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Review

The Protein 4.1 family: Hub proteins in animals for organizing membrane proteins ☆

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ABSTRACT

Proteins of the 4.1 family are characteristic of eumetazoan organisms. Invertebrates contain single 4.1 genes and the *Drosophila* model suggests that 4.1 is essential for animal life. Vertebrates have four paralogues, known as 4.1R, 4.1N, 4.1G and 4.1B, which are additionally duplicated in the ray-finned fish. Protein 4.1R was the first to be discovered: it is a major mammalian erythrocyte cytoskeletal protein, essential to the mechanochemical properties of red cell membranes because it promotes the interaction between spectrin and actin in the membrane cytoskeleton. 4.1R also binds certain phospholipids and is required for the stable cell surface accumulation of a number of erythrocyte transmembrane proteins that span multiple functional classes; these include cell adhesion molecules, transporters and a chemokine receptor. The vertebrate 4.1 proteins are expressed in most tissues, and they are required for the correct cell surface accumulation of a very wide variety of membrane proteins including G-Protein coupled receptors, voltage-gated and ligand-gated channels, as well as the classes identified in erythrocytes. Indeed, such large numbers of protein interactions have been mapped for mammalian 4.1 proteins, most especially 4.1R, that it appears that they can act as hubs for membrane protein organization. The range of critical interactions of 4.1 proteins is reflected in disease relationships that include hereditary anaemias, tumour suppression, control of heartbeat and nervous system function. The 4.1 proteins are defined by their domain structure: apart from the spectrin/actin-binding domain they have FERM and FERM-adjacent domains and a unique C-terminal domain. Both the FERM and C-terminal domains can bind transmembrane proteins, thus they have the potential to be cross-linkers for membrane proteins. The activity of the FERM domain is subject to multiple modes of regulation via binding of regulatory ligands, phosphorylation of the FERM associated domain and differential mRNA splicing. Finally, the spectrum of interactions of the 4.1 proteins overlaps with that of another membrane-cytoskeleton linker, ankyrin. Both ankyrin and 4.1 link to the actin cytoskeleton via spectrin, and we hypothesize that differential regulation of 4.1 proteins and ankyrins allows highly selective control of cell surface protein accumulation and, hence, function. This article is part of a Special Issue entitled: Reciprocal influences between cell cytoskeleton and membrane channels, receptors and transporters. Guest Editor: Jean Claude Hervé

Contents

Abbreviations: FA, FERM adjacent domain; SAB, spectrin–actin binding domain; CTD, C-terminal domain

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1. Introduction

This paper focuses on a group of proteins descended from ancestors that appear to have emerged early in animal evolution as part of a mechanism to allow tissues to form and to function in the context of whole organism physiology.

During the evolution of animals from simpler ancestors (those were probably colonial protists) new adaptations emerged that resulted in differentiated cells forming selective cell–cell interactions, leading to formation of tissues. Further adaptations allowed formation of signalling complexes positioned at points on cell surfaces to receive or send signals. Consequently, animal cell plasma membranes, far from being simply populated by transmembrane proteins floating like icebergs in a sea of phospholipids, as envisaged in simple cartoon representations of the fluid mosaic model [e.g. 1], have a very high degree of molecule-scale organization that allows protein–protein complexes to form and turn over.

With the evolution of mammals, the specialisation of cell types has been taken to an extreme with, for example, the advent of the

