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Regulation of Protein 4.1R Interactions with Membrane Proteins by Ca^{2+} and Calmodulin

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1. ABSTRACT

Red blood cell protein 4.1 (4.1R) is essential for maintaining erythrocyte shape and controlling membrane mechanical properties, such as deformability and stability. The importance of 4.1R has been demonstrated by the dramatic erythrocyte alterations observed in patients lacking this protein. Indeed, 4.1R null red blood cells adopt an elliptical shape and are characterized by unstable membranes. The key role of 4.1R likely results from multiple protein-protein interactions: lateral interactions with the spectrin/actin network and vertical interactions with the cytoplasmic domain of transmembrane proteins glycophorin C (GPC), Band 3 (anion exchanger 1, *AE1*), and CD44. 4.1R promotes the formation of a ternary complex with GPC and p55 through its 30kDa membrane-binding domain. Based on the primary structure of the prototypical 80kDa isoform of 4.1R, functional domains and sites for binding partners have been identified. The others and we have been focusing on the structure and function of the 30kDa NH_2-terminal domain of 4.1R, which is responsible for 4.1R interaction with the transmembrane proteins described above. A major finding is that Ca^{2+}, in association with calmodulin (CaM), plays a critical role in regulation of the interaction of the 30kDa domain with its various binding partners. This review is a detailed report of our current knowledge regarding 4.1R, and more specifically, 4.1R 30kDa domain: its primary structure, functions and modulation by Ca^{2+} and CaM. Emphasis is given on the relationships between structure and function that we have been able to establish through X-ray crystal structure analysis of the 30kDa membrane-binding domain in 4.1R. Finally, we give insights into the potential roles of 4.1R in the dynamic organization of the membrane skeleton viewed as a complex system.

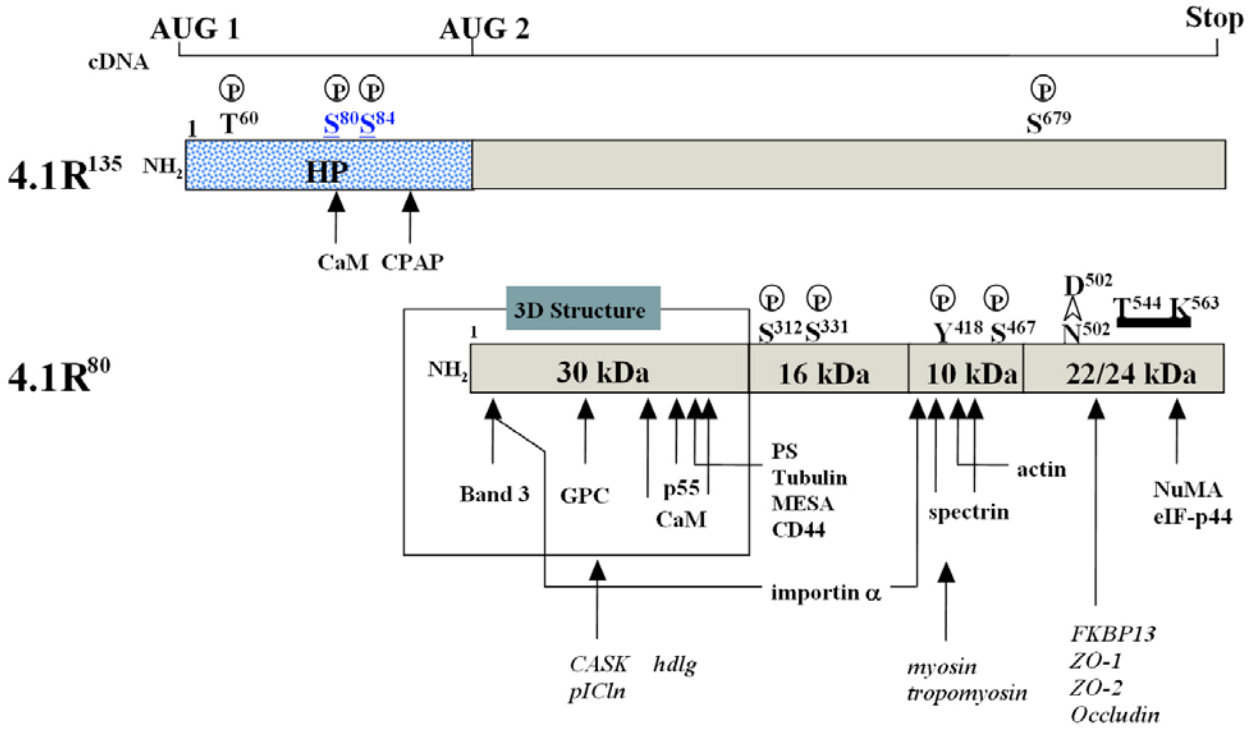

Figure 1. Primary structure of 4.1R isoforms and map of known binding partners for 4.1R. Translation of the prototypical red blood cell 80kDa 4.1R isoform ($4.1R^{80}$) is initiated at AUG-2, which is located in exon 4. Translation of the 135 kDa 4.1R isoform ($4.1R^{135}$), an isoform expressed in early erythroblasts and other nucleated cells, is initiated at AUG-1, which is located in exon 2'. An updated list of the binding partners identified for each domain of 4.1R is displayed. Confirmed (*black font*) and putative (*blue font*) phosphorylated residues are shown as circled "P"s. Glycosylation site is represented by a filled black box. An arrowhead indicates location of the deamidation site (Asn^{502}->N^{502}).

2. INTRODUCTION

Red blood cell protein 4.1, designated 4.1R to distinguish it from three recently characterized homologues, plays a critical role in maintaining cell morphology and membrane mechanical properties (1, 2). This property results from the fact that 4.1R binds to various transmembrane and membrane skeletal proteins (1, 2). Indeed, the elasticity and mechanical stability of the erythrocyte membrane is regulated by 4.1R interaction with the spectrin-actin network, with transmembrane proteins, such as Band 3, or glycophorin C (GPC) and with p55, a palmitoylated membrane protein, which belongs to the membrane-associated guanylate kinase (MAGUK) family (reviewed in 3). Hereditary defects in 4.1R result in erythrocytes with abnormal shape and decreased membrane mechanical stability, these alterations resulting clinically in a hemolytic anemia (1).

Three main structural/functional domains have been identified so far in 4.1R. A 30kDa NH_2-terminal membrane-binding domain possesses binding sites for the cytoplasmic tail of integral membrane proteins such as Band 3, GPC and CD44 (1, 4-6). This domain also binds to p55 (7), and calmodulin (CaM) (8, 9). Tubulin binds to this domain in nonerythroid cells (10). A 10kDa internal domain contains the critical spectrin-actin binding activity required for membrane mechanical stability (1), while the COOH-terminal 22/24kDa domain has recently been reported to bind to immunophilin FKBP13 (11), NuMA (12) and tight junction protein ZO-2 (13) (Figure 1).

Two ternary protein complexes involving 4.1R have been identified in the erythrocyte membrane: spectrin/actin/4.1R, and GPC/4.1R/p55 (Figure 2). The spectrin/actin/4.1R ternary complex plays a critical role in imparting mechanical stability to the erythrocyte membrane. Binding of CaM to 4.1R decreases its affinity for purified spectrin and actin in a Ca^{2+}-dependent manner *in vitro* (14), and destabilizes erythrocyte membrane mechanical integrity in intact cells (15). These data support an important regulatory role for 4.1R in the organization and function of the spectrin/actin/4.1R ternary complex.

In the present review, we will take advantage of our current knowledge of the dynamic organization of 4.1R 30kDa domain to address the three following questions:

1. Very little is known regarding the regulation of the various protein-protein interactions involved in the GPC/4.1R/p55 ternary complex. Which role is 4.1R playing in regulation of this ternary complex?

