R, appears to play a critical role in mediating 4.1R interaction with the negatively charged serine head group of PS.

Hydrophobic Nature of the Interaction between 4.1R and PS
Characterization of Protein 4.1R-PS Interaction

Liposomes—To further define the basis for the observed tight interaction between 4.1R and PS liposomes, we first quantitated 4.1R binding to PS liposomes. The amount of 4.1R bound to PS-liposomes increased with increasing 4.1R concentrations (Fig. 3A), and the measured maximal level of binding was 125 μg of 4.1R/mg of PS. Scatchard analysis did not generate a linear relationship between the ratio of bound to free 4.1R and bound 4.1R, and therefore, we could not derive a value for the dissociation constant (K_d) (Fig. 3B). We also explored the experimental conditions needed to release 4.1R from PS liposomes. High salt concentrations, which have been previously shown to dissociate 4.1R from IOVs prepared from red cell membranes, failed to dissociate 4.1R from PS liposomes. Even at a concentration of 2 M KCl, there was no release of 4.1R from PS liposomes. However, treatment of 4.1R-bound PS liposomes with 0.5% Tween 20 as well as other detergents resulted in the release of 4.1R (data not shown). These results strongly imply a hydrophobic interaction between 4.1R and PS liposomes.

Release of 4.1R from PS Liposomes by PLA_2—To further document the hydrophobic interaction between 4.1R and PS liposomes, we examined the ability of various phospholipases, PLA_2, PLC, and PLD, to release bound 4.1R. When PS liposomes with bound 4.1R were treated with PLA_2, 4.1R was released from the liposomes (Fig. 4A). It is noteworthy that the released 4.1R was found in fractions 9–11 (Fig. 4A), whereas free 4.1R was normally found in fractions 12–13 (see Fig. 1A). This finding suggested that the biochemical character of 4.1R released from the liposomes following PLA_2 treatment was altered. In marked contrast, neither PLC nor PLD could release 4.1R (Fig. 4B).

It should be noted that PLA_2 treatment resulted in hydrolysis of significant amounts of PS in the liposomes, resulting in the generation of large quantities of lyso-PS. However, these liposomes were intact. They did not pass through a polycarbonate membrane with a nominal pore diameter of 20 nm, implying that they were larger than 20 nm in size. In contrast, micelles composed of pure lyso-PS easily passed through the same polycarbonate membranes. These findings indicate that PLA_2-induced release of 4.1R from PS liposomes was not due to physical breakup of the liposomes.

Biochemical and Functional Characterization of PLA_2-released 4.1R—TLC analysis showed that FA was associated with released 4.1R, and fatty acid measurement showed that the FA:4.1R molar ratio was 6–8:1. Neither intact PS nor lyso-PS was found in association with the released 4.1R. Most of the 4.1R-associated FAs could be released by treatment with 0.5% Tween 20. Importantly, in terms of function, whereas native 4.1R interacted with band 3, GPC, CaM, and PS, 4.1R with associated FAs failed to interact with any of these binding partners. We then examined whether the ability of 4.1R to interact with its binding partners could be restored by removal of FAs from the FA-4.1R complex by Tween 20. As shown in Fig. 5, although FA-bound 4.1R did not bind to band 3 and GPC on IOVs, both native 4.1R and 4.1R from which bound FAs had been removed did bind to IOVs. Restoration of binding ability following removal of FAs was also observed for 4.1R binding to CaM and to PS liposomes (data not shown).