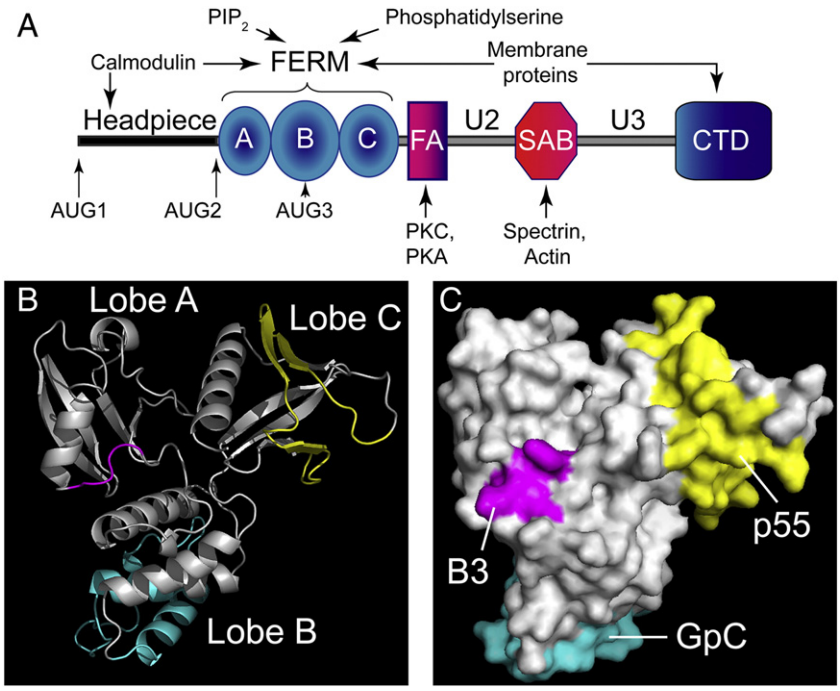

Fig. 1. Domain structure of 4.1 proteins. (A) Overall domain structure of a canonical vertebrate 4.1, showing major regions of interaction and regulation. Key: FA, FERM adjacent domain; SAB, spectrin–actin binding domain; CTD, C-terminal domain. (B) Cartoon representation of the fold of the FERM domain taken from PDB:1GG3. The three lobes are indicated (A, B, C). (C) Space filling model of the FERM domain showing binding sites for transmembrane proteins Band 3 (B3), glycophorin C (GpC) and p55. The colours in (C) and (B) represent the same sequences.

non-nucleated mammalian red blood cell. These and other adaptations of animal life seem to require a number of proteins that emerged during eumetazoan evolution.

One such protein is known as 4.1. It is a membrane-cytoskeleton cross-linker and adaptor that can bridge between cytoplasmic spectrin–actin filament complexes, and a wide variety of transmembrane proteins (Fig. 1A); further components of such complexes include other adaptor proteins such as membrane-associated guanylate kinases.

We explore here the nature of the 4.1 proteins, their interactions and regulation, and how their functions are reflected in the physiology of animals.

2. The protein 4.1 family: genes, proteins and domains

2.1. Discovery and general organization

Protein 4.1 was discovered in human red blood cells. Its name derives from its identification as a particular band on an SDS gel of erythrocyte membranes ("band 4.1 protein") [2]. Subsequently, gene cloning and genomics identified a total of four paralogues in vertebrates (with additional duplicates in ray-finned fish) [3]; other animals have single copies of 4.1 genes [4,5].

To distinguish the different paralogues, the proteins are now named 4.1R (the prototypical protein found first in red blood cells), 4.1N, 4.1G and 4.1B. Although the latter three were initially identified in neurons, generally, and brain, all four are relatively ubiquitously expressed. The corresponding genes are, respectively, EPB41, EPB41L1, EPB41L2 and EPB41L3 (see Table 1).

All 4.1 proteins, from the simplest invertebrates to mammals, have two common functional domains: a FERM domain and a C-terminal domain (CTD) that is unique to this group of proteins and define it (Fig. 1A, and Table 2). Additional exons characterise particular groups of animals in both invertebrates and vertebrates, with a particular gain of function in higher animals through the acquisition of a spectrin–actin binding domain (SAB) [5].

In mammals, the FERM domain is preceded on the N-terminal side by a variably spliced headpiece (also known as the U1 region) [6]. Splicing of the headpiece generates the two most abundant isoforms of 4.1R (apparent molecular mass on SDS gels 120–135 kDa and 80 kDa). However, the apparent molecular masses of 4.1R isoforms on SDS gels differ from the true masses derived from the sequence, and the true masses vary further because of differential splicing of small exons. Based on the amino acid sequence, 80 kDa forms are in the region of 66 kDa (e.g. Uniprot:P11171-4), and the 120/135 kDa isoforms are approximately 97 kDa (e.g. Uniprot:P11171-1).

Between the FERM domain and the spectrin–actin binding (SAB) domain lies a further variably spliced domain mostly of unknown function, and known as U2 [7,8]. Within this is a FERM-adjacent domain (FA) which contains regulatory phosphorylation sites [9]. A third variably spliced region (U3) lies between the SAB and C-terminal domains [7,8].

Of all these domains, only the FERM structure is known at atomic resolution [10]. The C-terminal domain is probably folded [11], but as yet no successful structural analysis has been reported. Structures of the other domains are unknown, although the headpiece [12] and SAB domains [9,13] are probably largely intrinsically unstructured.