2. Which transmembrane proteins and peripheral membrane proteins does 4.1R interact with in non-erythroid cells?

3. CaM has been shown to bind to the 30kDa domain of 4.1R at a molar ratio 1:1 in a Ca^{2+}-independent manner.

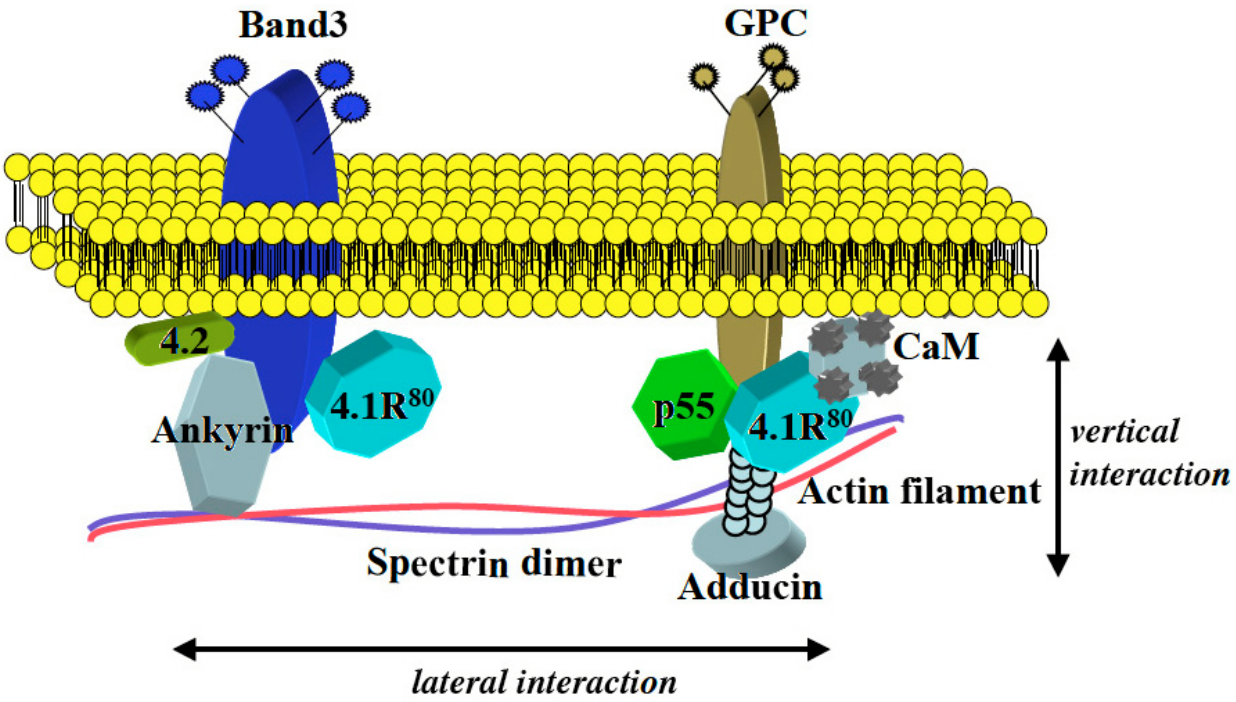

Figure 2. Organization of the erythrocyte plasma membrane. A protein network forming a "cytoskeleton" underlies the cytoplasmic surface of the red blood cell lipid bilayer. Spectrin (alphaand beta), actin, adducin, 4.1R and p55 are the main constituents of the skeleton. Interactions between these proteins are defined as lateral interactions. This protein network is anchored to the lipid bilayer through two vertical interactions, one involving ankyrin and Band 3, the other one involving $4.1R^{80}$, p55, and GPC. Band 3 also binds to protein 4.2 (*4.2*). This Figure does not show several additional actin-associated proteins, myosin, protein 4.9, adducin and tropomodulin.

However, there is an absolute Ca^{2+} requirement for CaM-induced regulation of 4.1R interactions with other membrane proteins. How can we reconcile these observations?

3. TECHNIQUES USED FOR MEASURING BINDING AFFINITY

Protein-protein and protein-peptide interactions can be analyzed using resonant mirror detection (RMD) (16-19), a method the IAsys system (LabSystem-Affinity Sensors, Cambridge, UK) is based on. RMD method relies on kinetic analysis of molecular interactions in a cuvette in which binding of a polypeptide in solution (*analyte*) to a polypeptide immobilized on the cuvette surface (*ligand*) is monitored by differential resonant mirror diffraction of a laser beam (16-18).

The advantage of this method is that it allows measurement of an association rate constant (k_a), and an off rate constant (k_d) (*see* Equation 1), from which an equilibrium dissociation constant $K_{(D)}$ can be calculated using Equation 3. Changes in k_a and k_d provide indirect evidence for changes in conformation and in distribution of surface charge within a molecule and within a complex, respectively. Importantly, proteins processed by RMD do not require any labeling with either radioisotopes or fluorescent probes, thus avoiding changes in protein conformation and/or distribution of surface charge that may result from such modifications. However, it is necessary to immobilize the analyte chemically on the cuvette, a procedure that sometimes results in masking or inactivation of critical residues necessary for protein-protein interaction.

Typical binding studies using the IAsys system involve the use of biotinylated peptides immobilized on either avidin-coated cuvettes or biotin-coated cuvettes complexed with avidin. However, we have observed that 4.1R 30kDa domain interacts non-specifically with streptavidin. Consequently, an avidin-biotin based approach cannot be employed for 4.1R binding assays. To overcome this problem, we developed a new method in which the synthetic peptide of interest is chemically linked to the surface of an aminosilane cuvette that has been pre-coated with bovine serum albumin (BSA) (6). In brief, BSA is first immobilized to the aminosilane cuvette with the cross-linking agent *bis* (sulfosuccinimidyl) suberate (BS^3). Immobilized BSA is then activated by addition of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and *N*-hydroxysuccinimide (NHS), then blocked with ethanolamine. Activated BSA is then able to bind to the synthetic peptide. The peptide/BSA-immobilized cuvette is finally probed with the analyte suspected to interact with the peptide. The advantage of this approach resides, first, in a significant increase in the amount of immobilized peptide, and, second, in an increase in analyte binding

specificity since the surface of the aminosilane cuvette is completely masked by BSA.

Binding of the protein in solution (polypeptide "A", *analyte*) to the polypeptide immobilized on the cuvette surface (polypeptide "L", *ligand*) is monitored by measuring a resonant mirror response, expressed in "*arc seconds*", using the IAsys system. The time-dependent changes in arc seconds provide information concerning real-time changes in surface concentration of protein complexes formed on the sensor surface (16-19). The resulting binding curve (arc seconds versus time) is analyzed using the software package FASTfit® (LabSystem-Affinity Sensors, Cambridge, UK). The rate of formation of the polypeptide complex [AL] on the surface at different concentrations of protein "B" added to the cuvette is given by:

$$dR/dt = k_a\,[A]\,R_{max} - (k_a\,[A] + k_d)\,R_t \quad \text{[Equation 1]}$$

where [A] is the concentration of protein "A" (*analyte*) used in deriving the binding curve, R_t is the response in arc seconds at time 't', R_{max} is the maximum value of R at saturating concentrations of protein "A", and k_a and k_d are association rate and off rate constants, respectively. Furthermore, the association of protein "A" with immobilized protein "L" (*ligand*) can be described by the following pseudo first order equation:

$$R_t = R_0 + R_{max}\,(1 - e^{-k_{on}\,t})$$

where R_0 is the initial response and k_{on} is given by:

$$k_{on} = k_a\,[A] + k_d \quad \text{[Equation 2]}$$

Using the above described relationships, the FASTfit® program uses an iterative curve fitting procedure to derive values for k_{on} as a function of [A] that best fit the arc seconds versus time binding data recorded at varying concentrations of protein "A". Based on the relationship outlined in equation 1, the slope of the plot of k_{on} versus [A] provides the value for k_a while the intercept provides the value for k_d (18). Dissociation constant from this form of kinetic analysis (termed "$K_{(D)\,kin}$") is then calculated as follows:

$$K_{(D)\,kin} = k_d / k_a \quad \text{[Equation 3]}$$

Dissociation rate constants can also be derived by measuring the loss of signal (arc seconds) as a function of time immediately following the replacement of the binding buffer containing protein B with a protein-free solution. The FASTfit® program is then used to derive the value of k_d by deriving the best fit of the dissociation curve data to the relationship (18):

$$R_t = R_{max}\,e^{-k_d t}.$$

For self-consistency, the values of k_d derived from these two different approaches must be very similar. This important criterion has been met in all the binding studies described in the present review.

Dissociation constant (termed "$K_{(D)\,Scat}$") can also be derived from the binding data by Scatchard analysis. The maximal extent of binding ($R_{eq.}$) at various concentrations of [A] is derived from binding curves as follows:

$$K_A(R_{max} - R_{eq}) = R/[A]$$

the slope of the plot of R_{eq} versus $R_{eq}/[A]$ providing the value of $-K_A$.

$K_{(D)\,Scat}$ is then calculated as follows:

$$K_{(D)\,Scat} = 1/-K_A$$

In all our studies, the $K_{(D)\,Scat}$, derived under a variety of experimental conditions, closely match the corresponding calculated $K_{(D)\,kin}$ values.

The IAsys system determines an apparent molar ratio of protein "A" (*analyte*) binding to protein "L". Maximum binding (B_{max}), expressed in arc seconds, is calculated by Scatchard analysis and titration assays (20, 21). The immobilized protein (protein "L") on the cuvette is represented by the response, also expressed in arc seconds.

The binding ratio of *ligand* to *analyte* was evaluated by using the Affinix® (Initium Co., Tokyo, Japan) technology based on the quartz crystal microbalance method (22). This method is based on the measurement of the change of frequency upon binding of a ligand to an analyte immobilized quartz crystal sensor chip. A decrease in frequency of 1Hz corresponds to the binding of 30pg of ligand to the chip. Estimation of the binding ratio was calculated using the theoretical molecular weights of the ligand and the analyte.

4. ORGANIZATION OF THE ERYTHROCYTE MEMBRANE

4.1. Maintenance of Erythrocyte Shape

The function of 4.1R has been deduced from the hematopoietic phenotype observed in human 4.1R deficient patients (23, 24), in transgenic 4.1R knock out mice (25) and in zebrafish (*Danio rerio)* subjected to chemical mutagenesis (26). 4.1R deficiency leads to hereditary elliptocytosis (HE), erythrocytes losing their typical biconcave disc shape to become elliptical. Thus, 4.1R acts in concert with other membrane proteins for maintaining normal erythrocyte shape (Figure 3).

