4.1 Proteins: Ion Transporters in Check

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Abstract

The classical function of 4.1R in erythrocytes is to contribute to the mechanical properties of the membrane by promoting the interaction between spectrin and actin. It is now well recognized that 4.1R is required for the stable anchorage of numerous cell surface erythrocyte membrane proteins. 4.1R is the prototypical member of the family of 4.1 proteins, which are expressed in many cell types, besides erythrocytes. The other members of the protein 4.1 family include 4.1N, 4.1G, and 4.1B. NHE1 (Na+/H+ exchanger isoform 1) has been reported to be hyperactive in 4.1R-null erythrocytes, supporting a functional interaction between NHE1 and 4.1R. We recently demonstrated that 4.1R binds directly to the cytoplasmic domain of NHE1 (NHE1cd). This interaction involves an EED motif in the 4.1R FERM (4.1Ezrin/radixin/moesin) domain and two clusters of basic amino acids in the NHE1cd. K+1-4R and R556FNKKYVKK, previously shown to mediate PIP2 (phosphatidylinositol-4,5-bisphosphate) binding. The affinity of this interaction is reduced in hypertonic and acidic conditions, demonstrating that this interaction is of electrostatic nature. The binding affinity is also reduced upon binding of Ca2+/CaM (Ca2+-saturated calmodulin) to the 4.1R FERM domain. We propose that 4.1R regulates NHE1 activity, through a direct protein-protein interaction that can be modulated by intracellular pH, as well as Na+ and Ca2+ concentrations. In this review, we discuss the increasing evidence for an important role for members of the protein 4.1 family of membrane skeletal proteins, in the regulation of various ion transporters in erythrocytes and in non-erythroid cells.

Keywords: 4.1 Proteins; NHE1; Erythrocyte; Transporters; Exchangers

Abbreviations: 4.1B: Band 4.1-like Protein 3 (Brain-Type); 4.1G: Erythrocyte Membrane Protein Band 4.1-like 2 (generally Expressed Type); 4.1N: Erythrocyte Membrane Protein Band 4.1-like 1 (Neuron-Type); 4.1R: Erythrocyte Membrane Protein Band 4.1; 4.1R*: 80kDa protein 4.1R; AE1: Anion Exchanger Isoform 1; Band 3cd: Cytoplasmic Domain of Anion Exchanger Band 3; CaM: Calmodulin; Ca2+/CaM: Ca2+-saturated CaM; pCl: Cl-channel-associated protein, FERM: Four. one/Ezrin/Radixin/Moesin; GPC: Glycophorin C; KCC2: Neuron-Specific K-Cl Cotransporter; NHE1: Na+/H+ exchanger isoform 1; NHE1cd: cytoplasmic domain of NHE1; PC: Phosphatidylycholine; PIP2: Phosphatidylinositol-4,5 bisphosphate; SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis.

Structure of the Erythrocyte Membrane and its Associated Cytoskeleton

The membrane skeleton which underlies the erythrocyte plasma membrane, consists of a spectrin/actin lattice anchored to various transmembrane proteins via two specialized cytoskeletal proteins, 4.1R and red cell ankyrin (ankyrin-R) [1,2] (Figure 1). 4.1R binds to the cytoplasmic domain of numerous cell surface proteins, such as tropomyosin, myosin, tropomodulin, and adducin, which are responsible for the dynamic organization of actin filaments. 4.1R interacts also with the transmembrane protein, glycophorin C (GPC), and with the membrane-associated guanylate kinase (MAGUK) protein p55, which also acts as an erythrocyte scaffolding protein (Figure 1) [1,2].