2.2. The FERM domain

2.2.1. Structure

The FERM domain was discovered by limited proteolysis of erythrocyte 4.1R [14]. A 30 kDa proteolytic fragment was found to bind erythrocyte membranes, via both protein and phospholipid sites [14–16]. Subsequent sequence comparison revealed similarity with a similar sized domain in the ERM proteins (ezrin, radixin and moesin), hence the name FERM (four-point-one, ERM) [17]. It is now clear that the FERM domain is widely spread in eukaryotic evolution, and appears to be a genetically mobile module that has been incorporated in a wide variety of genes [see e.g. 18]. It is very highly conserved among the 4.1 proteins: about 75% amino acid sequence identity between all paralogues in any mammalian species; the FERM domains of human 4.1R and fruit fly 4.1 (coracle) are about 65% identical.

The atomic structure of the human 4.1R and mouse 4.1B FERM domains have been determined by x-ray crystallography (Fig. 1B and C) [10,19]. They show a common three-lobed structure which is, in effect, a supra-domain [20]; in other words the folded structure is itself made up of three separately folded lobes. The N-terminal lobe (lobe A) has a fold analogous to ubiquitin. The central lobe (lobe B) has an α-helical fold like acyl-CoA binding protein. The C-terminal lobe (lobe C) has a fold like a pleckstrin homology domain [10]. This overall folding structure is conserved throughout known FERM domains, even though the level of sequence conservation is extremely low [18].

The lobes of the FERM domain are associated with ligand binding activities. In the case of 4.1R, Lobe A binds the erythrocyte anion exchanger and the rhesus complex proteins Rh; lobe B binds the

Table 1
Genes encoding 4.1 proteins and their corresponding phenotypes.

Vertebrates					
Protein	Gene	Organism	Uniprot	Physiological phenotype	Reference
4.1R	EPB41	Human	P11171	Hereditary elliptocytosis	[71,171,172]
		Mouse	P48193	Hereditary spherocytosis; fragile/misshapen cells. Heart: bradycardia, long QT, prolonged action potential. Learning and memory defects. Defective wound healing. Disorganisation of gastric glands. Defective Ca^{2+} absorption. Elevated humoral response to immunization.	[37,77,78,80,82,84,102]
4.1N	EPB41L1	Human	Q9H4G0	Nonsyndromic intellectual disability	[173]
		Mouse	Q9Z2H5		
4.1G	EPB41L2	Human	O43491		
		Mouse	O70318	Male infertility	[103]
4.1B	EPB41L3	Human	Q9Y2J2	Suppression of lung, meningioma, beta cell, breast, ovarian, prostate cancer progression/metastasis.	[86,87,89,95,174,175]
		Mouse	Q9WV92	Impaired gait and motility. Stabilisation of paranodes and juxtaparanodes; maintenance of axonal domains	[105,106]
Invertebrates					
Coracle	Cora	Fruitfly	Q9V8R9	Recessive embryonic lethality resulting from failure in dorsal closure. Required in embryonic morphogenesis; adult somatic muscle development; maintenance of imaginal disc-derived wing hair orientation; regulation of tube size, open tracheal system; establishment of glial blood–brain barrier.	[4,176,177]
FRM-1	frm-1	*C. elegans*	G5EEP9	Delayed hatching/temperature sensitive sterility (tm4168)	[178]
4.1		Leech		Impaired axon regeneration.	[179]

Table 2
Domain and structure database cross references.

Domain	PFAM	Interpro	PDB
FERM	PF09379 (Lobe A) PF00373 (Lobe B) PF09380 (Lobe C)	IPR000299 (Whole domain) IPR018979 (Lobe A) IPR019748 (Lobe B) IPR018980 (Lobe C)	4.1R: 1GG3, 2RQ1 3QIJ 4.1B: 2HE7, 3BIN (structure with cytoplasmic domain of TSLC1 bound)
FA	PF08736	IPR014847	
SAB	PF04382	IPR007477	
CTD	PF05902	IPR008379	

transporter XK, the chemokine receptor Duffy and glycophorin C; lobe C also binds the cell adhesion molecule CD44 and phospholipid phosphatidylserine [21–24]. The malaria parasite *Plasmodium falciparum* secretes a protein named Mesa which binds lobe C during the intra-erythrocytic phase of its life cycle [25]. Lobes A and C together form a binding site for the cytoskeletal adapter protein p55 [26] and the lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) [27].