In a similar fashion, zebrafish 4.1R mutants merlot (*mot*) and chablis (*cha*) exhibit severe hemolytic anemia characterized by abnormal cell morphology and increased osmotic fragility. The phenotypic analysis of merlot reveals a severe hemolysis of mutant red blood cells, consistent with the observed cardiomegaly, splenomegaly, elevated bilirubin levels and erythroid hyperplasia in the kidneys. Moreover, electron microscopic analysis demonstrates that mot red blood cells have membrane abnormalities and exhibit a severe loss of cortical membrane organization.

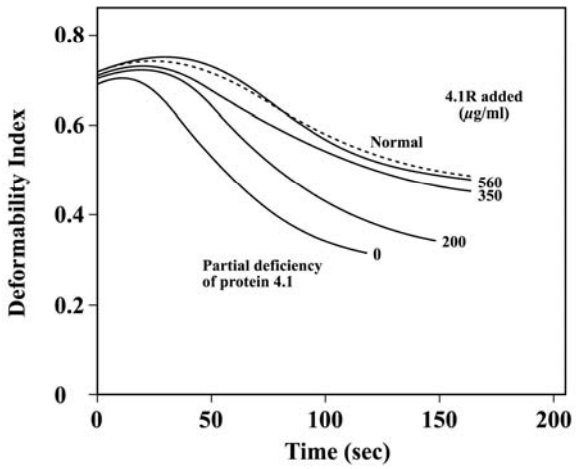

Figure 3. Restoration of membrane stability to partially 4.1R-deficient erythrocyte ghosts. Ghosts were incubated with 0 to 560 micro-g/ml purified 4.1R before resealing. Membrane stability was assessed by ektacytometry. In brief, the ghosts were suspended in dextran and subjected to constant shear stress of 750 dyn/cm^2. Change in laser diffraction pattern was measured by recording signal designated as deformability index (DI) as a function of time. When shear stress is initially applied the ghosts are deformed and adopt an ellipsoid shape with a narrow elliptical pattern that is characterized by a high DI. With time, as the ghosts become unable to withstand high shear stress, they begin to fragment. The resultant loss in membrane surface caused decrease in DI, the rate of which being a measure of membrane stability. Ghosts prepared from 4.1R-deficient red blood cells showed decreased membrane stability. Membrane stability was restored to nearly normal values at 560 micro-g/ml protein.

4.2. Maintenance of Erythrocyte Membrane Mechanical Properties

The membrane skeleton, which underlies the erythrocyte plasma membrane, is made of a spectrin-actin lattice anchored to various transmembrane proteins by two specialized cytoskeletal proteins, 4.1R and red blood cell ankyrin, ankyrin-R (Figure 2) (1). 4.1R stabilizes horizontal interactions between spectrin hetero-dimer (alpha$_2$-beta$_2$) and short actin (~14 molecules) filaments. Ektacytometry studies have revealed that 4.1R plays a key role in controlling erythrocyte membrane mechanical properties. Indeed, resealed membranes prepared from red blood cells totally and partially deficient in 4.1R showed a dramatic decrease in membrane stability (Figure 3) (23, 24). Interestingly, addition of either purified 4.1R (23) or purified 10kDa spectrin-actin binding domain of 4.1R (27) to unstable 4.1R deficient membranes was able to restore mechanical stability to such membranes. This demonstrated unequivocally an essential role for 4.1R, and more specifically for a 21 amino acid peptide encoded by exon 16 in the spectrin-actin binding domain, in maintaining membrane stability by promoting spectrin/actin interactions (28). In 4.1R mutant zebrafish, tubulin was diffusely distributed within red blood cells (26). Actin filaments interact with numerous accessory proteins, such as tropomyosin, myosin, tropomodulin, and adducin (1), which ensure reorganization of actin filaments. 4.1R also interacts with transmembrane protein GPC, and with p55. 4.1R participates in formation of two different ternary complexes, GPC/p55/4.1R, and spectrin/actin/4.1R. Membrane stability is also controlled in part by Band 3-ankyrin-spectrin interaction. The absence of 4.1R, ankyrin, or spectrin or selected mutations in these proteins results in alterations in erythrocyte shape and mechanical properties. A similar membrane skeleton structure is found in non-erythroid cells.

5. 4.1R AS A MODULATOR IN A COMPLEX MEMBRANE SYSTEM

5.1. Facilitation of p55 Binding to GPC

4.1R binds to both cytoplasmic domain of GPC and p55, thus promoting formation of a ternary complex (*see* also Figures 1 and 2). We performed a detailed molecular and functional characterization of the role of 4.1R in regulating organization of the GPC/4.1R/p55 ternary complex. Kinetic analysis of the binding data reveals that 4.1R interaction with p55 increases the binding affinity of p55 for GPC by an order of magnitude, supporting that 4.1R promotes interaction between p55 and GPC. In addition, binding of CaM to 4.1R decreases 4.1R binding affinity for both p55 and GPC in a Ca^{2+}-dependent manner, implying that this ternary complex can undergo dynamic reorganization in the membrane (*see* section III). These findings demonstrate an important role for 4.1R in regulating organization of the GPC/4.1R/p55 ternary complex. Whether p55 and/or Ca^{2+}/CaM binding to 4.1R modulate the interactions responsible for the formation of the GPC-4.1R-spectrin/actin complex is another subject of great interest. Such a study would allow us to establish the functional relevance of p55 in erythrocytes (3).

5.2. Modulation of Ankyrin Binding to Transmembrane Proteins

Distinct regions in the cytoplasmic domain of CD44 interact with 4.1R and ankyrin-R respectively. Preincubation of either full-length 4.1R or 4.1R 30kDa domain with recombinant cytoplasmic domain of CD44 (CD44cyt) impairs ankyrin-R binding to CD44cyt. These results indicate that, although 4.1R does not bind to the same site than ankyrin-R in the cytoplasmic domain of CD44, 4.1R is able to compete with ankyrin-R and may thus modulate ankyrin-R interaction with CD44 (6).

Similarly, 4.1R interaction with Band 3 ($K_{(D)}$ = ~0.1 micro-M) results in a decrease in ankyrin-R binding affinity for Band 3. Inversely, dissociation of 4.1R from Band 3 promotes ankyrin-R binding to Band 3. Thus, 4.1R is able to modulate ankyrin-R binding to Band 3. This competition is likely to modulate red blood cell membrane stability (4). In support of this hypothesis, displacement of 4.1R from Band 3 by addition of an excess of the synthetic peptide IRRRY (5 mM), the motif in Band 3 cytoplasmic domain responsible for binding of 4.1R, to red blood cell membranes, results in a decrease in membrane stability.

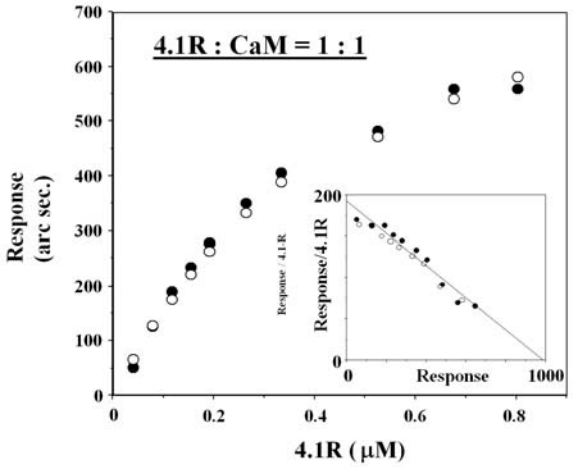

Figure 4. Scatchard plot analysis of 4.1R binding to CaM using the IAsys™ system. *A*. Concentration dependence of 4.1R binding to CaM. Increasing concentrations of purified 4.1R (up to 0.8 micro-M) were incubated with an aminosilane cuvette on which CaM has been immobilized, either in the presence (●) or absence (○) of Ca^{2+}. The amounts of 4.1R/CaM complex formed under equilibrium conditions are represented by the "Response expressed in arc seconds". *B.* Scatchard plot analysis of the data shown in panel *A*. Maximal binding (B_{max}) of 4.1R to CaM immobilized cuvette was 970 arc seconds, both in the presence and absence of Ca^{2+}. The amount of immobilized CaM on the aminosilane cuvette accounted for 200 arc seconds by it. The apparent molar ratio of 4.1R binding to CaM was 1:1, both in the presence and absence of Ca^{2+}.

Using the same strategy, 4.1R has been also shown to regulate cell volume change of skate (*Raja erinacea*) erythrocytes, through its interaction with Band 3 (29). The relevance of 4.1R interaction with Band 3 was recently re-inforced by the discovery that this interaction plays an important role in mitosis based on phenotypic changes observed in Band 3 mutant zebrafish (30).

5.3. Facilitation of Spectrin/Actin Interaction

4.1R binds to beta-spectrin and actin filaments, thus promoting formation of a 4.1R/spectrin/actin ternary complex essential for erythrocyte membrane stability. The ternary complex has a high affinity with a $K_{(D)}$ (equilibrium dissociation constant) of $\sim 10^{-12} M^{-2}$ (31). The effects of 4.1R may be modulated by Ca^{2+} and CaM (*see* section V).

