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Phosphatidylinositol-4,5 bisphosphate (PIP_2) inhibits apo-calmodulin binding to protein 4.1

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ABSTRACT

Membrane skeletal protein $4.1R^{80}$ plays a key role in regulation of erythrocyte plasticity. Protein $4.1R^{80}$ interactions with transmembrane proteins, such as glycophorin C (GPC), are regulated by Ca^{2+}-saturated calmodulin (Ca^{2+}/CaM) through simultaneous binding to a short peptide (pep11; A^{264}KKLWKVCVEHHTFFRL) and a serine residue (Ser^{185}), both located in the N-terminal 30 kDa FERM domain of $4.1R^{80}$ (H·R30). We have previously demonstrated that CaM binding to H·R30 is Ca^{2+}-independent and that CaM binding to H·R30 is responsible for the maintenance of H·R30 β-sheet structure. However, the mechanisms responsible for the regulation of CaM binding to H·R30 are still unknown. To investigate this, we took advantage of similarities and differences in the structure of Coracle, the *Drosophila* sp. homologue of human $4.1R^{80}$, i.e. conservation of the pep11 sequence but substitution of the Ser^{185} residue with an alanine residue. We show that the H·R30 homologue domain of Coracle, Cor30, also binds to CaM in a Ca^{2+}-independent manner and that the Ca^{2+}/CaM complex does not affect Cor30 binding to the transmembrane protein GPC. We also document that both H·R30 and Cor30 bind to phosphatidylinositol-4,5 bisphosphate (PIP_2) and other phospholipid species and that that PIP_2 inhibits Ca^{2+}-free CaM but not Ca^{2+}-saturated CaM binding to Cor30. We conclude that PIP_2 may play an important role as a modulator of apo-CaM binding to $4.1R^{80}$ throughout evolution.

1. Introduction

Protein 4.1, originally identified as an 80 kDa protein ($4.1R^{80}$) in human erythrocytes, plays a crucial role in the maintenance of erythrocyte morphology and mechanical integrity [1]. The importance of $4.1R^{80}$ in maintaining the structural integrity of erythrocytes is underscored by the abnormal erythrocyte phenotype observed in $4.1R^{80}$ deficient patients [2] and $4.1R^{80}$ null mice [3]. Limited chymotryptic digestion of $4.1R^{80}$ generates four structural domains (30, 16, 10, and 22/24 kDa) [4]. The N-terminal 30 kDa domain of $4.1R^{80}$, the so-called FERM (four-one, ezrin, radixin, moesin) domain, referred to as H·R30 here, mediates $4.1R^{80}$ binding to plasma membrane via transmembrane proteins, such as glycophorin C (GPC) and the anion exchanger band 3 [5].

We have previously characterized two calmodulin (CaM) binding sites in H·R30, a Ca^{2+}-sensitive site, serine residue Ser^{185}, and a Ca^{2+}-independent site, pep11 (A^{264}KKLWKVCVEHHTFFRL) (Fig. 1) [6], and established that both sites are required for Ca^{2+}-dependent regulation of $4.1R^{80}$ binding to transmembrane proteins [6]. We have shown that the binding ratio of CaM to $4.1R^{80}$ is 1:1 [6]. We have also documented the biological significance of Ca^{2+}-independent CaM binding to H·R30 in maintaining H·R30 β-sheet structure [7] and that, reciprocally, the structure of CaM is stabilized through its binding to H·R30 [8]. We have extended our initial findings in man to zebrafish by showing that, like human 4.1R, zebrafish 4.1R also binds to CaM in a Ca^{2+}-independent manner [9]. This led us to propose that the interaction of $4.1R^{80}$ and CaM may fulfill a chaperon-like function in human erythrocytes [10].

Protein (4.1R) is expressed and evolutionarily conserved in *Drosophila* sp. in which 4.1R is expressed as *coracle* (Cor) [11].

Abbreviations: $4.1R^{80}$, 80 kDa erythrocyte type protein 4.1; CaM, calmodulin; Ca^{2+}/CaM, Ca^{2+}-saturated CaM; Cor30, FERM domain of Coracle; H·R30, FERM domain of human $4.1R^{80}$; FERM, four.one/ezrin/radixin/moesin; GPC, glycophorin C; PIP_2, phosphatidylinositol-4,5 bisphosphate; PS, phosphatidylserine.