GPC is a 32kDa single-transmembrane protein expressed at ~50,000-100,000 molecules/erythrocyte. The cytoplasmic domain consists of 47 amino acid residues (ID: P04921). The R44HK sequence has been identified as the 4.1R binding sequence in the cytoplasmic domain of GPC [3-6]. This RHK motif is highly conserved in the cytoplasmic domain of Neurexin IV, Paranodin and TSLC1 (Tumor Suppressor Lung Cancer 1) [7]. GPC possesses a p55 binding motif, EFPI, in its C-terminal region (Figure 1). p55 is a 55 kDa erythrocyte scaffolding protein that belongs to the MAGUK family of proteins (ID: Q00013). This protein is characterized by the presence of a PDZ (Post-synaptic density protein-95), Dlg (Drosophila disc large tumor suppressor), ZO-1 (Zonula Occludens-1) domain, a SH (src-homology) 3 domain, and a catalytic inactive guanylate kinase-like (GUK) domain, all of which function as protein-protein interaction modules (Figure 1) [8]. The number of p55 copies in the human erythrocytes is ~80,000. Although the function of p55 in erythrocytes has not been elucidated, p55 seems essential for maintenance of polarity in neutrophils [9]. Thus, 4.1R participates in the formation of two different ternary complexes in erythrocytes, the 4.1R/p55 complex and the 4.1R/spectrin/actin complex.

Ektacytometry studies have revealed that 4.1R plays a key role in controlling erythrocyte membrane mechanical properties. Indeed, resealed membranes prepared from erythrocytes, totally or partially deficient in 4.1R, show a dramatic decrease in membrane stability [10,11]. Interestingly, addition of either purified full-length 4.1R or purified 10 kDa spectrin-actin binding domain of 4.1R to 4.1R-deficient membranes restores their mechanical stability. This demonstrates 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 through promotion of spectrin-actin interactions [1,2].

Erythrocyte membrane stability is also controlled in part by the band 3-ankyrin-spectrin interaction (Figure 1). Band 3 is a 102 kDa protein with 14 transmembrane domains. It is expressed at 1,200,000 molecules/cell. It forms dimers that assemble into tetramers, each tetramer binding to one molecule of ankyrin. This is the basis for the organization of the band 3-ankyrin-spectrin complex [1]. Band 3 mediates exchange of HCO₃⁻ and CI⁻, and is therefore referred to as Anion Exchanger 1 (AE1) [1] (ID: P02730).

4.1R binds to the L⁺RRRRY and L⁺RRRY sequences in band 3 cytoplasmic domain [12,13]. Although the crystal structure of the N-terminal cytoplasmic domain of band 3 has been reported, this structure is still putative as the N-terminal 55 residues, including the N-terminal 55 residues, the spectrin and actin binding (SAB) domain. The actual stoichiometry of band 3 binding to 4.1R is still unknown. The importance of band 3 in membrane architecture results from its role in anchoring the spectrin network, through interaction with the scaffold protein ankyrin. We have characterized a similar function for4.1R in modulating ankyrin interaction with CD44, a single transmembrane protein, which acts as a receptor for hyaluronic acid [15,16]. Relevant to this review, we show in the following that the sensitivity of 4.1R binding to band 3 to the microenvironment (pH, salt and Ca²⁺ concentrations), differs greatly from that of 4.1R binding to the sodium-proton exchanger NHE1.

Salomo et al. [17] have documented that protein 4.1R can bind in vitro to additional erythrocyte transmembrane proteins, bearing well characterized antigenic features, such as Kell, XK, Rh and Duffy. These interactions remain to be validated in vivo. The function of 4.1R has been inferred from the hemotopoietic phenotype observed in human 4.1R deficient patients, in transgenic 4.1R knockout mice [18], and in zebrafish (Danio rerio) [19], subjected to chemical mutagenesis [20,21]. 4.1R deficiency leads to hereditary elliptocytosis (HE), a condition in which erythrocytes lose their typical biconcave disc shape to adopt an elliptical shape. Thus, 4.1R acts in concert with other membrane proteins, to maintain the normal shape of erythrocytes [10,11,22,23].