6. Ca^{2+}/CaM-DEPENDENT REGULATION OF 4.1R INTERACTIONS

Ca^{2+} is a key signal transduction messenger in cells activated by various agonists. Ca^{2+} acts also as a regulatory factor for numerous cell functions, mostly through its interaction with CaM. Among other functions, Ca^{2+}, in combination with CaM, is able to modulate 4.1R interaction with various binding partners, thus regulating functions fulfilled by 4.1R-containing protein complexes.

6.1. Variations in Intracellular Ca^{2+} Level

Intracellular Ca^{2+} concentration is normally maintained at sub- micro molar levels (<0.1 micro-M). Increase in intracellular Ca^{2+} occurs under physiological and pathological conditions. For example, normal erythrocytes undergo transient elevation of cytosolic Ca^{2+} upon shear stress in the circulation. In pathological red blood cells, such as sickle, thalassemic, or damaged cells, Ca^{2+} may increase up to 100 micro-M (1). When intracellular Ca^{2+} concentration reaches at least 1 micro-M, activation of various Ca^{2+}-dependent enzymes, such as PKC, CaM-dependent kinase, phospholipase C, calpain, and transglutaminase, takes place. This results in changes in cell shape and deformability, in alterations of membrane stability and ion fluxes, in proteolysis, and in cross-linking of membrane proteins. Except for Ca^{2+}-activated proteolysis mediated by calpain, most Ca^{2+}-induced changes are reversible, depending on the extent and duration of the increase in intracellular Ca^{2+}.

6.2. 4.1R, a CaM-Binding Protein with Unique Features

CaM is a highly conserved Ca^{2+}-binding protein that binds to various Ca^{2+}-dependent enzymes, transporters, and structural proteins, thus modulating their activity (32). In most cases, Ca^{2+} is essential for the initial binding of CaM to these proteins and, consequently, for subsequent modulation of protein function by CaM. 4.1R is unique in that it binds to CaM independently of Ca^{2+} but that it still requires Ca^{2+} for undergoing functional alteration.

6.3. Ca^{2+}/CaM-Dependent Regulation of Membrane Protein Interactions

Human red blood cell CaM concentration is ~1 micro-M. At this physiological concentration, membrane-bound Ca^{2+}-ATPase is activated to maintain Ca^{2+} at a sub-micro-molar level (<0.1 micro-M) (33). CaM binds to 4.1R 30kDa domain in a Ca^{2+} -independent manner (Figure 4) and to a MARCKS homologue sequence in adducin (34). In concert with Ca^{2+}, CaM modulates protein-protein interactions involving either 4.1R or adducin. More specifically, Ca^{2+}/CaM reduces binding affinity of 4.1R for the spectrin-actin complex, thus decreasing membrane mechanical stability (Figure 5).

We have previously mentioned that 4.1R was competing with ankyrin-R to bind to CD44cyt. However, addition of 100 micro-M Ca^{2+} and 1 micro-M CaM to a preformed 4.1R 30kDa - CD44 complex, restores approximately 70% of ankyrin-R binding to CD44. These data suggest that 4.1R can modulate ankyrin-R interaction with cytoplasmic domain of CD44 but that Ca^{2+}/CaM controls this effect of 4.1R. Unlike 4.1R, ankyrin-R does not bind to CaM, either in the presence or absence of Ca^{2+}. Furthermore, while Ca^{2+}/CaM accelerates the dissociation of 4.1R from CD44, it has no effect *per se* on CD44-ankyrin-R interaction (6). At Ca^{2+} concentrations greater than 0.1 micro-M, 4.1R 30kDa domain binding to CD44 starts to decline. Maximal inhibition of binding occurs at 100 micro-M of Ca^{2+}, while the half-maximal effect is observed at 7 micro-M of Ca^{2+} (7). Similarly, full length 4.1R and 4.1R 30kDa domain binding to Band 3 is also inhibited by Ca^{2+}/CaM interaction with 4.1R 30kDa domain (35, 36).

To determine whether Ca^{2+}/CaM could also regulate interactions of 4.1R with GPC and p55, binding of 4.1R 30kDa

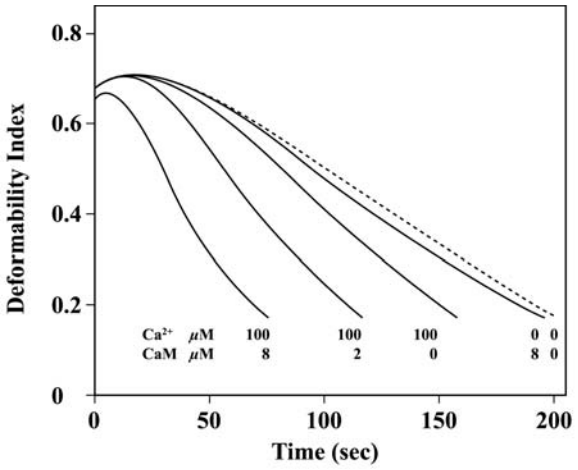

Figure 5. Ca^{2+}/CaM -induced decrease in membrane stability. Resealed ghosts were prepared in the presence or absence of Ca^{2+} (0 or 100 micro-M). Various concentrations of CaM (0, 2, 4, and 8 micro-M) were added and membrane stability was measured as indicated in Figure 3. Membrane stability decreased with increase in Ca^{2+} only when CaM was present.

domain to GPC and p55 has been quantitated in the presence or absence of Ca^{2+}/CaM. Addition of either 5 micro-M CaM or 100 micro-M Ca^{2+} has no effect on the association and dissociation rate constants of the interactions of 4.1R 30kDa domain with either GPC or p55. However, in the presence of both Ca^{2+} and CaM, the affinity of these interactions decreases by a factor of 10. The Ca^{2+}/CaM-induced increase in the $K_{(D)\ kin}$ value for 4.1R 30kDa domain-p55 interaction is due to a decrease in k_a value, i.e. decreased association between 4.1R and p55, while the Ca^{2+}/CaM-induced increase in the $K_{(D)\ kin}$ value for 4.1R 30kDa domain-GPC interaction is due to an increase in k_d value, i.e. increased dissociation between 4.1R and GPC. These changes may be caused by conformational changes of 4.1R 30kDa domain induced by Ca^{2+}/CaM. Similar effects of Ca^{2+} and CaM are observed when full length 4.1R is used in similar binding assays. These results demonstrate that Ca^{2+}/CaM binding to 4.1R 30kDa domain can down regulate 4.1R interaction with both p55 and GPC (Figure 6). The Ca^{2+} concentration dependence of CaM-regulated binding of 4.1R to GPC and to p55 has been investigated. Binding of recombinant 4.1R 30kDa domain to GPC starts to decrease at Ca^{2+} concentrations greater than 1 micro-M, while its binding to p55 is altered at 4 micro-M (36).

Low and high-shear viscometry and binding assays reveal that CaM can also modulate organization of the 4.1R/spectrin/actin complex in a Ca^{2+}-dependent manner. Interestingly, at low Ca^{2+} concentrations, Ca^{2+}/CaM inhibits the 4.1R-potentiated actin cross-linking activity, while the actin binding activity of spectrin is not (14). Based on these results, we propose a model for regulation of two 4.1R-containing ternary complexes by Ca^{2+}/CaM in the red blood cell membrane skeleton (Figure 6).

Interestingly, two CaM inhibitors, W-7 and W-13, used at concentrations up to 100 micro-M, fail to inhibit Ca^{2+}/CaM-induced dissociation of 4.1R from either CD44 (6) or red blood cell *inside-out-vesicles* (IOVs) (37). These results suggest that the profile of CaM binding to 4.1R may be different from the Ca^{2+}-dependent CaM binding to other target proteins. The W-7 binding site in CaM has been recently mapped by NMR analysis of the W-7/CaM complex (38, 39). The results demonstrate that W-7 shields completely the side-chains of key residues in two hydrophobic pockets, Phe^{19}, Met^{36}, and Met^{72} in the NH_2-terminal half, and Phe^{92}, Met^{109}, and Met^{145} in the COOH-terminal half of CaM, respectively (38, 39). Recent data from our laboratory suggest that 4.1R-binding site(s) in CaM may be located in the middle region of the CaM molecule (our unpublished data).

7. FUNCTIONAL STRUCTURE OF 4.1R

7.1. Overview of 4.1R

4.1R forms multi-molecular complexes with transmembrane proteins and membrane-associated proteins, such as spectrin and actin (Figure 2). Such complexes, which are critical for maintaining structural stability in red blood cells, could well be involved in other functions in non-erythroid cells, such as for example signal transduction at sites of cell-cell and/or cell-matrix contacts.