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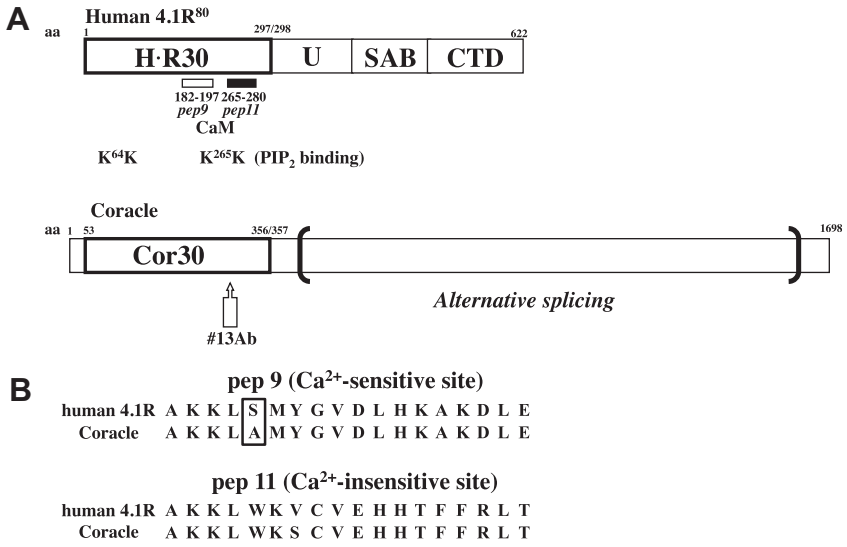

Fig. 1. Domain organization of human 4.1R^{80} (accession ID: P11171) and *Drosophila* Coracle (accession ID: Q9V8R9). Human 4.1R^{80} consists of four chymotryptic domains [5]: an N-terminal 30 kDa domain (*H·R30*), a 16 kDa unique region (*U*), a spectrin-actin binding (*SAB*) domain, and a C-terminal domain (*CTD*). The N-terminal FERM homologue domain of Cor is presented as *Cor30*. Amino acid sequence identity between human H·R30 and Cor30 is 59%. The CaM binding sites in H·R30 are represented by an open rectangle (Ca^{2+}-sensitive CaM binding peptide encoded by exon 9; sequence shown in (B)) and by a closed rectangle (Ca^{2+}-insensitive CaM binding peptide encoded by exon11; sequence shown in (B)). The recognition site for monoclonal specific antibody #13Ab in Cor30 is shown with an arrow [21]. (B) Alignment of the CaM binding sequences in human 4.1R^{80} and Coracle. Top panel: sequences of the Ca^{2+}-dependent CaM binding site (*pep9*); bottom panel: sequences of the Ca^{2+}-independent CaM binding site (*pep11*).

Based on the homology of the pep11 CaM binding sequence in 4.1R and Cor and based on previous reports [11], one might predict that the N-terminal FERM homologue domain of Cor (Cor30) also binds to CaM in a Ca^{2+}-independent manner. Strikingly, Ser^{185} in H·R30 (that acts as a Ca^{2+}-sensitive CaM binding site) is replaced by an alanine in Cor30 (Fig. 1) [11]. This suggests that, unlike 4.1R, Cor30 interaction with CaM may be strictly Ca^{2+}-independent. If this is the case, which factor does regulate this interaction? Therefore, investigating Cor30 binding to CaM may provide us with important mechanistic insights.

Coracle binds to neurexin IV, a member of the Glycophorin/Neurexin/Paranodin (GNP) family of transmembrane proteins [12]. The cytoplasmic domain of these proteins is highly conserved in mammals while the sequence of their extracellular domain is very unique [12]. The cytoplasmic domain of *Drosophila* neurexin IV shares a 4.1R binding motif, R^{1244}HK, with that of human GPC (GPCcyt) [12–14].

4.1R^{80} is a well-documented phosphatidylserine (PS) and phosphatidylinositol-4,5 bisphosphate (PIP_2) binding protein, this interaction being mediated by H·R30 [15–19]. The binding of 4.1R to GPC is enhanced while that to band 3 is inhibited by PIP_2 [15–19]. The PIP_2 binding sites in H·R30 have been mapped as two unique tandem lysine residues, K^{63}K and K^{265}K [18]. Of particular note, the K^{265}K motif, which is conserved in *coracle*, lies in the pep11 Ca^{2+}-independent CaM binding sequence (Fig. 1A). A logical hypothesis that arises from these observations is that PIP_2 (but not Ca^{2+}) modulates CaM binding to *coracle*.

In the present study, we measured the binding affinity of Cor30 for CaM in the presence and absence of Ca^{2+} and for human GPC by using biosensor-based techniques (kinetic analysis) and by performing binding studies with human erythrocyte inside-out vesicles (IOVs). We show that, in absence of Ca^{2+}, PIP_2 inhibits binding of apo-CaM to both H·R30 and Cor30. We also demonstrate that, in contrast to H·R30 (and as predicted), the regulation of Cor30 binding to GPC is strictly Ca^{2+}-independent as demonstrated by the inability of the Ca^{2+}/CaM complex to modulate Cor30 binding to GPC. Notably, in presence of Ca^{2+}, whereas PIP_2 (but not Ca^{2+}) inhibits CaM binding to Cor30, in contrast, Ca^{2+} inhibits CaM binding to H·R30 but PIP_2 fails to further inhibit this latter interaction. The results presented here provide further evidence for an involvement of both Ca^{2+} and PIP_2 in modulating CaM binding to 4.1R^{80}, the extent at which Ca^{2+} and PIP_2 each promotes, and potentially competes for, this regulation being determined by key structural features of the CaM binding sites in the FERM domain of 4.1R isoforms.