**Protein 4.1R**

**Structure**

4.1R forms multi-molecular complexes with transmembrane proteins and membrane-associated proteins, such as spectrin and actin [1,2] (Figure 2A). Such complexes, which are critical for maintaining the structural properties of the red blood cell membrane, exist in non-erythroid cells, where they are involved into other functions, such as, for example, signal transduction at sites of cell-cell, and/or cell-matrix contacts. 4.1R (ID: P11171), which is expressed at approximately 200,000 copies per erythrocyte, can be extracted by high salt treatment. The resulting erythrocyte membranes depleted of spectrin and actin are called Inside-Out Vesicles (IOVs). Based on its 622 amino acid...
composition [22,23], the predicted molecular weight of 4.1R is only ~70 kDa, the discrepancy with the apparent molecular weight (80 kDa), resulting in part from the unstructured structure of some domains of 4.1R [23]. Limited a-chymotryptic digestion of 4.1R generates four polypeptides: a 30 kDa N-terminal membrane binding domain, a 16 kDa domain, a 10 kDa spectrin-actin binding domain and a 22/24 kDa C-terminal domain [22,23]. A 4.1R isoform expressed in erythroblasts, but not in mature erythrocytes, contains an extra N-terminal 209 amino acid headpiece (HP) region. The inclusion or exclusion of the HP region is under the control of complex splicing events extensively documented by Parra et al. [24-26]. The apparent molecular weight of this 4.1R isoform in SDS-PAGE is ~135 kDa, and it is therefore, referred to as 4.1Rα. However, its theoretical molecular weight is ~100 kDa. We have demonstrated that this discrepancy results from the unstructured structure of the HP region [23,27]. Using the PrDOS software (http://prdos.hgc.jp/), we have determined that the HP region (amino acids 1-209) adopts a highly disordered structure, which contrasts with the highly ordered structure of the 30 kDa (FERM) domain (amino acids 210-507) [28-30]. Structural differences between these two domains was confirmed by dynamic light scattering (DLS) measurements, the hydrodynamic diameters of the 4.1RHP region, and of the 4.1R FERM domain differing greatly, 7.6 and 5.6 nm, respectively, in spite of their molecular weight of 23 kDa and 30 kDa, respectively. We have recently extended these findings by establishing by small angle X-ray scattering that, as it was the case for 4.1R, the 209 amino acid HP region of 4.1G, another member of the protein 4.1 family, adopted also an intrinsically disordered structure [26,27].

In contrast, the consistency between the theoretical and the apparent molecular weights of the 4.1R FERM domain illustrated the ordered (folded) nature of this domain. Importantly, PrDOS-based analysis of full length 4.1Rα predicted that the 30 kDa domain would be the only region in the protein to adopt an ordered (folded) structure. The crystal structure of the 4.1R 30 kDa domain is reminiscent of the shape of a cloverleaf or of a propeller, with three clearly distinct lobes (PDB: 1gj3) [31]. First, the N-lobe, which corresponds to the first 78 amino acids, and which includes the band 3 binding motif LHEEDY, consists of 4 double-stranded β-strands. Second, the α-lobe, which corresponds to the following 90 amino acids, and which includes the GPC binding site, consists of 4 α-helices. Third, the COOH-terminal lobe (C-lobe), which contains the p55 binding surface, consists of seven β-strands, and ends with a α-helix (Figure 2B). Our findings contribute not only to a better understanding of the structure of membrane skeletal proteins, but also to the function of intrinsically disordered proteins.