4.1R, present at approximately 200,000 copies per red blood cell, can be extracted by high salt treatment of IOVs, which correspond to red blood cell membranes depleted of spectrin and actin. 4.1R can then be purified by successive anion exchange-column chromatography, using a Q-Sepharose column, and cation exchange-column chromatography, using a SP-Sepharose column (40-43). Purified 4.1R migrates as two bands on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with apparent molecular weight of 80kDa and 78kDa, respectively (*see* "*22/24kDa (CTD) domain*" in this section). Based on its 622 amino acid composition (44, 45), the predicted molecular weight of 4.1R is only ~70kDa, the discrepancy with the apparent molecular weight resulting from extensive post-translational modifications of 4.1R. Limited □-chymotryptic digestion of 4.1R generates four polypeptides: a 30kDa NH_2-terminal membrane binding domain, a 16kDa domain, a 10kDa domain (SAB: spectrin/actin binding domain) and a 22/24kDa COOH-terminal domain (CTD) (40). The apparent molecular weight of these fragments has been deduced from their migration on SDS-PAGE.

7.2. 30kDa (FERM) Domain

The NH_2-terminal 30kDa domain of 4.1R, also named FERM (protein four point one, ezrin, radixin, and moesin) domain (46), is made of 298 amino acids. Interestingly, most of the Cys residues in 4.1R are concentrated in this domain (7 out of 10 Cys), the three remaining Cys being located within the NH_2-terminal headpiece characteristic of the 135kDa 4.1R isoform (*see* section VII). The amino acid sequence of the 30kDa domain is about 30% identical to that of the NH_2-terminal half region of ezrin, radixin and moesin, which are also peripheral membrane associated proteins interacting with actin filaments (reviewed in 47).

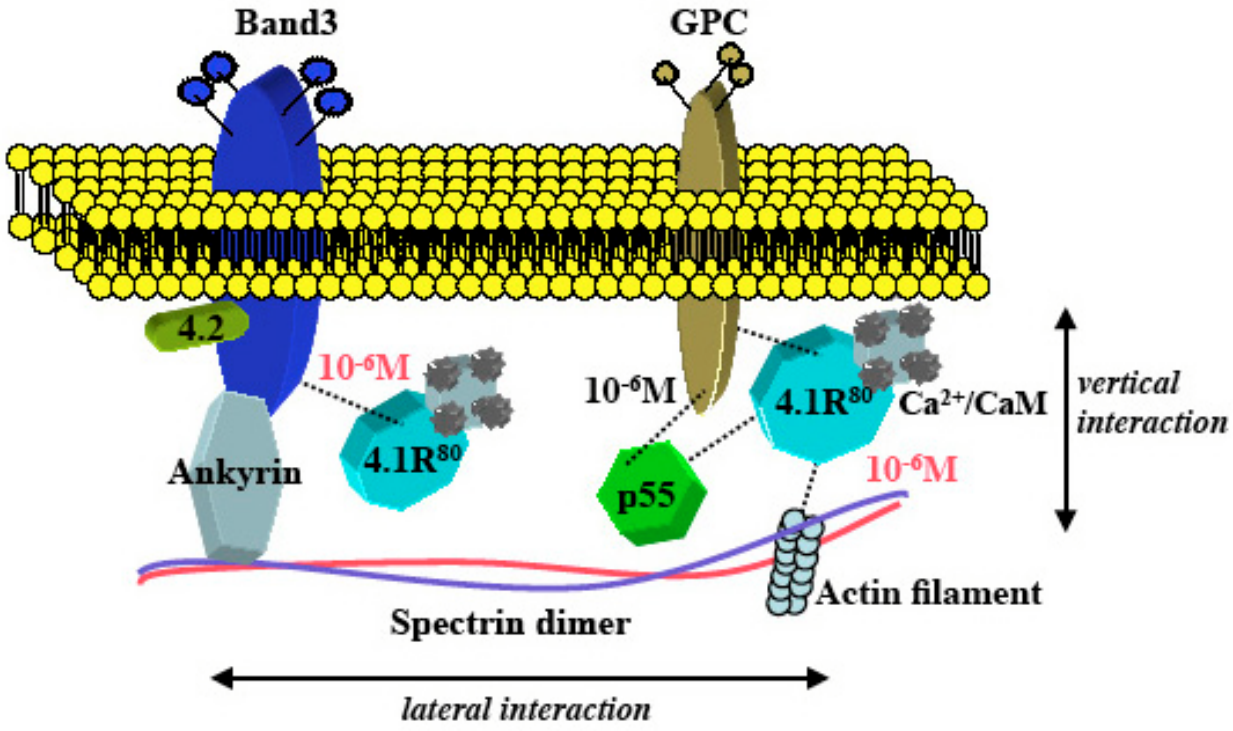

Figure 6. Model proposed for Ca^{2+}/CaM-dependent regulation of 4.1R binding to membrane proteins. Red blood cell intracellular Ca^{2+} concentration is normally maintained at less than 1.0 micro-M (10^{-6} M). At this Ca^{2+} concentration, CaM is bound predominantly to the Ca^{2+} -independent site located in peptide 11 of 4.1R 30kDa domain (*see* sections V and VI). At higher Ca^{2+} concentrations, CaM binding affinity for the Ca^{2+}-dependent site, located in peptide 9 of 4.1R 30kDa domain, is increased. This results in a conformational and/or electric surface change which alters 4.1R binding sites, 4.1R interacting consequently with lower affinity with its binding partners p55, GPC, and spectrin/actin. This model implies that Ca^{2+}/CaM-dependent effect on protein 4.1R binding to transmembrane proteins can only take place when CaM binds to both Ca^{2+}-independent and Ca^{2+} -dependent binding sites in 4.1R.

As shown in Figure 1, the 30kDa domain contains multiple binding sites for transmembrane proteins Band 3, GPC (1, 2), and CD44 (6). It also interacts with p55, and other members of the PDZ-domain containing protein family (3), with CaM (8, 9), with the subunit alpha of the nuclear shuttle protein importin (importin alpha) (48), and with pICln (49). Interestingly, this domain also binds to phosphatidylserine (PS) (50). Tubulin binding site was identified in the exon 10 encoded region of 30kDa domain. The binding motif contains a unique Leu rich sequence (51).

The binding motif in 4.1R for the cytoplasmic domain of Band 3 has been mapped as L^{37}EEDY in a region encoded by exon 5. The corresponding binding sites for 4.1R in Band 3 cytoplasmic domain are I^{386}RRRYand L^{343}RRRY (52).

The GPC binding site in 4.1R 30kDa domain was narrowed down to a region encoded by exon 8, spanning from Gly^{120} to Arg^{166} (36). The binding site for 4.1R in GPC corresponds to a 12-amino acid segment within the cytoplasmic domain of GPC (5, 53) that includes the unique motif R^{86}HK (reviewed in 54). This motif is present in other transmembrane proteins, such as Neurexin IV in *Drosophila*, Paranodin, a brain-specific protein of the neurexin family in rat (55), or Syndecan-2 in human (56). PKC-dependent phosphorylation of 4.1R prevents 4.1R binding to GPC (57).

4.1R and ankyrin-R bind to distinct motifs in the cytoplasmic domain of CD44, 4.1R binding to N^{628}SRRRCGQKKKLVI (6), while ankyrin-R binds to N^{642}SGNGAVE DRKPSGL (6). These data imply that the basic amino acid clusters RRR and KKK in the cytoplasmic domain of CD44 are involved in high affinity interactions with 4.1R. The same motif is present in the cytoplasmic domain of Band 3. 4.1R interaction with CD44 has been confirmed *in vivo*, both proteins co-localizing in the plasma membrane of native keratinocytes. The binding site for CD44 in 4.1R 30kDa domain has yet to be identified. Interestingly, ezrin, radixin, and moesin, which all belong to the protein 4.1 superfamily, also interact with the 4.1R-binding motif in CD44 (47).

The 30kDa domain interacts also with p55, through a region encoded by exon 10, spanning from Tyr^{214} to Glu^{246} (36). The 4.1R-binding site in p55 is a positively charged 39 amino acid stretch, located between the SH3 domain and the MAGUK domain. This 4.1R binding domain in p55, named 'HOOK domain', is mostly

conserved in PDZ domain-containing proteins Dlg and CASK (56). Its consensus sequence would be S/TX_3 $(K/R)_4$ (54). As described earlier, 4.1R forms a ternary complex with the cytoplasmic domain of GPC and with p55 in erythrocytes (36, 58, 59). p55 binds to the very last three amino acid residues of GPC cytoplasmic domain, $Y^{126}FI$, a sequence that is typical of PDZ domain targeting motifs (53). Given that Syndecan-2 contains a 'GPC-like 4.1R binding motif' and that it interacts with PDZ domain-containing proteins, which are able to bind to 4.1R, it is tempting to speculate that similar ternary complexes, such as CASK/4.1R/Syndecan-2, could exist in epithelial cells (56).

In vitro protein binding assays enabled us to identify two distinct CaM binding sites within 4.1R 30kDa domain: a Ca^{2+}-insensitive binding site, A^{264}KKLWKVCV EHHTFFRL, encoded by exon 11 (pep11), and a Ca^{2+}-sensitive binding site, A^{181}KKLSMYGVDLHKAKD, encoded by exon 9 (pep9) (35). The binding molar ratio of full-length 4.1R or 4.1R 30kDa domain to CaM is 1:1, either in the presence or absence of Ca^{2+} (14, 35). The key amino acid residue for Ca^{2+}-sensitive CaM binding in pep9 is Ser^{185}, while those for Ca^{2+}-insensitive CaM binding in pep11 are aromatic amino acids Trp^{268}, Phe^{277}, and Phe^{278}. The topology of pep9 and pep11 within 4.1R 30kDa domain is described in the following section. CaM is a highly conserved Ca^{2+}-binding protein that modulates functions of various structural and transport proteins, and activities of many Ca^{2+}-dependent enzymes (reviewed in 60, 61). More specifically, in the context of this review, binding of 4.1R 30kDa domain to the cytoplasmic domain of transmembrane proteins, Band 3, GPC, or CD44, and to membrane-associated proteins, such as p55, is regulated by Ca^{2+} and CaM (*see* section V).