2. Material and methods

2.1. Materials

The cDNA sequence encoding *coracle* N-terminal FERM domain (Cor30; amino acids 1–356) was amplified by PCR, using coracle sequence-specific primers including a 5′ SmaI restriction site (forward primer) and a 3′ XhoI restriction site (reverse primer), and cloned into the pGEX-4T2 vector (GE Healthcare Sciences (Piscataway, NJ). The resulting plasmid DNA was validated by DNA sequencing (Operon Biotechnology Co., Ltd., Tokyo) and used to transform Rosetta® BL21 (DE3) *Escherichia coli* (Novagen Inc., Madison, WI). Methods for protein expression and purification have been previously described [6,9]. Protein purity was assessed by SDS–PAGE using 15% acrylamide gels. Protein concentrations were determined by UV spectroscopy [6,9].

Generation of constructs encoding the cytoplasmic domains of human GPC (GPCcyt) and band 3 (band 3cyt), purification of bovine brain CaM and production of the mouse monoclonal antibody to H·R30, mAb#13, have been previously described [6,20,21].

2.2. Methods

2.2.1. Preparation of IOVs

IOVs depleted of 4.1R^{80} were prepared from phorbol 12-myristate 13-acetate (PMA)-treated human erythrocyte membranes as previously described [9] and based on a previous report describing PMA treatment of erythrocytes resulting in protein kinase C (PKC) activation [22]. IOVs protein concentration was determined using

the Bradford protein assay (Pierce Inc., Rockford, IL) and bovine serum albumin as standard.

2.2.2. Binding analysis using a CaM-Sepharose 4B column

A mixture of Cor30 and GST was dialyzed against 50 mM Tris–HCl, pH7.5 containing 0.1 M NaCl and either 1 mM $CaCl_2$ (Buffer A) or 5 mM EGTA (Buffer B) and loaded onto a 1.6 × 8 cm CaM-Sepharose 4B column (GE Healthcare Sciences, Piscataway, NJ) pre-equilibrated with Buffer A (or Buffer B) at a 0.2 ml/min flow rate. The column was then washed with an excess of Buffer A or Buffer B to remove unbound proteins. Bound proteins were eluted stepwise with Buffer B followed by Buffer B containing 0.5 M NaCl.

2.2.3. Resonant mirror detection (RMD) binding assays

Protein–protein interactions were analyzed by IAsys® system resonant mirror detection using aminosilane-coated cuvettes (Thermodynamics Affinity Sensors, Cambridge, UK) as previously described [9]. The protein immobilized on the cuvette is referred to as the "*ligand*" and the protein in solution added to the cuvette as the "*analyte*". All binding assays were carried out at 25 °C under constant stirring. In experiments aimed at investigating the effects of Ca^{2+} and CaM on the binding of Cor30 to immobilized human GPCcyt or band 3cyt, Cor30 (50 nM–1 μM) was pre-incubated for 30 min at 25 °C with 5 μM CaM in Buffer C (20 mM imidazole HCl, pH 7.2, 0.1 M NaCl) supplemented with either 1 mM EGTA or 1.1 mM $CaCl_2$ and 1.0 mM EGTA prior to performing the binding assay [6,7].

The kinetic analysis of analyte binding to ligand was performed as previously described [6,9]. Software-based Scatchard plot analysis of the binding data enables to calculate the maximal binding value (B_{max}) and to derive dissociation constant (K_d) values for various molar concentrations of analyte [6].

2.2.4. Sedimentation assay of Cor30 with IOVs

Various concentrations of Cor30 were incubated with IOVs in 10 mM Tris–HCl, pH7.4 containing 0.15 M NaCl (TBS) for 20 min at 25 °C. The Cor30-IOVs mixture was laid on 150 μl of a 3.3% sucrose cushion equilibrated in TBS containing 1 mM EDTA and 1 mM DTT. The sample was centrifuged at 60,000 rpm for 10 min at 4 °C in a TL-100 ultracentrifuge using a TLA-100 rotor (Beckman Coulter, Inc. Fullerton, CA). Supernatant and precipitate fractions were subjected to SDS–PAGE.

2.2.5. Immunoblot assay

The protocol for immunoblot assay has been previously described [21]. Densitometric analysis and calculation of photodensity were carried out with the Digital Science EDAS290 System® (Kodak, Rochester, NY) [21].