Effect of Ca^{2+}-CaM on 4.1R Binding to PS Liposomes—Previous studies identified two distinct CaM binding motifs in the 30-kDa membrane binding domain of 4.1R, one encoded by exon 10 that binds CaM with high affinity in the absence of Ca^{2+} and another encoded by exon 9 that binds CaM with high affinity in the presence of Ca^{2+}. Because Ca^{2+}-CaM has been shown to regulate the interactions of 4.1R with various membrane proteins including band 3 and GPC (10, 12, 13, 29–31), we explored whether Ca^{2+}-CaM could also regulate interaction of 4.1R with PS. We found that preincubation of 4.1R with CaM, in either the absence or presence of Ca^{2+}, had no effect on 4.1R binding to PS liposomes. Importantly, when 4.1R-CaM complex was added to PS liposomes, CaM rapidly dissociated from 4.1R following binding of 4.1R to PS-liposomes (data not shown). In another series of experiment, we found that CaM could not bind to PS-bound 4.1R. These findings imply the existence of a potential overlap between binding sites for CaM and for PS in the 30-kDa membrane binding domain of 4.1R. An examination of the recently generated crystal structure of the 30-kDa domain (38) provides direct validation for this suggestion. Although in the linear protein sequence, the YKRS motif (which was documented to be important for 4.1R intercalation into PS liposome) in exon 10 and the high affinity Ca^{2+}-insensitive CaM binding motif in exon 11 of the 30-kDa domain are separated by 29 amino acids, as shown in Fig. 6, in the three-dimensional structure these two motifs are physically adjacent to each other, with potential overlap of the binding pockets.

4.1R Binding to PS-loaded IOVs—Having demonstrated an interaction between 4.1R and PS liposomes, we further explored the nature of 4.1R interactions with membrane proteins and PS in a biological membrane system. For these studies, we employed erythrocyte IOVs from which all peripheral proteins were stripped. 125I-4.1R bound to IOVs in a dose dependent manner (Fig. 7A) with a maximal binding capacity of 275 μg/mg of IOV proteins. The bound 4.1R could not be dissociated by PLA_2 treatment (Fig. 7A), indicating that 4.1R binds to band 3 and GPC but not to PS in such IOV membranes. This is most likely due to loss of PS-rich domains in these IOV membranes during their preparation. However, when exogenous PS (1.6 μg/μg IOV proteins) was preloaded into these IOV membranes, the amount of bound 4.1R increased progressively with increasing concentrations of added 4.1R. More importantly, all additionally bound 4.1R could be dissociated by PLA_2 treatment of PS-loaded IOVs (Fig. 7A). Scatchard analysis of these binding data indicated that whereas 4.1R bound to protein components of IOVs with a dissociation constant (K_d) of 2.5 × 10^{-7} M (Fig. 7B), as with PS liposomes, no saturable binding of 4.1R to PS-loaded IOVs could be demonstrated (Fig. 7B).

Abnormal Cellular Distribution of 4.1R Deficient in PS Binding—Our in vitro binding assay showed that mutation of the YKRS sequence to AAAA abolished the ability of 4.1R to bind PS liposomes (Fig. 2). To further explore the biological significance of 4.1R-PS interaction in vivo, COS-7 cells were transfected with either GFP-wild type 4.1R or YKRS-AAAA mutant 4.1R fusion protein. Expression of each fusion protein was confirmed by Western blot analyses of transfected cells using a monoclonal anti-GFP antibody. Both wild type GFP-4.1R and mutant GFP-4.1R were expressed at similar levels and migrated at the expected size of ~110 kDa (Fig. 8A). Immunofluorescence analyses revealed that whereas wild type GFP-4.1R was broadly expressed in the nucleus, cytoplasm, and plasma membrane (Fig. 8B, a and b) as previously reported, the cellu-
lar distribution of mutant GFP-4.1R was strikingly different. Mutant 4.1R accumulated in the perinuclear region (Fig. 8B, c and d). Staining of the transfected cells with the ER probe rhodamine R6 confirmed the accumulation of mutant 4.1R in the ER (Fig. 8B, e-h). This discrepancy was neither the result of differences in the levels of expression of mutant GFP-4.1R compared with the wild type GFP-4.1R (Fig. 8A) nor the result of differences in protein solubility (data not shown). Taken together, these data strongly suggest that impairment of 4.1R binding to PS dramatically alters 4.1R sorting within the cell.