Ca2+-dependent regulation of 4.1R binding to membrane proteins through calmodulin

Binding of calmodulin (CaM) to target proteins usually depends strongly on the extent of saturation of CaM with Ca2+ [32-35]. However, in human erythrocytes, saturation of CaM with Ca2+ (Ca2+/ CaM) destabilizes instead the mechanical stability of membranes [36-39]. Consistent with this observation, we have previously shown that the binding affinity of the 4.1R FERM domain for the 4.1Rα/GPC/p55 and the 4.1Rα/spectrin/actin ternary complexes decreases when Ca2+/ CaM binds to the 4.1R FERM domain, despite the fact that CaM binds to the 4.1R FERM domain in a Ca2+-independent manner [38,39]. These unusual characteristics of CaM binding to the 4.1R FERM domain can be attributed to the fact, that although the stoichiometry of 4.1R FERM domain binding to Ca2+/CaM is 1:1 [39], 4.1R FERM domain possesses two CaM binding sites: a Ca2+-independent CaM binding motif, A280KKLWKCVEHHTFFRL (pep11), and a Ca2+-dependent CaM binding motif, A318KKLSMYGVDLHKAKDL (pep9) [39]. Although it is known that Sec24 in pep9 is critical for its function, the structure-function relationships for pep9 are still unclear. The dissociation constant in equilibrium (Kd) of CaM binding to the 4.1R FERM domain is in the sub-micromolar range, in either the presence or absence of Ca2+ [37,39]. However, whereas the Kd of Ca2+/CaM binding to pep11 is in the sub-micromolar range, that of Ca2+/CaM binding to pep9 is in the micromolar range [39]. These findings suggest that Ca2+/ CaM may undergo conformational changes upon pep11 binding. We reason that such conformational changes in Ca2+/CaM may account for the complex and unusual characteristics of the Ca2+/CaM-dependent regulation of 4.1R FERM domain binding to its binding partners. Of particular note, the sequence of pep11 is very different from any of the sequences currently listed in the “Calmodulin Target Database” [40], suggesting that pep11 binding characteristics may be unique. We have previously demonstrated that apo-CaM stabilizes the β-sheet rich C-terminal region (100 C-terminal residues) of the 4.1R FERM domain, in either the presence or absence of Ca2+ [39,41]. Since CaM intracellular concentration (2 – 8 μM) is much higher than that of Ca2+, [Ca2+]i, being maintained at ~ 10 nM in physiological conditions, one can predict that nearly all CaM molecules are in a Ca2+-free state, i.e. in an “apo-” state [36,41].

NHE1 in Erythrocytes

Na+/H+ exchanger (NHE1) is the prototypical member of a family of electrophoretic antiporters that play an essential role in the regulation of intracellular pH and cell volume, and that mediate transepithelial Na+ and HCO3- absorption [42-45]. NHE1 is the most ubiquitously expressed member of the NHE family, and is therefore, often considered the “housekeeping” NHE. NHE1 participates in the regulation of intracellular volume and pH in all cell types, including erythrocytes [42]. It is the major isoform expressed in mammalian erythrocytes [46]. The topology of the transmembrane domain of NHE1 has been well documented [43-46]. The C-terminal domain of NHE1 has been identified as a pH sensor [47]. NHE1 is activated by a decrease in intracellular pH, NHE1 activation resulting in a 1:1 export of H+ and import of Na+. The 3D structure of the cytoplasmic domain of NHE1 has not been resolved, except for the CaM binding sites and the calcineurin B homologous protein (CHP) domain [48-51]. A recent study reported that the distal part of the carboxyl cytoplasmic domain of NHE1 is intrinsically disordered, but that it contains conserved regions of transient α-helicity that play an important role in NHE1 trafficking [49].

NHE1 is the only resident plasma membrane NHE isoform [42-45]. There is now a large body of evidence for the membrane skeleton being involved in regulating the basal rate of NHE1-mediated ion transport, and in the transmission of signals that modulate NHE1 activity, in response to physical stimuli [52-55]. Thus, protein 4.1R-null mouse erythrocytes exhibit a dramatic NHE1 phenotype, characterized by cell dehydration and high intracellular Na+, resulting from hyperactivity of NHE1 [56]. This latter observation demonstrates unequivocally a role for 4.1R in modulating NHE1 activity. Other cytoskeletal proteins participate in the regulation of NHE1 activity. For example, a recent study showed that β-adducin (Add2) null mouse erythrocytes, which exhibit an abnormal spherocytic shape, lack a-adducin and SLCA9A1/NHE1 [46].