2.2.2. Regulation

The FERM domain represents a point where numerous forms of regulation converge on the interaction of 4.1 with membrane-bound ligands. Regulation has been extensively characterised for 4.1R; much less is known about the regulation of the other three 4.1 proteins.

Ser-312 and Ser-331 in the 4.1R FA domain are substrates for protein kinase C; their phosphorylation weakens the interaction of lobe B with glycophorin C, Duffy and XK [28], and this is associated with weakening membrane mechanical stability [29].

PIP_2 promotes binding of glycophorin C, but inhibits binding of the erythrocyte anion exchanger [27]. As with the ERM proteins, PIP_2 causes a conformational change in the FERM domain, although the atomic structure of a 4.1 FERM domain with bound PIP_2 has not been determined thus far.

The presence of the 4.1 headpiece weakens the interaction with glycophorin C [12]. The headpiece also contains a Ca^{2+}/calmodulin binding site [12,30,31]. Binding of Ca^{2+}/calmodulin to the headpiece completely inhibits the binding of 4.1R to glycophorin C, and greatly inhibits binding to the erythrocyte anion exchanger [31].

Ca^{2+}/calmodulin is also known to control erythrocyte mechanical properties: calmodulin in the presence of physiological Ca^{2+} concentrations (but not in its absence) reduces membrane mechanical stability [32]. 4.1R seems to be one of the effector proteins for Ca^{2+}/calmodulin function in this regard, even though the major (80 kDa) isoforms of 4.1R in the erythrocyte lack the headpiece. However, Ca^{2+}/calmodulin and apo-calmodulin also bind to the FERM domain. There are two separate binding sites: a Ca^{2+} -dependent one (PDB:1GG3, residues 181–197) linking the end of lobe B to the first β-strand of lobe C and a Ca^{2+}-independent one (PDB:1GG3, residues 264–280) located in the C-terminal α-helix of lobe C [33]. The Ca^{2+} bound form inhibits interaction with the anion exchanger [23,33].

2.3. The C-terminal domain

Like the FERM domain, the C-terminal domain was first discovered by its resistance to limited proteolysis of erythrocyte 4.1R. The apparent molecular mass of the fragments is 22/24 kDa, although as with whole 4.1R the true mass differs from that reported by SDS gels: mass spectrometry confirms prediction from sequence that the molecular mass is 17 kDa. [11,14]. No high resolution structure has been determined yet, but protease resistance, as well as its globular properties indicate it is folded. It is highly conserved in evolution, and, because it is unique to 4.1 proteins, effectively defines them [11].

The CTD binds to a number of membrane proteins. For example, the cytoplasmic C-terminal regions of glutamate receptors GluR1 and GluR3 interact with 4.1 CTD [34,35]. A similar interaction is reported in fruit flies where the CTD of coracle interacts with GluRIIA, -B and -D, which are homologous with mammalian ionotropic glutamate receptors [36]. The plasma membrane Ca^{2+} pump, PMCA1b, interacts with the CTD via its cytoplasmic C-terminal region [37].

The CTD also mediates interaction with certain cytoplasmic proteins, including NuMA, a protein associated with mitotic spindles [38] and with FKBP13 [39,40].

2.4. Developmental regulation of 4.1 by mRNA splicing

22 exons code for the sequence of 4.1R. Of these approximately half can be subject to differential mRNA splicing [e.g. 7,41,42]. A similar scale of splice variation is noted with the other 4.1 paralogues [8]. The main consequences are as follows.

Since the headpiece has such a significant effect on FERM domain interaction, and confers a level of Ca^{2+}/calmodulin regulation on FERM activities, it is important to note that splicing of this region is developmentally regulated. The headpiece is encoded by exons 2 and the beginning of exon 4; exon 2 is variably spliced. During erythropoiesis, early-stage isoforms of 4.1R include exon 2 and therefore have an extended headpiece [43,6]. During terminal differentiation the headpiece is lost [44].

The functional significance of the headpiece has not yet been evaluated fully in the other 4.1 proteins. For example, in 4.1G the headpiece is encoded by two exons, one of which contains a calmodulin binding sequence [8,12,45].

Further functional variation comes from differential splicing of the SAB. This is comprised of exons 16 and 17 in vertebrate 4.1 proteins, each of which can be differentially spliced [44,46]. Exon 17 alone can bind spectrin and actin, but high affinity binding requires both exons [47,48].