2.2.6. Analysis of Cor30 and H·R30 binding to phospholipids

PIP Strip™-membrane type (Echelon Biosciences Inc., Salt Lake City, UT) was used for determination of Cor30-GST and H·R30-GST binding to phospholipids. Non-specific blocking was performed as previously described [21]. Recombinant glutathione-*S*-transferase (GST) was used as a negative control.

2.2.7. Measurements of PIP_2-mediated effects on CaM binding to Cor30

Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2; PIP_2) HCl salt and inositol 1,4,5-trisphosphate (IP_3) (Sigma–Aldrich (St. Louis, MO) at 1 μM were pre-incubated with 0.14 μM of Cor30 in Buffer A or B for 10 min at 25 °C. The mixture was loaded onto a CaM-immobilized aminosilane-coated cuvette and subjected to binding analysis.

2.2.8. Modeling and visualization of Cor30

The 3D structure of Cor30 was generated using the Swiss-Model software package [23]. Protein 3D structure was visualized using the MolFeat® software (Ver.4.6, FiatLux Co., Tokyo, Japan).

3. Results

3.1. Ca^{2+}-independent CaM binding to Cor30

We compared and H·R30 and Cor30 binding properties given key structural differences in CaM binding domains between these two highly conserved proteins. Cor30 binding to CaM was first assessed after loading of a mixture of Cor30 onto a CaM Sepharose CL-6B column in the presence or absence of Ca^{2+}. The elution profile showed a large peak corresponding to the unbound fraction, followed by a small peak appearing after addition of 5 mM EGTA and a third peak eluting at 0.6 M NaCl (Fig. 2A). SDS–PAGE analysis of these three elution fractions confirmed that GST was recovered in the unbound fraction whereas Cor30 was eluted in the high salt (0.6 M NaCl) fraction (Fig. 2A). No protein was detected in the

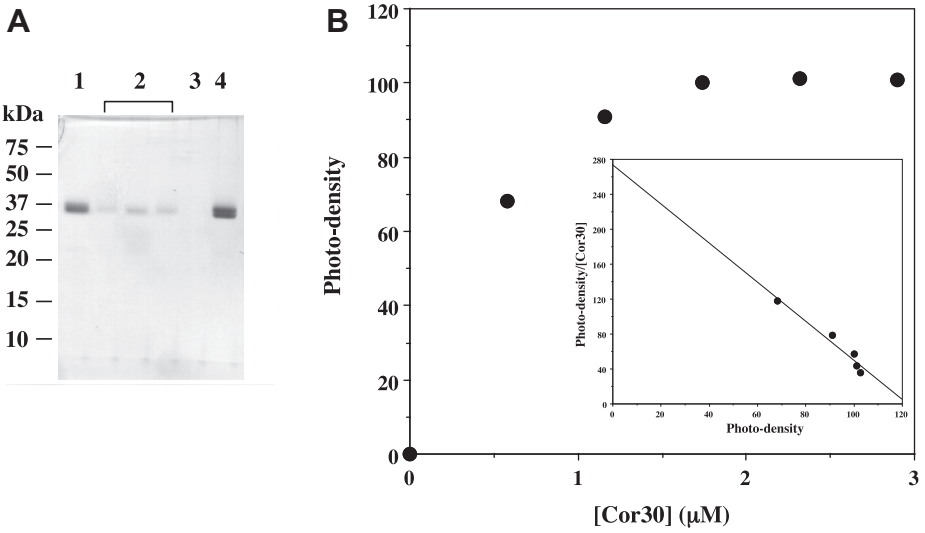

Fig. 2. Cor30 binding to human erythrocyte IOV. (A) SDS–PAGE analysis of Cor30 binding to CaM Sepharose CL-6B (Coomassie blue-stained gel). *lane 1*: Cor30 input, *lanes 2*: pooled flow-through fractions with buffer A, *lane 3*: pooled elution fractions with Buffer B, *lane 4*: pooled elution fractions with 0.6 M NaCl (final concentration) in Buffer B. (B) Binding profile and Scatchard plot analysis (*insert*) of Cor30 binding to human IOVs. After sedimentation assay of Cor30 with human IOVs, Cor30 binding to IOVs was assessed by immunoblotting.

5 mM EGTA fraction, indicating that Cor30 was still able to bind to the CaM-Sepharose column in the absence of Ca^{2+} (Fig. 2A). Cor30 bound to CaM with a K_d of ~280–300 nM both in presence and absence of Ca^{2+}, a K_d value very similar to that for H·R30 (Table 1). We could not calculate the Cor30:CaM binding ratio due to the inability of quantifying the amount of CaM immobilized on the cuvette.