Two clusters of basic amino acids in the C-terminal domain of NHE1, K185R and R206FKKYYVKK, have been previously shown to
mediate (phosphatidylinositol 4,5-bisphosphate (PIP2) binding) [57]. The interaction of the NHE1 cytosolic domain with phospholipids present in the plasma membrane promotes NHE1 activity [58]. Activity of NHE1 has also been shown to be enhanced upon interaction of Ca2+-saturated CaM (Ca2+/CaM) with the C-terminal domain of NHE1, and more specifically with the R95-A50 and N94-L140 domains [55,56].

**Regulation of NHE1 Activity by 4.1Rn**

**Characterization of 4.1Rn binding to NHE1 through in vitro binding assays**

Our previous finding that NHE1 is hyperactive in 4.1R-null mouse erythrocytes [56] strongly supports a functional interaction between 4.1R and NHE1. In testing this prediction, we recently showed that this phenotype results from an actual physical interaction between 4.1Rn and NHE1. IAsys-based in vitro binding assays enabled us to identify the L192-Q227 peptide as the minimal region in NHE1cd interacting with 4.1Rn. Of particular note, the binding affinity of 4.1Rn for NHE1 was very similar (Kd ~100-200 nM) to those previously reported for 4.1R binding to its two major transmembrane binding partners in erythrocytes, GPC and band 3 [5,15,39]. Having confirmed a direct interaction between 4.1Rn and NHE1, we mapped the motifs responsible for this interaction in both 4.1Rn and NHE1. The use of various 4.1Rn recombinant proteins enabled us to show that the 4.1R FERM domain, and more specifically a 35 amino acid peptide encoded by alternative exon 5 within this domain, mediated 4.1Rn interaction with NHE1. Although the 4.1R FERM domain bound to NHE1cd, with an affinity similar to that of full-length 4.1Rn, a variant 4.1R FERM domain lacking the exon 5-encoded peptide failed to interact with NHE1. Furthermore, mutation of the EED motif within the exon 5-encoded peptide, a motif previously reported to participate in 4.1Rn interaction with band 3 [12,13,59], resulted also in a significant decrease in 4.1Rn binding affinity for NHE1cd (Kd ~1400 nM), illustrating the importance of this peptide in 4.1Rn-NHE1 interaction. Notably, none of the three other domains of 4.1Rn, 16 kDa domain, 10 kDa spectrin/actin binding domain or C-terminal 24 kDa domain, bound to NHE1cd.

Alignment of the amino acid sequences of the juxta-membrane regions of rat band 3 (anion exchanger 1 (AE1)) and NHE1 cytosolic domains revealed the presence of a common cluster of positively charged residues, i.e. RRR and KKK, respectively. Site-directed mutagenesis of key lysine residues (K->A), in either the K305-KKQETKR-A310AAQETAA (M1) or the R306FNNKYYVKA-A310FNAAYVAA (M2) motif, resulted in a loss of 4.1Rn-NHE1 interaction. These data led us to conclude that both M1 and M2 sequences are necessary for NHE1 interaction with 4.1Rn. Of particular note, these two motifs are absent in the same region of the band 3 cytoplasmic domain, an observation which strongly suggests a different type of interaction between 4.1Rn and band 3, and between 4.1Rn and NHE1 IAsys binding analysis estimated the binding ratio of 4.1R FERM domain to NHE1 at approximately 1:1. Binding affinity was calculated by Scatchard plot analysis, with maximal binding being observed in the 10^-7 M range. These results indicate that a single molecule of 4.1R FERM domain interacts simultaneously with the two NHE1cd binding sites described above.

We predict that the spacing of the two basic residue clusters in NHE1cd, K305KKQETKR (M1) and R306FNNKYYVKA (M2), would enable trapping of the 4.1R EED motif, despite the presence of phenylalanine and valine hydrophobic residues. In that respect, 4.1R FERM domain-NHE1 interaction may resemble a key-keyhole structure. Crystal structure studies will enable further characterization of these interactions at the atomic level.