Two distant topological sorting signals are required for efficient nuclear import of 4.1R. Indeed, 4.1R bears two importin alpha binding motifs (48), a low affinity binding site, E^{38}ED, located within the region encoded by exon 5, and a high affinity binding site, K^{406}KKRER, located within the region encoded mostly by exon 16 (*see* "*10kDa (SAB) domain*" in this section). Neither full-length 4.1R nor 4.1R 30kDa domain is able to bind to the beta-subunit of importin, importin beta.

Recently, a direct association between 4.1R and pICln, an actin-binding protein that may play a key role in regulation of cell volume, has been characterized (40). This observation supports that pICln, in association with 4.1R, may link cytoskeletal elements to an unidentified volume-sensitive chloride channel.

We observed that purified 4.1R polymerizes above 1 micro-M, suggesting that 4.1R is subject to intermolecular and/or intramolecular interactions. Upon cell activation, proteins get phosphorylated, this modification resulting in protein unfolding and unmasking of residues allowing their interaction with binding partners (reviewed in 47).

Interestingly, 4.1R is able to interact with phospholipids PS, and possibly PIP_2. Binding of 4.1R 30kDa domain to PS has been demonstrated by the use of liposome (62), or of carboxyfluoresceine (63). It has been reported that PS initially recognizes an Y^{233}KRS motif encoded by exon 10 (50). This sequence is reminiscent of the F^{153}KKS motif involved in MARCKS protein interaction with PS (64). Although the physiological significance of 4.1R interaction with PS remains to be investigated, the YKRS motif has recently been shown to be required for proper sorting of 4.1R in nucleated cells (50). It remains to be determined whether YKRS-mediated sorting of 4.1R depends on 4.1R interaction with PS and/or with proteins involved in protein sorting. It has been shown that phosphatidylinositol-4, 5 bisphosphate (PIP_2) could promote 4.1R binding to GPC *in vitro* (65). This was supported by a later study showing decrease in 4.1R binding to red cell membranes depleted of a large pool of their endogenous PIP_2 (66). However, another study, based on the use of IOVs prepared from GPC deficient (Leach phenotype) erythrocytes, suggested that PIP_2 affected mostly 4.1R binding sites other than GPC, such as possibly Band 3 (67).

The *Plasmodium falciparum* mature parasite-infected erythrocyte surface antigen (MESA), a protein exported from the parasite to the infected red blood cell (IRBC) membrane skeleton, binds also to 4.1R 30kDa domain. This interaction involves a 19-residue motif in MESA and a 51-residue region encoded by exon 10 in 4.1R (68). The 3D structure of this region reveals that the MESA binding site overlaps the region of 4.1R involved in formation of the p55/GPC/4.1R ternary complex. Further binding studies using p55, 4.1R, and MESA showed competition between p55 and MESA for 4.1R, implying that MESA interaction with the IRBC membrane skeleton may modulate 4.1R/p55 interaction that occurs in normal erythrocytes *in vivo*. Defining the minimal binding domains involved in critical protein interactions in IRBCs may aid the development of novel therapies for *Plasmodium falciparum* (69).

7.2.1. Crystal Structure of 4.1R 30kDa (FERM) Domain

As shown in Figures 7 and 8, crystal structure of 4.1R 30kDa domain is reminiscent of the shape of a cloverleaf, or of a propeller, with three clearly distinct lobes (70, 71). First, the N-lobe, which contains the first 78 amino acids and which includes Band 3 binding motif L^{37}EEDY, is formed by 4 double-stranded beta-strands. Next, the alpha-lobe, which contains the following 90 amino acids and which includes GPC binding site, is formed by 4 alpha-helices. Last, the COOH-terminal lobe (C-lobe), which contains the p55 binding surface, is made of seven beta-strands, and ends with an alpha-helix. The Ca^{2+}-sensitive CaM binding site is included within a loop and beta-strands located between the α-lobe and the C-lobe. The Ca^{2+}-insensitive CaM binding site is located in an alpha-helix structure within the C-lobe. Interestingly, the Tyr^{41} residue in 4.1R Band 3 binding motif and the Trp^{268}, Phe^{277}, and Phe^{278} residues in the Ca^{2+}-insensitive CaM binding motif in pep11, form a hydrophobic surface. The Ca^{2+}-sensitive CaM binding site is formed by an extended structure (Figure 7), which, compared to the alpha-helical structure of the Ca^{2+}-insensitive CaM binding site, has somewhat a higher temperature factor,

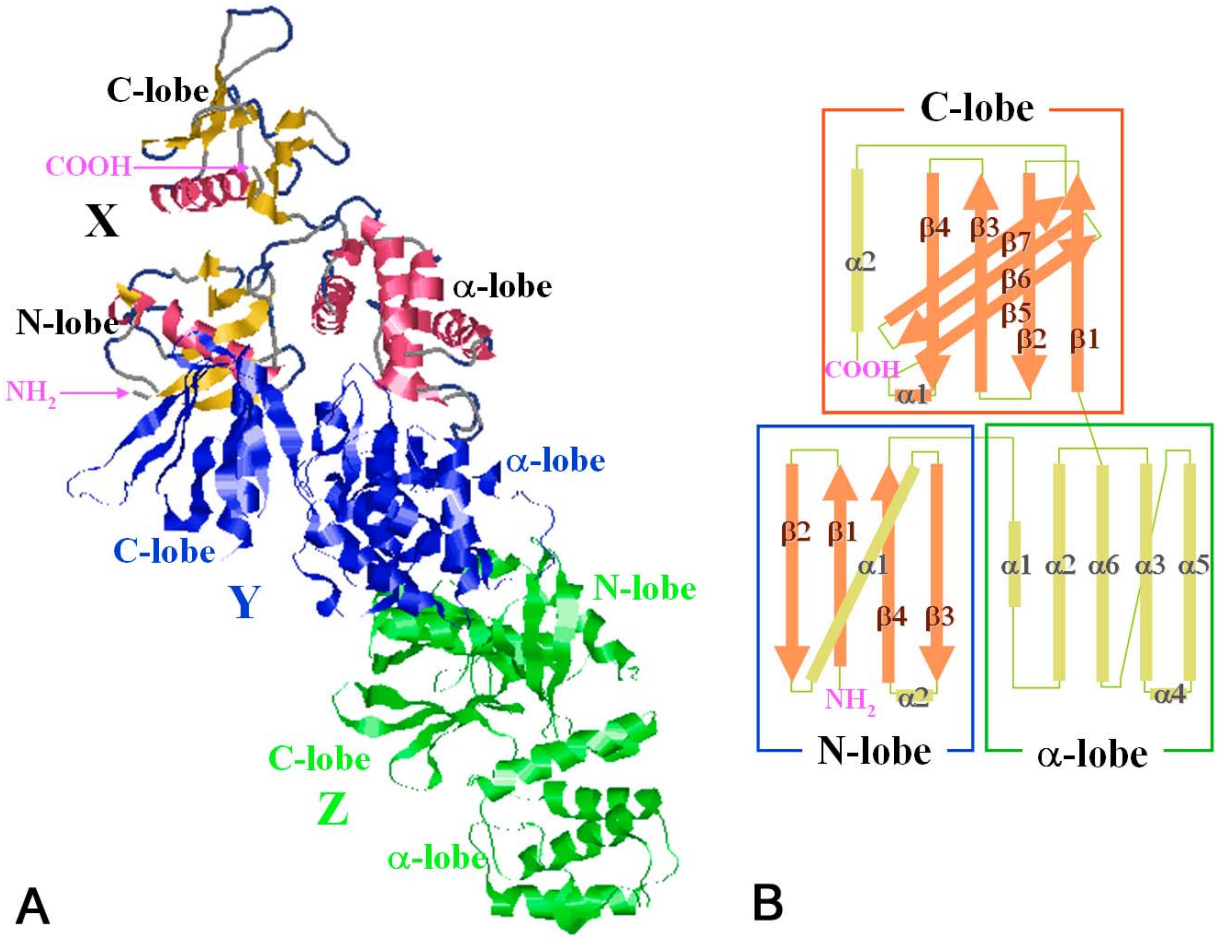

Figure 7. Overall structure of 4.1R 30kDa domain. *A*. Visualization of 4.1R 30kDa domain structure by ribbon representation with secondary structure elements. Letters in the N-lobe and in the C-lobe represent beta-strands while numbers in the alpha-lobe represent alpha-helices. 4.1R 30kDa domain crystallized as a triplicate represents as X (yellow and red represents as alpha-helices and beta-strands, respectively), Y (blue), and Z (green). The crystallographic data of 30kDa domain of 4.1R were treated with molecular visualization program RASMOL. *B*. Topological diagram of 4.1R 30kDa domain. Boxes and arrows represent alpha-helices and beta-strands, respectively.

indicating that the Ca^{2+}-sensitive CaM binding site is a more flexible region than the Ca^{2+}-insensitive one (71). Two loops, one spanning from His^{136} to Asp^{139} within the GPC binding site, and one spanning from Ile^{242} to Tyr^{250} within the p55 binding site, display the highest temperature region factor and constitute therefore the most flexible regions (Figure 7). Ca^{2+} binding to CaM possibly leads to conformational changes in the three lobes in 4.1R 30kDa domain, in particular in these highly flexible regions, causing an alteration in the overall capacity of 4.1R to bind to its partners.