3.2. Analysis of Cor30 binding to human erythrocyte IOVs

IOVs prepared from human erythrocytes were used to assess Cor30 binding to transmembrane proteins taking advantage of the optimized isolation of human IOVs and of the sequence similarity between *Drosophila* Neurexin IV and human GPC cytoplasmic domains. Cor30 binding features, highlighted by the above RMD binding assays, were confirmed *in vivo* by Cor30 sedimentation assays with human IOVs (data not shown). Cor30 bound to IOVs with a K_d of ~100 nM (Fig. 2B), a value similar to that previously observed for human $4.1R^{80}$ or H·R30 (K_d = ~150 nM) [24]. As shown in Table 2, Cor30 bound to human GPCcyt with a K_d of 400–700 nM. These results supported important structural and functional differences between Cor30 and H·R30 and unique species-specific characteristics for GPC-4.1R interaction.

We have previously shown that $4.1R^{80}$ binding to GPCcyt is regulated by Ca^{2+}/CaM through CaM binding to pep11 and a Ser 185 residue in pep9 in H·R30 [6]. However, using the same assays, we show here that Ca^{2+}/CaM did not change the affinity of Cor30 binding to human GPCcyt and band 3cyt (Table 2). These findings could not be extended to *Drosophila* since we could not identify the *Drosophila* homologue of human band 3 in protein databases. Dubreuil et al. have reported the existence of an anion exchanger in *Drosophila* that, unlike human band 3, does not function as a spectrin anchor, leading the authors to conclude that this anion exchanger in *Drosophila* is functionally very different from band 3 in mammalians [25].

Table 1
Characteristics of human 4.1R and coracle FERM domains binding to CaM in absence and presence of Ca^{2+}.

Analyte	Ligand	Condition	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_d (nM)
HR·30	CaM	EDTA	$6.5 \pm 0.19 \times 10^4$	$2.0 \pm 0.21 \times 10^{-2}$	294 ± 24
		Ca^{2+}	$3.1 \pm 0.21 \times 10^4$	$1.1 \pm 0.15 \times 10^{-2}$	331 ± 19
Cor30	CaM	EDTA	$5.3 \pm 0.20 \times 10^4$	$1.7 \pm 0.13 \times 10^{-2}$	306 ± 11
		Ca^{2+}	$1.4 \pm 0.10 \times 10^5$	$4.1 \pm 0.20 \times 10^{-2}$	290 ± 26

K_d values for the interactions of human $4.1R^{80}$ and Coracle 30 kDa FERM domains (*H·R30* and *Cor30*, respectively) and to CaM immobilized aminosilane-coated cuvettes in the presence (*Ca^{2+}*) and absence of Ca^{2+} (*EDTA*) are shown. Each analyte was incubated at various concentrations (50 nM–1 μM) with the identified ligand immobilized on the aminosilane cuvette as described in the Section 2. From the binding curves obtained by the resonant mirror detection method, K_d values (mean ± S.D) were determined from the binding curves obtained by the resonant mirror detection method in 3–5 independent experiments using the software package FAST-Fit™.

3.3. PIP_2 regulates Cor30 and H·R30 binding to apo-CaM but not to Ca^{2+}/CaM

We then compared qualitatively and quantitatively the binding of H·R30-GST and Cor30-GST fusion proteins to various phospholipids (Fig. 3A). As observed for H·R30-GST, Cor30-GST bound not only to phosphatidylserine and to PIP_2 but also to phosphatidic acid, cardiolipin, phosphatidylinositol (PtdIns) (4)P and PtdIns (3,4,5)P_3. Specificity of these interactions was confirmed as GST alone did not bind to any of these phospholipids (Fig. 3A).

The involvement of PIP_2 was further confirmed by showing that Cor30-GST and H·R30-GST preincubated with an excess of PIP_2 could no longer bind to a CaM-immobilized cuvette in the absence of Ca^{2+} (apo-CaM; Fig. 3B). In contrast, whereas H·R30-GST binding to CaM was decreased in presence of Ca^{2+} (Ca^{2+}/CaM) but not inhibited further by PIP_2, Cor30-GST binding to Ca^{2+}/CaM, that was insensitive to Ca^{2+} as shown above, was partially inhibited by PIP_2 (Fig. 3B). Indeed, the B_{max} was reduced by 58–62% and the K_d by 17% (~240 nM) in presence of PIP_2 compared to the B_{max} and K_d measured in absence of PIP_2.

4. Discussion

In the present study, we demonstrate that, as it is the case for H·R30, Cor30 binds to CaM in a Ca^{2+}-independent manner. We have previously demonstrated that the interaction of apo-CaM with H·R30 is of high biological significance since apo-CaM stabilizes the β-sheet structure of the C-lobe of H·R30 (Fig. 4). Based on the results presented here and on the prediction that the 3D structure of Cor30 is similar to that of H·R30 (Fig. 4), we predict that the interaction of apo-CaM with Cor30 fulfills a similar function [7]. Consistent with our observations, the Ca^{2+}-independent CaM binding site in H·R30, pep11, is conserved in Cor30 (Fig. 1). We have also previously shown that pep11 maintains the thermal stability of CaM in both presence and absence of Ca^{2+} [6,7], leading us to suggest that CaM plays a chaperon-like function through its binding to H·R30 [10].