**Factors responsible for the dynamic association of 4.1R with NHE1**

**pH:** We have shown that 4.1R FERM domain binding to NHE1cd, as a function of pH, adopted a deep parabola shape, the maximum binding (minimum Kd ~ 150 nM) being observed at pH 6.5 [60]. The affinity of 4.1R FERM domain binding to NHE1cd was similar between pH 6.0 and 7.2. In contrast, binding affinities decreased dramatically at more basic (~ pH 8.0), or more acidic pH (~ pH 5.5), the dissociation constants (Kd) increasing more than 20 times at pH 5.5 and 8.5 [60]. In contrast, Kd for the 4.1R FERM domain binding to band 3 were not significantly different over a large range of pH (5.0-8.5), illustrating once again the different nature of the 4.1R-NHE1 and the 4.1R-band 3 interactions.

**NaCl:** We also found that the 4.1R FERM domain interaction with NHE1cd is sensitive to Na+ concentration. The binding of 4.1R FERM domain to NHE1cd at pH 7.5 was already reduced by 50% at NaCl concentrations as low as 0.2 M, the binding being totally inhibited at 0.3 M NaCl [60]. 4.1R FERM domain binding to NHE1cd is, therefore, very sensitive to variations in NaCl concentration, in particular in the physiological range of 0.1 M to 0.2 M NaCl. In contrast, the 4.1R-band3 interaction was more resistant to variations in NaCl concentration, the binding decreasing by only 50% at 0.5 M NaCl. These observations support once again that the interactions of 4.1R with NHE1 and band 3 are of very distinct nature.

**Ca2+/CaM:** We previously showed that CaM binds to the 4.1R FERM domain in the absence of Ca2+, and decreases the binding affinity of 4.1R FERM domain for its binding partners in a Ca2+-dependent manner [5,15,39]. We investigated whether Ca2+/CaM could regulate the binding of the 4.1R FERM domain to NHE1cd. The NHE1cd construct used in all our experiments, corresponding to the most N-terminal 70 amino acids of NHE1cd (L192-Q227), a peptide that included both M1 and M2 motifs (i.e. the PIP2 binding sites), but that lacked the two previously described CaM binding sites, i.e. R95-A50 and N94-L140 [55,56]. In the absence of CaM, the Kd observed for 4.1R-NHE1 interaction, at a physiological pH of 7.5, was similar to that of previously characterized partner binding. At pH 6.0, 4.1R FERM domain could still bind to NHE1cd, and to Ca2+/CaM with a Kd ~ 100 nM. Ca2+/CaM binding to 4.1R FERM domain reduced the binding affinity of 4.1R FERM domain for NHE1cd, the inhibitory effect of Ca2+/CaM being more pronounced at pH 7.5 than at pH 6.0. These results indicated that the 4.1R FERM domain-NHE1 interaction is regulated by CaM in a Ca2+-dependent manner, and that this regulation is more pronounced at physiological pH values. At Ca2+ concentrations greater than 0.01 µM (pCa=8), the extent of 4.1R binding to NHE1 started to decline, the maximal inhibition being reached at Ca2+ concentrations of 100 µM and higher (pCa=4). Half-maximal inhibition was observed at a Ca2+ concentration of ~ 0.1 µM (pCa=7) [60].

We also compared PIP2 binding to NHE1cd at different pH values, and in the presence or absence of Ca2+. Consistent with a previous report [57], PIP2/PC liposomes bound more strongly to NHE1cd at pH 6.0, than at pH 6.8, or at pH 7.5. Furthermore, we found that the PIP2/NHE1cd interaction was not significantly affected by Ca2+.