Crystal structure of the FERM domains of moesin and radixin has been recently solved (71-76). The X-ray crystal graphic analysis indicates that the structure of both domains is very similar. Interestingly, co-crystallization of the polar head group of PIP_2, inositol triphosphate (IP_3), and of radixin FERM domain has allowed mapping of the PIP_2 binding surface in radixin FERM domain. Importantly, a consensus sequence for the PIP_2 binding motif has been identified (72).

Co-crystallization of moesin FERM domain and COOH-terminal domain, this latter acting as the actin binding domain, has also confirmed the existence of intramolecular interactions which mask functional regions of these domains (73).

Crystal structure of Band 3 cytoplasmic domain has been recently reported (77). $4.1R^{80}$ binds to the L^{343}RRRY sequence in Band 3 cytoplasmic domain, a region that is part of an alpha-helix structure that is exposed at the surface of the two molecules of the Band 3 dimer.

Based on immunochemical and biochemical studies, the positively charged L^{343}RRRY sequence appears as a possible binding site for the negatively charged L^{37}EEDsequence in 4.1R. However, since the side chains of Ile^{343}, Arg^{346}, Tyr^{347} in the IRRRY sequence, are on less accessible surfaces of the alpha-helix structure in Band 3 cytoplasmic domain, any model that pairs I^{386}RRRY with L^{37}EEDY would require either partial unfolding of the

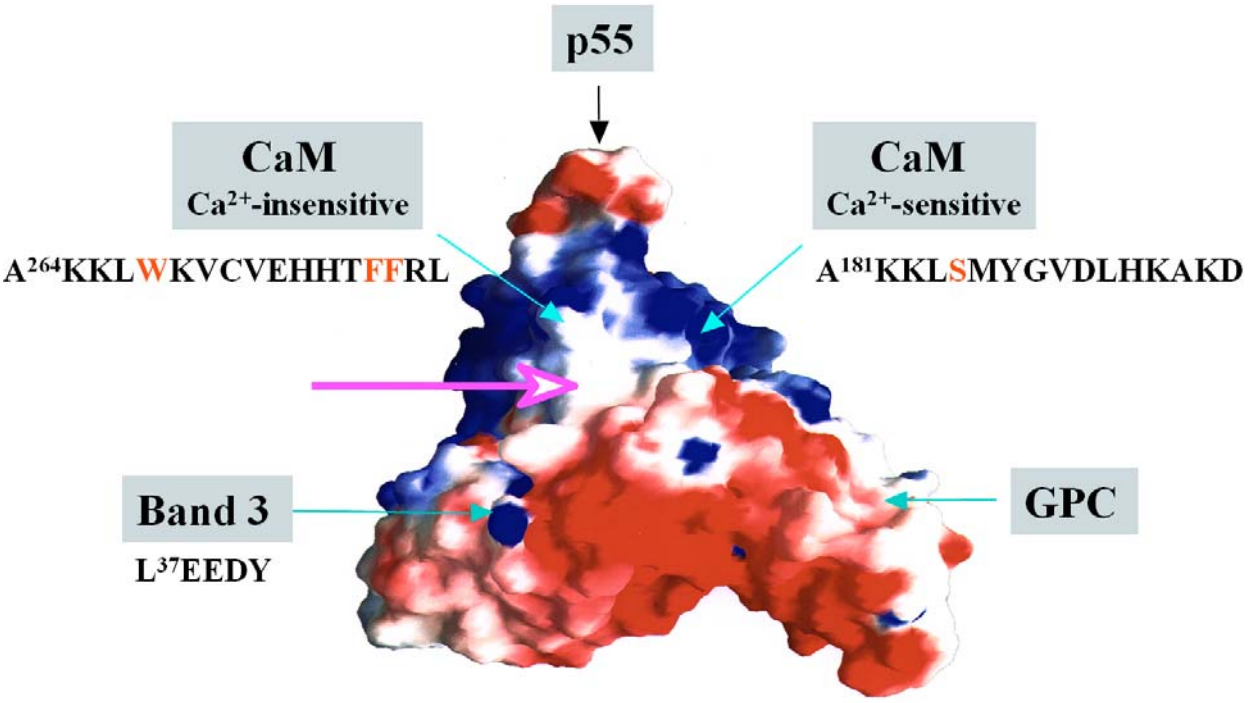

Figure 8. Electric potential surface map and crystal structure of 30kDa domain of 4.1R. An electric potential surface map of 4.1R 30kDa domain is shown (71), the red regions corresponding to negatively charged regions, the blue regions corresponding to positively charged regions, and the white regions corresponding to hydrophobic regions. The Ca^{2+}-insensitive CaM binding site, which contains predominantly hydrophobic residues (indicated by the pink arrow), is located in an indentation between the N-lobe and the C-lobe (*see* ribbon structure in Figure 7). Interestingly, the Band 3 binding motif, LEEDY, is located near this Ca^{2+}-insensitive CaM binding site. The Ca^{2+}-sensitive CaM binding site is located between the alpha-lobe and the C-lobe. It contains a hydrophobic stretch flanked by charged residues. Key residues in each CaM binding site are shown in red (*see* section VI). GPC binding sequence, spanning from G^{120} to R^{166}, is located in the alpha-lobe that is predominantly negatively charged. The p55-binding site, spanning from Tyr^{214} to Glu^{258}, is present in the C-lobe.

alpha-helix in Band 3 cytoplasmic domain or interactions restricted to more masked residues. Co-crystallization of Band 3 cytoplasmic domain and 4.1R 30kDa domain will be invaluable for detailed characterization of the interaction between Band 3 and 4.1R (Figure 8).

7.3. 10kDa (SAB) Domain

4.1R 10kDa domain contains 67 amino acids. The predicted molecular weight of ~8kDa is similar to the apparent molecular weight. The 21 amino acids encoded by exon 16, from Lys^{407} to Glu^{427}, have been originally identified as the primary spectrin/actin binding (SAB) sequence (27). However, part of this sequence, the very NH_2-terminal basic amino acid cluster K^{406}KKRER, has been identified as a high affinity binding site for importin alpha (*see* "*30kDa (FERM) domain*" in this section) (48). Two sequences in the 10kDa domain, G^{414}ENIYIRHSNLMLE and V^{454}PEPRPSEWDKRLSTHS, named "SAB1" and "SAB2" respectively, have been shown to be required for 4.1R interaction with spectrin (27). Recently, the actin-binding motif within the 10kDa domain has been narrowed down to a unique 8 amino acid stretch, L^{446}KKNFMES, located in exon 17 immediately upstream of SAB2 (78).

In skeletal muscle, an 110kDa 4.1R isoform containing the NH_2–terminal head-piece (HP) region (*see* section VII), has been shown to mediate interaction with myosin, and tropomyosin, through the 21 amino acids encoded by exon 16 (79). Importantly, 4.1R has been reported to modulate actin-activated Mg^{2+}-ATPase activity of rabbit skeletal muscle myosin (80). Recently, the interaction of cardiac myosin with 4.1R was also reported (81). To date, such an interaction between 4.1R and myosin, and/or tropomyosin, has not been reported in erythrocytes.

PKA and epidermal growth factor receptor tyrosine kinase (82, 83) phosphorylate Ser^{467} and Tyr^{418} residues, respectively, in the 10kDa domain. The biological significance of phosphorylation of these residues has not been established yet.

Recently, importance of PIP_2 for 4.1R binding to CH (calponin homologue) domain of beta-spectrin (residues 1~131) was reported (84).

7.4. 22/24kDa (CTD) domain

4.1R COOH-terminal domain (CTD) contains 150 amino acids. The apparent molecular weight of this

domain, estimated by SDS-PAGE, is 22/24kDa. The existence of two bands differing only by 2kDa, results from the extent of deamidation of Asn^{502} to Asp^{502} (85). It has been also reported that the region from Thr^{544} to Lys^{563} of the CTD is glycosylated by a cytosolic enzyme in erythrocytes (86). The extent of various modifications explains the dramatic difference between the apparent molecular weight of 4.1R CTD (22/24kDa) and its predicted molecular weight of ~16.1kDa. The functional impact of these post-translational modifications remains unknown.