We have previously demonstrated that the regulation of human $4.1R^{80}$ binding to GPC by Ca^{2+}/CaM requires CaM binding to a Ser^{185} residue in pep9 (that acts as a Ca^{2+}-dependent CaM binding site) and to pep11 (that acts as a Ca^{2+}-independent CaM binding site) [6]. We show here a distinct behavior between Cor30 and H·R30 in respect to the effect of the Ca^{2+}/CaM complex on the regulation of their binding to GPC and band 3. Although the

Table 2
Unique characteristics of Ca^{2+}/CaM dependency for coracle FERM domain interaction with human membrane proteins.

Analyte		Condition	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_d (nM)
GPCcyt		EDTA	$3.2 \pm 0.20 \times 10^4$	$1.5 \pm 0.20 \times 10^{-2}$	468 ± 54
		Ca^{2+}	$2.7 \pm 0.10 \times 10^4$	$1.8 \pm 0.20 \times 10^{-2}$	656 ± 27
	+CaM	EDTA	$3.8 \pm 0.21 \times 10^4$	$2.0 \pm 0.11 \times 10^{-2}$	526 ± 15
		Ca^{2+}	$5.3 \pm 0.20 \times 10^4$	$2.7 \pm 0.20 \times 10^{-2}$	511 ± 21
Band 3cyt		EDTA	$3.3 \pm 0.20 \times 10^4$	$2.1 \pm 0.22 \times 10^{-2}$	634 ± 25
		Ca^{2+}	$4.3 \pm 0.20 \times 10^4$	$2.6 \pm 0.20 \times 10^{-2}$	607 ± 28
	+CaM	EDTA	$3.0 \pm 0.10 \times 10^4$	$1.8 \pm 0.20 \times 10^{-2}$	600 ± 48
		Ca^{2+}	$4.9 \pm 0.20 \times 10^4$	$2.7 \pm 0.20 \times 10^{-2}$	570 ± 17

K_d for the interaction of Coracle 30 kDa FERM domain (Cor30) with the cytoplasmic domain of human GPC (*GPCcyt*) and Band 3 (*Band 3cyt*) in the presence and absence of Ca^{2+} and CaM are shown. Cor30 (50 nM–1 μM) was preincubated with CaM (5 μM) and either 0.1 mM EDTA (*EDTA*) or 1.1 mM $CaCl_2$ and 1.0 mM EDTA (*Ca^{2+}*) for 30 min at 25 °C in buffer A. The complex of CaM and Cor30 was incubated with GPCcyt or Band 3cyt immobilized on the aminosilane cuvette as described in the Section 2. From the binding curves obtained by the resonant mirror detection method, K_d values (mean ± S.D) were determined from the binding curves obtained by the resonant mirror detection method in 3–5 independent experiments using the software package FAST-Fit™.