We have shown that 4.1R binds directly to NHE1 in vitro, and we have identified the motifs in 4.1R and NHE1 mediating this interaction (Figure 3). We also documented that the 4.1Rn-NHE1 interaction...
Proposed model for regulation of NHE1 activity by 4.1R

is modulated by changes in pH, Na⁺ concentration, and Ca²⁺/CaM complex formation. Our in vitro data clearly demonstrate that at acidic pH, 4.1R dissociates from NHE1cd, whereas binding of PIP₂ to NHE1cd is rather increased. This distinct behavior may be heightened by variations in intracellular CaM and Ca²⁺ concentrations, as the regulatory effect of Ca²⁺-CaM on 4.1R⁻⁻⁻⁻NHE1 interaction contrasts dramatically with its inability to regulate the PIP₂-NHE1 interaction. We hypothesize that the antagonistic effects of 4.1R⁻⁻⁻⁻ and PIP₂ on NHE1 activity [50,57], play an important role in the regulation of NHE1 activity, and that in absence of 4.1R⁻⁻⁻⁻, sustained binding of PIP₂ to NHE1cd results in an increase in NHE1 activity. This phenotype may be heightened in erythrocytes, because their PIP₂ content is higher than in other cell types [60].

Protein 4.1 Family of Cytoskeletal Proteins

Three protein 4.1 genes sharing high homology with 4.1R, have been characterized. They have been named 4.1G, 4.1N [61], 4.1N [62] and 4.1B [63], based on the predominant tissue expression pattern of their mRNAs. The primary structure of 4.1G, 4.1N and 4.1B is similar to that of 4.1R⁻⁻⁻⁻, in the sense that these three other 4.1 proteins contain an NH₂-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 30 kDa membrane binding domain, followed by a second unique region (U2 region, which corresponds to the 16 kDa domain in 4.1R), the conserved SAB domain (except for 4.1 N), a third unique region (U3 region), and finally a highly conserved COOH-terminal domain [64].

4.1 proteins and regulation of ion transporter activity

The 4.1 proteins are cytoskeletal adaptor proteins that are responsible for the control of the mechanical stability of cellular membranes, and for the proper anchorage of diverse transmembrane proteins at the cell surface. 4.1R has recently been shown to be required for the normal function of several ion transporters in heart [65-69].

Although 4.1R knockout mice had a generally normal morphology, their hearts exhibited bradycardia and prolongation of both action potentials and QT intervals. Through electrophysiological studies, Baines et al. [65] have identified anomalies in various ion channel activities in 4.1R null mice. Furthermore, the immunoreactivity of voltage-gated Na⁺ channel NaV1.5 was reduced, indicating a role for 4.1R in proper cellular localization (i.e. membrane anchorage) of this ion channel [66].

A striking parallel exists between the effects of 4.1R on NaV1.5, and its effects on another ion channel that interacts with 4.1 in epithelia, the store-operated channel TRPC4 [65]. Although the 4.1R binding sites for voltage-gated Na⁺ channels have not been mapped yet, the phenomena observed in 4.1R knock-out mice suggest that 4.1R regulates Na⁺/Ca²⁺-exchanger activity in the heart [67]. Deletion of part of the C-terminal cytoplasmic domain of TRPC4, that contains a sequence reminiscent of a 4.1-binding motif, leads to inactivation of this channel [68]. This supports an important role for 4.1R in the proper cellular localization and regulation of function of these ion channels [65].