Although no binding partner for 4.1R CTD has been identified yet in erythrocytes, the recent characterization of various binding partners for this domain in nucleated cells gives us insights into unexpected cellular functions for 4.1R. Thus, 4.1R may play an important role in organization of tight junctions, through interaction of 4.1R CTD with tight junction proteins ZO1, ZO2, and occludin (13). In addition, this domain may also be important for mediating a role for 4.1R in regulation of cell division through organization of centrosomes and of the mitotic spindle. Indeed, the nuclear mitotic apparatus protein (NuMA) binds to a three Val residue motif present in the CTD (12). Last, 4.1R may play a key role in linking the cytoskeleton to the protein translation machinery through interaction of 4.1R CTD with eIF3-p44, a subunit of the eIF3 complex, which plays an essential role in initiation of protein translation (87).

8. AT LEAST TWO ALTERNATIVE TRANSLATION INITIATION SITES IN ONE SINGLE GENE

A key feature of the 4.1R gene is the presence of two potential translation initiation sites, both located in alternative exons (88-91). Indeed, an upstream site, named AUG1, is present in an alternatively spliced region at the 5'end of exon 2 (exon 2'), while a downstream site, named AUG2, is located in exon 4. The existence of a third potential translation initiation site, located in exon 8, has been reported (92), but its physiological relevance has still to be investigated. The use of AUG1 results in generation of a 135kDa 4.1R isoform ($4.1R^{135}$), while the use of AUG2 generates the shorter 80kDa isoform $4.1R^{80}$ (Figure 1). Thus, compared to $4.1R^{80}$, the $4.1R^{135}$ isoform contains an additional NH_2-terminal extension, also referred to as headpiece (HP) (90). The choice between various non-coding exons (exons 1) located far upstream of the coding region of the 4.1R gene dictates the downstream alternative events that govern inclusion or exclusion of exon 2' and therefore translation initiation at either AUG1 or AUG2 (90). Interestingly, while both $4.1R^{135}$ and $4.1R^{80}$ are expressed in erythroblasts, only $4.1R^{80}$ is detected in mature erythrocytes.

4.1R NH_2-terminal HP region contains 209 amino acids. Its apparent molecular weight is ~50kDa according to SDS-PAGE, while its predicted molecular weight is only ~23.4kDa. The 4.1R isoform, which contains the headpiece, has an apparent molecular weight of 135kDa and a predicted molecular weight of only 93kDa based on the weight of its 831 amino acid residues. This discrepancy may suggest that $4.1R^{135}$ may not have a globular shape. Unlike 4.1R 30kDa domain, 4.1R HP, either isolated from *Xenopus* sp. or used as a synthetic peptide of human origin, binds to CaM exclusively in the presence of Ca^{2+} (93, 94). The CaM binding sequence in 4.1R HP has been identified as S^{76}RGLSRLFSSFLKRPKS. This sequence contains serine residues that can be phosphorylated by both PKA and type II CaM-dependent kinase. Phosphorylation of the synthetic peptide, corresponding to this CaM binding domain, results in a reduction in CaM binding affinity (95). Another difference between $4.1R^{135}$ and $4.1R^{80}$, thus likely attributable to HP, is the poor ability of $4.1R^{135}$ to be shuttled to the nucleus (96). This likely results from the fact that the HP impairs 4.1R interaction with importin alpha, the binding affinity of $4.1R^{135}$ for importin alpha being in the sub-micromolar range (unpublished data), while that of $4.1R^{80}$ is ~30 nM (48).

Although the function of 4.1R headpiece remains largely unknown (91), a recent study has provided a first clue about a potential role that this region may confer upon 4.1R. Using the yeast two-hybrid system, it has been established that 4.1R HP interacts specifically with a novel centrosomal protein, CPAP (centrosomal protein 4.1-associated protein) (97). This interaction, along with that of the COOH-terminal domain of 4.1R with NuMA, further supports an important role for 4.1R in centrosome organization and consequently in control of cell division (98). The HP region has been recently shown to contain a phosphorylation site for cdc2 kinase, the level of phosphorylation of 4.1R being subjected to variations during cell cycle and being strongly related to mitotic state in HeLa cells (99).

9. 4.1R HOMOLOGUES

Three novel protein 4.1 genes, sharing high homology with 4.1R, have been recently characterized. They have been named 4.1G (100), 4.1N (101), and 4.1B (102), based on the predominant expression pattern of their mRNAs. The primary structure of 4.1G, 4.1N and 4.1B is similar to that of $4.1R^{135}$, in the sense that these three new 4.1 proteins contain an NH_2-terminal HP region. Since this region is poorly conserved among 4.1 proteins, it has been named first unique region (U1 region). This region is located immediately upstream of the highly conserved 30kDa membrane binding domain, followed by a second unique region (U2 region, which corresponds to the 16kDa domain in 4.1R), the conserved SAB domain (except for 4.1N), a third unique region (U3 region), and finally a highly conserved COOH-terminal domain.

Interestingly, the amino acid sequences responsible for CaM and p55 binding in 4.1R 30kDa domain are very conserved in the corresponding regions of 4.1G (103), 4.1N and 4.1B (104). By contrast, other members of the protein 4.1 superfamily, such as ezrin, radixin and moesin, show poor homology with 4.1R in the CaM binding motifs (35). This evolutionary conservation

pattern predicts that all four 4.1 proteins, but not other ERM proteins, can potentially bind to both CaM and p55, and functionally regulate p55 interactions with integral proteins in non-erythroid cells in a Ca^{2+}/CaM-dependent fashion. Moreover, p55 is a representative of a large family of MAGUK proteins that share two conserved domains of particular relevance to this model. First, these proteins possess PDZ domains that mediate interaction with the cytoplasmic tail of various integral membrane proteins. For example, Syndecan, which contains an YFI COOH-terminal peptide similar to that in GPC, has been shown to interact with the MAGUK protein hCASK (56). Second, MAGUK proteins bear a so-called HOOK domain, which plays a key role in mediating their interaction with 4.1R. Indeed, in addition to p55, other MAGUK proteins, such as hDlg and hCASK, have been reported to bind to 4.1R (3, 56).

The FERM domain of 4.1N has been recently involved in formation of a ternary complex with Syndecan 2 and CASK, in a similar fashion to formation of the GPC-p55-4.1R complex in erythrocytes (105). In addition, the CTD of 4.1N has been shown to interact with inositol (1, 4, 5)-triphosphate (IP_3) receptor Type 1 in rat brain synaptosomes (106). This strongly suggests that 4.1N may bring together multiple protein complexes through simultaneous interactions of various binding partners with distinct domains of 4.1N. Thus, a Syndecan 2-CASK-4.1N-IP_3receptor Type1 complex could be formed in synapses. The FERM domain of 4.1N has also been recently shown to interact with nectin like protein 1, a cell adhesion molecule that shares a highly conserved amino acid sequence motif responsible for GPC interaction with 4.1R (107). Once again, this interaction seems important for proper recruitment of 4.1N to plasma membrane. The FERM domain of 4.1B/DAL1 has been reported to bind to the cytoplasmic domain of tumor suppressor lung cancer (TSLC) 1, a transmembrane protein that shares also the GPC motif responsible for interaction with 4.1R. Therefore, interaction of the FERM domain with transmembrane proteins is likely to play an important role in cell differentiation and in control of cell proliferation (108).

Interaction of 4.1 proteins with receptors does not only mediate their proper positioning in plasma membrane but can also regulate their function. Thus, 4.1G does not only regulates cell surface expression but also modulates the activity of the A1 adenosine receptor (A1AR) through binding of its CTD to the third intracellular loop of the receptor (109). Indeed, 4.1G interferes with A1AR signal transduction by reducing A1AR-mediated inhibition of cAMP accumulation and intracellular calcium release. 4.1G has also been recently shown to promote clustering of LFA-1 and PTA-1 on the plasma membrane of T-cells, thus playing an important role in T cell activation (110).

Taken together, these observations suggest that Ca^{2+}/CaM modulation of interaction of 4.1R, and possibly of its homologues, with transmembrane and other membrane skeletal proteins may play a major role in dynamic cytoskeletal reorganization during cell signaling in erythroid as well as non-erythroid cells.

10. CONCLUSION

Now that completion of the human genome sequence is almost achieved, scientists have become increasingly interested in studying protein 3D structure in order to gain an understanding of the organization of protein complexes and of the regulation of their functions based on characterization of molecular interactions. In that context, deciphering the mechanisms underlying specific molecular recognition and self-organization of a complex system appears as a very attractive and innovative field of investigation. Integration of biophysical, biochemical and cell biology approaches will warrant success in understanding the structure and function of such "complex systems". In that respect, the erythrocyte membrane is a model of choice, given the extensive knowledge of the architecture and functions of its cytoskeleton. 4.1R, which plays a key role in organization and function of the red blood cell cytoskeleton through its interaction with various protein and lipid partners, represents a very valuable protein for pursuing studies on mechanisms of molecular recognition and organization of functional protein complexes.

11. APPENDIX

We recently showed that 4.1R actually regulates ankyrin binding to CD44cyt in HeLa cells (111). Indeed, immunostaining studies indicated that 4.1R and CD44 were expressed along peripheral plasma membranes, while ankyrin was predominantly cytosolic in HeLa cells. In contrast, transfected HeLa cells over-expressing the ezrin FERM domain, this domain being able to interact with high affinity with CD44cyt, showed instead predominant 4.1R distribution in the cytosol and ankyrin expression in the membrane along with CD44 (our unpublished data).

12. ACKNOWLEDGEMENTS

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