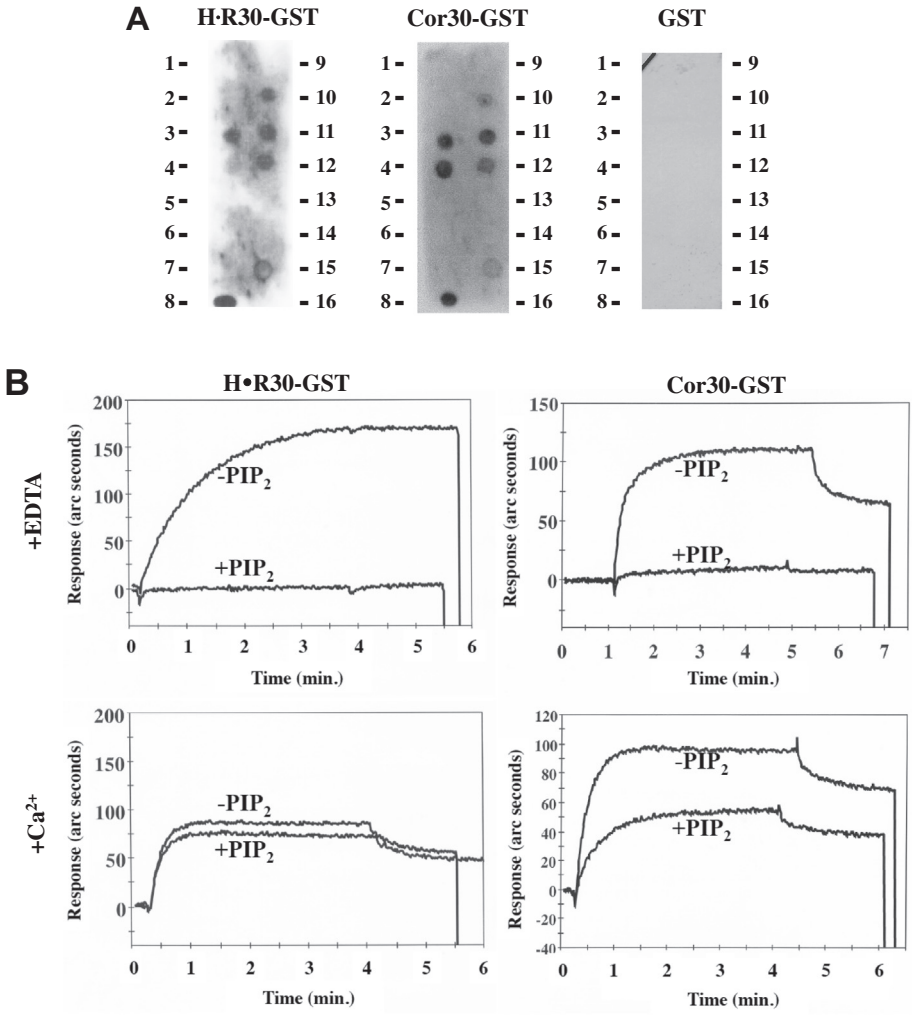

Fig. 3. Ca^{2+} and phospholipid dependency of Cor30 binding to CaM. (A) The binding of Cor30-GST, H·R30-GST or GST alone (50 μg) to phospholipid-immobilized PIP Strip™-membrane was detected with a primary anti-GST antibody conjugated with horseradish peroxidase and visualized by chemiluminescence. 1, triglyceride; 2, diacylglycerol; 3, phosphatidic acid; 4, phosphatidylserine; 5, phosphatidylethanolamine; 6, phosphatidylcholine; 7, phosphatidylglycerol; 8, cardiolipin; 9, phosphatidylinositol (PtdIns); 10, PtdIns(4)P; 11, PtdIns (4, 5)P_2; 12, PtdIns(3,4,5)P_3; 13, cholesterol; 14, sphingomyelin; 15, 3-sulfogalactosylceramide; 16, solvent blank. (B) IAsys binding profiles of 0.14 μM Cor30 (left panels) and H·R30 (right panels) to apo-CaM (+EGTA, top panels) or Ca^{2+}/CaM (+Ca^{2+}, bottom panels) in the presence and absence of 1.0 μM PIP_2, (+PIP_2 and −PIP_2, respectively).

predicted 3D structure of Cor30 is very similar but not identical to that of H·R30, the amino acid sequence and the spatial organization of pep11 are identical in both proteins (Fig. 4). The conservation of pep11 is consistent with the similar affinity for Ca^{2+}-independent CaM binding to H·R30. Furthermore, the structure of the GPC binding domain in the α-lobe of H·R30 is also very similar to the neurexin IV binding domain in Cor30 [13,14]. In contrast, one residue in the amino acid sequence of pep9, Ser^{185}, differs in human $4.1R^{80}$ and Coracle (Fig. 1). This residue constitutes the key component for Ca^{2+}/CaM-dependent regulation of H·R30 binding to transmembrane proteins [5]. Strikingly, this residue is replaced by a proline in zebrafish (*Danio rerio*), a threonine in frog (*Xenopus* sp.) and an alanine in fly (*Drosophila* sp.) [13]. This feature may well account for the ability of zebrafish and *Xenopus* 4.1R as well as *Drosophila* coracle to bind to CaM solely in a Ca^{2+}-independent manner and for the fact that, unlike human 4.1R, interactions of these three 4.1 proteins with GPC and band 3 are not regulated by Ca^{2+}/CaM [9]. This raises an interesting evolutionary question about the relevance of the functional gain of Ca^{2+}-dependent CaM binding in mammalian 4.1R as a result of the acquisition of the Ser^{185} residue.

Another important finding is that PIP_2 inhibits CaM binding to both H·R30 and Cor30 in the absence of Ca^{2+} but only CaM binding to Cor30 in the presence of Ca^{2+}. This key observation suggests two hypotheses: (i) a competition between Ca^{2+} and PIP_2 in regard to regulatory function. In this scenario, the regulatory role of Ca^{2+} prevails in the presence of a functional Ca^{2+}-dependent CaM binding site (i.e. when Ser^{185} is present in pep9), the regulatory function of PIP_2 being "unmasked" when such a Ca^{2+}-dependent CaM binding site is not present (i.e. when Ser^{185} is not present in pep9); (ii) in presence of Ca^{2+}, Ca^{2+} forms a complex with PIP_2 that precludes PIP_2 from fulfilling its regulatory function.