FIG. 3. Concentration dependence of 4.1R on its binding to PS liposomes. Various concentrations of 4.1R were incubated with PS liposomes, and the amount of 4.1R bound to the liposomes was quantitated as described under “Experimental Procedures.” As 4.1R concentration increased, the amount of bound 4.1R increased. The maximal binding capacity of 4.1R was 125 μg of PS liposomes (A). Scatchard plot (B) failed to document an equilibrium between 4.1R and PS liposomes.

FIG. 4. Elution profile of PS liposome-bound 4.1R following phospholipase treatment. PS liposome-bound 4.1R was treated with various phospholipases and analyzed by gel filtration column chromatography as described under “Experimental Procedures.” 4.1R still bound to PS liposomes eluted into early fractions (fractions 5–7), whereas free 4.1R dissociated from liposomes eluted into later fractions (fractions 9–11). PLA2 treatment dissociated 4.1R from PS liposomes (A), whereas PLC treatment failed to dissociate 4.1R from PS liposomes (B).

FIG. 5. Binding of 4.1R to IOV as a function of its association with FAs. Native 4.1R (lane 1), FA-bound 4.1R (lane 3), and 4.1R from which FAs had been removed by Tween 20 (lane 5) were incubated with IOVs. Following centrifugation, proteins bound to IOVs were collected and analyzed by SDS-PAGE. Native 4.1R and 4.1R from which FAs had been removed bound to IOVs (lanes 2 and 6), whereas FA-bound 4.1R did not (lane 4).

FIG. 6. Crystal structure of the membrane binding domain of 4.1R with an emphasis on YKRS motif. YKRS residues (233–236) are displayed in space filling representation, whereas CaM (Ca2+-sensitive and Ca2+-insensitive), GPC, and band 3 binding regions are presented as ball and stick models. Hydrophobic residues near the YKRS motif are displayed in a translucent space filling representation.

In the present study we performed detailed characterization of the interaction of 4.1R with PS by studying the binding and release of 4.1R from PS liposomes and PS-loaded erythrocyte membrane IOVs. A major finding of the study is that 4.1R binding to PS liposomes appears to be a two-step process. During the first step, 4.1R intercalates into PS liposomes through interaction with the serine head group of PS. Positively charged residues YKRS in the 30-kDa membrane binding domain of 4.1R and negatively charged serine head group of PS play a critical role in this initial interaction. During the second step, 4.1R forms a tight hydrophobic association with the fatty acid moieties. Importantly, binding of fatty acids to 4.1R abolishes its ability to interact with all of its protein binding partners.

A number of additional insights regarding 4.1R-PS interaction were also garnered. The finding that 4.1R bound to negatively charged PS liposomes and phosphatidic acid liposomes but not to
uncharged PC liposomes implies that negatively charged head group of phospholipid molecules is critical for the initial interaction between 4.1R and the phospholipid. Furthermore, the requirement for 30% or more of PS in PS/PC liposomes for the initial interaction to occur indicates that a significant surface density of negative charges in the liposome is needed to initiate the phospholipid interaction with 4.1R. This charge interaction appears to be important only during the first stage of this interaction because subsequent hydrolysis of the head groups by either PLD or PLC failed to dissociate 4.1R from PS liposomes.

The YKRS motif in the N-terminal 30-kDa domain of 4.1R appears to play an important role in initiating the interaction between 4.1R and the negatively charged surface of PS because mutation of this sequence motif markedly diminished the ability of 4.1R to bind PS. The YKRS motif in 4.1R is similar to FKKS, the previously identified consensus PS binding sequence motif in myristoylated, alanine-rich protein kinase C substrate (38). It should also be noted that the YKRS sequence is highly conserved in all three homologues of 4.1R, namely 4.1G, 4.1N, and 4.1B (39), implying that these other 4.1 proteins also are likely to be PS-binding proteins.