Protein 4.1R is not the only 4.1 protein expressed in heart [70]. Protein 4.1G and protein 4.1N were also detected in this tissue by immunochemistry. Only protein 4.1B was not. In contrast with 4.1R that was expressed as several spliceforms, only one splice variant of 4.1N and of 4.1G could be identified. 4.1R⁻⁻⁻⁻ was present in subcellular fractions enriched in intercalated discs, as part of a complex resistant to solubilization under non-denaturing conditions. At the intercalated discs, 4.1R did not co-localize with the adherens junction protein, β-catenin, but overlapped with two other plasma membrane proteins, the Na⁺/K⁺-ATPase and the Na⁺/Ca²⁺ exchanger NCX1. Like 4.1R, 4.1N was present in intercalated discs, but it was not detected in lateral plasma membranes. Both 4.1R and 4.1N were expressed in internal structures that at the level of resolution of light microscopy were in proximity of the Z-discs (possibly T-tubules). 4.1G was also expressed in intracellular structures, some of which were coincident with the sarcoplasmic reticulum. 4.1G could be immunoprecipitated as a complex with spectrin and SERCA2, an intracellular pump located in the sarcoplasmic or endoplasmic reticula, and that possesses Ca²⁺-ATPase activity [66]. This thorough analysis indicates that various 4.1 gene products and spliceforms can co-exist in a tissue, such as the heart, where they are differentially compartmentalized, and where they form specific complexes with proteins involved in various aspects of cardiomyoocyte Ca²⁺ transport.

Based on their findings, Baines et al. [65] have suggested that the spectrum of transmembrane proteins that interact with 4.1 proteins overlaps, with that of transmembrane proteins that interact with ankyrin. Whether 4.1R acts sterically or, for example, by inducing conformational changes in the cytoplasmic domain of these channels, remains to be investigated. In support of the latter, it has been reported that in erythrocytes, 4.1R is required for normal conformational presentation of epitopes of band 3 and Kell [17].

Na⁺ channels are not the only class of channel that binds 4.1 proteins. Thus, 4.1R interacts with the Rhesus complement protein Rh, that Besides its well known antigenic attributes, acts as a CO2/Na⁺ channel [70,71]. Functional regulation of the Ca²⁺-activated K⁺ channel (Gardos channel) KCNN4, through interaction with 4.1R has been reported. Indeed, in 4.1R-null erythrocytes, the sensitivity of KCNN4 to Ca²⁺ is increased [72]. In neurons, the ionotropic glutamate receptors, GluR1 and GluR4, which function as ligand-gated cation channels, interact with 4.1N or 4.1G, 4.1N or 4.1G being required for proper cell surface expression of GluR1 and GluR4 [73,74]. Similar structural and functional complexes have been identified between acetylcholine receptors and 4.1N [75,76], or adenosine receptors, and 4.1G [77].
presence of a ternary complex between 4.1B, p55 and the bicarbonate co-transporter 1 (NBC1) in nephron proximal tubules has been reported [78]. The existence of complexes organized around 4.1B in the proximal tubule region of the nephron is consistent with our report of compartmentalized expression of 4.1B, 4.1R, and 4.1N along the nephron [79]. We are currently investigating the potential involvement of 4.1R and 4.1N in regulation of NHE1 cellular localization and activity in more distal regions of the tubule, i.e. thick ascending limb and distal convoluted tubule. The neuron-specific K-Cl cotransporter KCC2 has been shown to interact with 4.1N [80]. Interestingly, overexpression of KCC2 resulted in abnormal cellular localization of 4.1N [81]. This suggested that perturbations of any components of these multiple protein complexes (protein 4.1, transporter, or other associated membrane protein), have ripple effects on all components.

Lastly, protein 4.1R and perhaps, other members of the protein family, have been reported to interact with a volume sensitive chloride channel pICln [82,83]. However, this chloride transport function mediated by pICln has been more recently challenged, and there is an increase consensus for pICln, rather acting as a regulator of spliceosome function [84]. An involvement of 4.1R in organization and regulation of spliceosomes is consistent with reports of 4.1R expression in discreet regions of the nucleus [85,86].

Conclusion

The primary function of the protein 4.1-mediated interactions described in this review is to promote and maintain the cell surface expression of selected ion transporters/channels, the absence of any of these 4.1 proteins resulting in a decrease or even a complete absence of expression at the cell surface. These observations are consistent with the concept first documented in erythrocytes that 4.1 proteins act as key scaffolding proteins, whose interactions are finely tuned by their microenvironment. We anticipate that future studies will document further the complex organization and the exquisite regulation of protein 4.1-based protein complexes.

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