A third mechanism is likely to come into play *in vivo*, especially as erythrocytes age. That is the Ca^{2+}-dependent activation of phospholipases A_2 and C that catalyze hydrolysis of membrane phospholipids, such as PIP_2 or PS [18,19]. This mechanism has been shown to play a key role in regulating PIP_2-dependent interaction of 4.1R with GPCcyt and band3cyt [15–19]. Similarly, hydrolysis of PS by phospholipase A_2 has been shown to impair the ability of PS to promote H·R30 interaction with membrane binding partners [19]. Highly relevant to our study, it has been reported that, upon PS binding to H·R30, CaM dissociates from H·R30 both in presence and absence of Ca^{2+} [22]. Importantly, PIP_2 exhibits unique features, as PIP_2 can regulate CaM binding to H·R30 in absence of Ca^{2+} whereas PS cannot [19]. However, one can rule

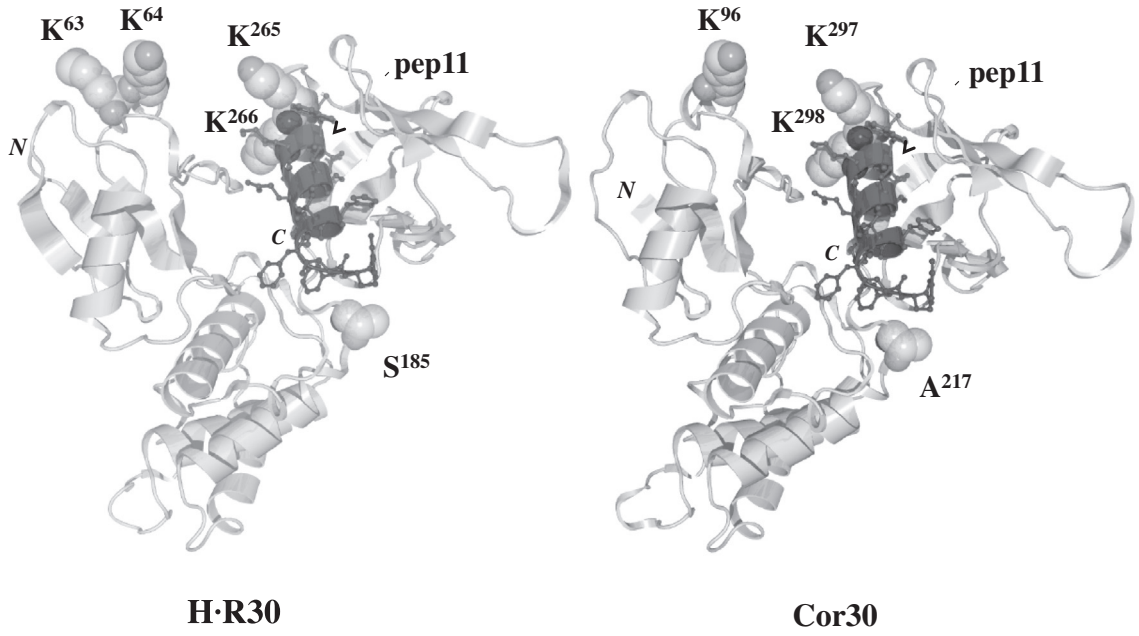

Fig. 4. Comparison of 3D structure of H·R30 domain and predicted 3D structure of Cor30 domain. The 3D structure of H·R30 (accession ID: 1GG3) and predicted 3D structure of Cor30 were generated using the Swiss-Model [23]. The Ca^{2+}-dependent CaM binding site Ser^{185} and the corresponding residue in Cor30 (Ala^{217}, Fig. 1B), the Ca^{2+}-independent CaM pep11 binding site [6] as well as the two PIP_2 binding sites in H·R30 ($K^{63}K$ and $K^{265}K$) [18] and corresponding sites in Cor30 (K^{95} and $K^{297}K$), are shown. N and C represent the N- and C- terminal ends of H·R30 and Cor30, respectively.

out Ca^{2+}-dependent phospholipase-mediated hydrolysis of phospholipids to explain our findings *in vitro* since phospholipases are absent in these reconstituted binding assays.

Of particular note, three of the four Lys residues that constitute the two PIP_2 binding sites identified in H·R30 and their spatial organization are conserved in Cor30, lysine residue K^{63} being replaced with a serine residue in Cor30. This suggests that K^{63} is not essential for PIP_2 binding to protein 4.1 FERM domains. Consistent with this, a previous report has shown that mutation of the $K^{265}K$ in H·R30 results in a more dramatic decrease in the binding affinity of PIP_2 for mutant H·R30 than mutation of the $K^{63}K$ motif [18].

Our study provides compelling evidence for PIP_2 acting as an efficient modulator of CaM-4.1R interaction in absence of a Ca^{2+}-dependent mechanism of regulation. Further investigation is needed to decipher the complex events underlying PIP_2-dependent regulation of the CaM-$4.1R^{80}$ interaction in the absence of Ca^{2+}.

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