Although the binding of 4.1R with its protein binding partners, such as band 3, GPC, p55 and CaM, was saturable (and we could therefore derive a value for the dissociation constant (K_D)), 4.1R binding to PS was not saturable. The amount of 4.1R bound to PS liposomes increased with increasing 4.1R concentrations, and under the experimental conditions employed, the measured maximal level of binding was 125 μg of 4.1R/mg of PS, corresponding to a molar ratio of 4.1R to PS of 1:800. Scatchard analysis of these binding data failed to gen-

**Fig. 7. Binding of 4.1R to IOVs and PS-loaded IOVs.** A, various concentrations of 125I-4.1R were incubated with IOVs (open circles) and IOVs preloaded with PS (filled circles). IOVs were collected by centrifugation, and 125I counts associated with IOVs were quantitated as described under "Experimental Procedures." As 4.1R concentration increased the amount of 4.1R bound to IOVs increased. Compared with native IOVs, a significantly higher amount of 4.1R bound to IOVs preloaded with PS. Although PLA_2 treatment did not dissociate 4.1R bound to native IOVs (open squares), PLA_2 treatment dissociated 4.1R from PS-loaded IOVs (filled squares). In fact, following PLA_2 treatment, the amount of 4.1R associated with PS-loaded IOVs was the same as that found in association with native IOVs. B, Scatchard analysis of 4.1R binding to native IOVs (filled squares) and PS-loaded IOVs (filled circles).

**Fig. 8. Expression of wild type and YRKS-AAAA mutant 4.1R-GFP fusion proteins in transfected COS-7 cells.** A, Western blot analyses of transfected COS-7 cells. COS-7 cells were transfected with cDNAs encoding either GFP alone, GFP-wild type 4.1R, or GFP mutant 4.1R fusion protein and processed for Western blot as described under "Experimental Procedures." Lane 1, nontransfected cells; lane 2, GFP-transfected cells; lane 3, GFP-wild type 4.1R-transfected cells; lane 4, GFP-YRKS-AAAA mutant 4.1R-transfected cells. B, distribution of GFP-wild type 4.1R and mutant 4.1R in transiently transfected COS-7 cells. COS-7 cells were transfected with GFP-tagged wild type 4.1R or YRKS-AAAA mutant 4.1R, fixed with 4% paraformaldehyde, and processed for immunofluorescence analyses as described under "Experimental Procedures." Two representative fields of cells transfected with each 4.1R construct are shown. Wild type GFP-4.1R was expressed in the nucleus, cytoplasm, and plasma membrane (a and b). In marked contrast, mutant GFP-4.1R accumulated around the nucleus (c and d). Staining of ER in mutant 4.1R-transfected cells with rhodamine R6 is shown in e and f. The colocalization of GFP-mutant 4.1R with the ER probe is shown in g and h, confirming the accumulation of mutant GFP-4.1R in the ER. Scale bar, 20 μm.
erate a value for the dissociation constant \( (K_D) \). This is in contrast to earlier findings of Cohen et al. (24), who obtained a \( K_D \) value of \( 3.3 \times 10^{-7} \) m for 4.1R binding to PS. It should also be noted that our binding data are consistent with the data of Rybicki et al. (23).

The observed differences in binding characteristics of 4.1R to membrane proteins and to PS are most likely a reflection of the differences in the nature of these interactions: electrostatic between membrane proteins and 4.1R and hydrophobic between PS and 4.1R. This suggestion is further supported by the finding that although 4.1R can be dissociated from its protein binding partners under high ionic strength conditions, 4.1R cannot be dissociated from PS liposomes in such conditions. Dissociation of 4.1R from PS liposomes requires PS hydrolysis by PL-A2. Furthermore, the documentation of the association of several FAs with the released 4.1R implies that sn-2 acyl chains of PS are responsible for the documented hydrophobic interaction. Because FAs do not directly bind 4.1R in solution (data not shown), hydrophobic environment of acyl chains in PS liposomes must be playing a key role in inducing the interaction of 4.1R with FAs. Once formed, the head group of PS does not seem to be necessary for sustaining the tight hydrophobic interaction because removal of head groups by PLC and PLD does not dissociate 4.1R from PS liposomes.