Moreover, during erythropoiesis a wide range of splice variants of 4.1R are produced. Direct analysis by RT-PCR of full-length splice variants revealed no less than seven major and 11 minor splice variants [43]. Exon 16 is tightly regulated such that it is excluded in early erythroid progenitor cells but efficiently included in late erythroblasts [49,50]. The range of variation includes splicing in both FERM and CTD regions, indicating that, in addition to regulation by factors such as phosphorylation, calmodulin and PIP_2, post-transcriptional mechanisms contribute to regulation of 4.1R-membrane protein interaction. Exon 5 in the FERM domain encodes both the AE1 binding site and a site for binding of protein p55: this too can be differentially spliced [26,41,43]. Additional, tissue-specific splicing generates variation in the U3 region in epithelia where exon 17A is muscle specific and exon 17B is differentially spliced during epithelial development [51].

3. Structural organization at the membrane

3.1. 4.1 function independent of high affinity spectrin–actin binding

4.1 proteins are often considered as spectrin and actin binding proteins, because of the importance of this activity to erythrocyte membrane properties [47,48]. However, it is important to note that the fruitfly 4.1 protein coracle does not have a spectrin–actin binding domain [4], and this applies to other simple invertebrates [52]. Therefore the activities of 4.1 proteins that evolved earliest are independent of spectrin–actin binding.

The SAB of vertebrate 4.1 proteins can be differentially spliced [6,7]. Indeed, analysis of the expression pattern of the SAB of 4.1R indicates that the full SAB (comprised of exons 16 and 17 together) is relatively selectively expressed. In erythropoiesis, for instance, expression of exon 16 of 4.1R only occurs late in the process [53,54]. Thus, high affinity spectrin–actin binding is probably only required at the point when the erythrocyte membrane cytoskeleton complex assembles.

Even in tissues where it might be expected that the mechanical stabilisation of membranes by SAB activity would be essential, exons 16 and 17 are not constitutively expressed. For example, comprehensive analysis of cDNA sequences from human and mouse heart revealed that exon 16 could be differentially spliced from 4.1R [55]; evidently, high affinity spectrin–actin binding was not a feature of all 4.1R splice variants.

A further consideration is that the SAB of 4.1N does not appear to encode a high affinity spectrin–actin binding activity. Gimm and co-workers [13] analysed the activities of the SAB domains of all four human 4.1 proteins, and found that 4.1R, 4.1G and 4.1B could all promote cross-linking of spectrin tetramers and actin into a gel. 4.1N was the exception and showed no such activity. It would appear that the SAB of 4.1N has lost this activity in evolution. Alternative residual or newly evolved activities of 4.1N SAB are not known.

All these considerations indicate that the 4.1 proteins of all animals probably have functions that are dictated by the membrane-interactive domains (i.e. the FERM domain and CTD). Even if the erythrocyte paradigm indicates that spectrin–actin binding is critical in certain cell or developmental situations, the common activity of 4.1 proteins is their interaction with multiple membrane proteins and their assembly into macromolecular complexes.

3.2. The 4.1-spectrin–F-actin interaction

What we know about the 4.1-spectrin–F-actin interaction at the plasma membrane mostly comes from studies on the erythrocyte. In the erythrocyte model, 4.1R is linked to a short filament of F-actin that contains 12–17 monomers and has a length approximately equivalent to a single tropomyosin molecule [56,57] (Fig. 2A). The minus end of the F-actin is capped by tropomodulin [58], and the plus end by adducin–spectrin [59,60] or 4.1R-spectrin complexes [61].

The binding site for the 4.1 SAB on spectrin is in the N-terminal CH1 and CH2 domains of the actin-binding site of β-spectrin [62]. CH1 and CH2 also bind PIP_2, and this regulates spectrin-4.1 interaction [62]. The SAB also appears to bind tropomyosin [63].

Stoichiometries of actin and 4.1R in red cell membranes suggest that there should be 5–7 4.1R molecules per actin filament, and the same number of spectrin molecules [56]. Spectrin itself can bind to lipids [62,64–67] so that the whole complex is closely associated with the membrane. Thus, 4.1R has the potential to gather together multiple transmembrane proteins at F-actin–spectrin cytoskeleton junction points (Fig. 2B). In the erythrocyte these junctions occur all over the cell surface connected by the spectrin tetramer lattice [57,68].

4. Functional interaction of 4.1R with membrane proteins

4.1. Physiological and disease conditions

Table 1 shows the phenotypes that have been associated with different 4.1 genes in mouse, man and invertebrates. The main conclusions are discussed in the following sections.