A significant finding of the present study is the observation that once 4.1R is associated with FAs, it cannot bind to band 3, GPC, CaM, or PS. This is, however, a reversible process because removal of FAs from 4.1R restores its ability to interact with its binding partners. The functional reversibility of 4.1R interactions implies that the loss of function of 4.1R upon FA binding is most likely due to induced conformational changes and not due to irreversible denaturation of the protein. Because FA binding to 4.1R completely abolishes its ability to interact with its binding partners, whereas Ca\(^{2+}\)-CaM decreases the affinity of 4.1R interactions with its binding partners by only an order of magnitude (from about 0.1 to 1 \( \mu \)M), we suggest that the conformational changes induced in 4.1R by FA binding are likely to be much larger than those induced by Ca\(^{2+}\)-CaM.

We previously documented that CaM can bind 4.1R and modulate the interactions of 4.1R with band 3, GPC, CD44, and p55 in the presence of Ca\(^{2+}\) (9–13). In the present study, we document that CaM cannot bind to PS-bound 4.1R. As the YKRS sequence responsible for the initial interaction of 4.1R with PS and the CaM binding sites in 4.1R are in very close physical proximity to each other in the C-lobe of the crystal structure of 30-kDa membrane binding domain (38), it is likely that PS binding to 4.1R physically impairs the access of CaM to its binding site. An alternative possibility is that binding of PS to 4.1R induces a conformational change(s) that alters CalM binding sites.

We have shown that although CaM cannot bind to PS-bound 4.1R, 4.1R preincubated with CaM (either in the absence or presence of Ca\(^{2+}\)) can bind to PS liposomes. However, following this binding, CaM rapidly dissociates from 4.1R. This finding is consistent with the fact that the hydrophobic nature of 4.1R-PS interaction is much stronger than the dynamic CaM-4.1R interaction, with a dissociation constant in the order of 0.1 \( \mu \)M.

Our finding that 4.1R can associate with PS that had been incorporated into the outer layer of erythrocyte IOVs raises the possibility that 4.1R is likely to bind to the PS-rich domains occurring in the inner leaflet of native erythrocyte membranes. Moreover, the finding that a threshold concentration of PS is needed for binding of liposomes to 4.1R suggests that 4.1R binding may indeed be restricted to PS-rich domains in the membrane.

The biological significance of 4.1R-PS interaction was further supported by the fact that whereas wild type GFP-4.1R displayed a broad distribution within transfected COS-7 cells as previously reported (40), YRKS-AAAA mutant GFP-4.1R accumulated in the ER. Because this mutant 4.1R cannot bind to PS, it is tempting to propose that impairment in 4.1R sorting may result from the lack of 4.1R interaction with PS. It is interesting to note that the YRKS motif in 4.1R is similar to the YRFF sequence, which is present in the \( \beta_2 \) integrin cytoplasmic domain and which has been previously shown to act as a plasma membrane sorting signal (43). It should be noted, however, that 4.1R localizes not only to plasma membrane but also to other subcellular structures, such as cytoplasm and nucleus, emphasizing the complexity of 4.1R cellular sorting. It remains to be investigated whether the YRKS motif in 4.1R is responsible for protein sorting through direct interaction with PS or indirectly due to interaction with other proteins involved in protein sorting.

In light of our data and of other recent findings regarding the functional relevance of the interaction of anionic phospholipids, such as PS and PIP\(_2\), with cytoskeletal and microtubule-associated proteins (25–28, 41), it is tempting to propose that protein-phospholipid interaction may play an important role in protein sorting, in appropriate cellular localization and in regulation of its function. Considering the expression of 4.1R and its homologs in various cell types, the potential role of the 4.1R-PS interaction in modulating cell function should be further explored.

REFERENCES