4.1.1. Anaemias

Early indications of the physiological requirement for 4.1R function came from the analyses of the human hereditary anaemia elliptocytosis (HE) [69,70]. Several different mutations in the human EPB41 gene have been described, although homozygous cases, which show the most extreme phenotype, are extremely rare. About 5% of cases of HE result from quantitative deficiency of 4.1R, or defects in its activity [71,72]. Activity defects are best described in relation to mutations in the SAB domain, which lead to decreased mechanical stability of the cell membrane. This weakness can be reversed by addition of recombinant SAB domain to isolated red cell membranes (ghosts), indicating that the membrane interactions of 4.1R are not essential for the mechanical properties of the cell [48].

However, some elliptocytoses reveal an additional role for 4.1R. In quantitative deficiency of 4.1R, various membrane proteins are lost or significantly reduced. Glycophorin C, CD44, CD47 are among these [73–75]. A peripheral protein, membrane associated guanyate kinase (MAGUK), p55 (MPP1) is also lost [76]. Thus, a significant role for 4.1R emerges in relation to correct accumulation of various membrane proteins.

4.1.2. The broad phenotype of 4.1R knockout mice

A strain of knockout mice has been made and is well characterised [77,78]. This has the first two initiation codons (AUG1 and AUG2) deleted. Since these two initiation codons are required for expression of the two forms of 4.1R (120/135 kDa and 80 kDa) that are expressed at various stages of erythropoiesis, the entire red blood cell lineage of these animals lacks 4.1R. Nevertheless, they can still produce a third isoform of 4.1R from AUG3 in certain tissues: this isoform is initiated in exon 8, and yields truncated forms (50–65 kDa) that lack lobe A and most of lobe B of the FERM domain [43,79], see also [80].

As with human EPB41 mutations, the mice are very anaemic [77,78]. Depending on strain background, this can reach approximately 30% reticulocytosis. Nevertheless, the mice are viable, do not have a substantially reduced lifespan, and can be bred as homozygotes.

The erythrocytes are mechanically weak and shed membrane, giving a comparatively spherocytic phenotype [77]. As with human red cells, some membrane proteins are lost or reduced in the membranes, indicating a conserved role for 4.1R in this respect. Additionally, the conformation of a portion of the anion exchanger (band 3) is altered [24]. The activity of the Na^+/H^+ exchanger is misregulated [81], because there is a direct functional binding between 4.1R and the Na^+/H^+ exchanger, NHE1 [82].

Beyond red cells numerous phenotypic alterations have been observed in other tissues.

In the heart, there is a bradycardia, and the QT interval and action potential duration are prolonged [80]. Ca^{2+} transients are larger and slower to decay and there is increased sarcoplasmic reticulum Ca^{2+} content and frequency of Ca^{2+} sparks in 4.1R KO mice. In addition, several ion transporters have altered activity: The Na^+/Ca^{2+} exchange current density is reduced, the transient inward current (I_{to}) inactivation is faster and the persistent Na^+ current (I_{Na}) density is increased. These hearts also show reduced expression of the voltage-gated Na^+ channel NaV1.5 and increased expression of protein 4.1G. A relationship between 4.1R and elements of Na^+ linked signalling was also suggested by the quantitative colocalisation of 4.1R and the Na^+/K^+-ATPase and the Na^+/Ca^{2+} exchanger NCX1 [83].

A further phenotypic manifestation recently reported is that 4.1R −/− mice develop hyperparathyroidism and osteoporosis due to impaired intestinal Ca2 + absorption [37]. This reflects association with the intestinal Ca^{2+} pump PMCA1b. In the absence of 4.1R, PMCA1b protein expression in small intestine was reduced to about one quarter of the wild type level, although mRNA expression was unchanged. Two cytoplasmic regions of PMCA1b were found to interact with the FERM domain of 4.1R: lobe A bound cytoplasmic loop 2 of PMCA1b; lobe C bound to the C-terminal cytoplasmic region. Presumably 4.1R interaction is required to retain PMCA1b at the enterocyte basolateral membrane.

4.1R KO mice also display enhanced humoral response to antigens such as nitrophenyl keyhole limpet hemocyanin (NP-KLH) [84]: production of both IgM and IgG in response to immunization with NP-KLH was increased. As we note below in Section 6, this derives from altered T-cell signalling.

The mice also tend to have skin blisters that derive from poor wound healing [85]. The migration of keratinocytes into wounds is reduced, and this is associated with defective integrin transport to the cell surface.

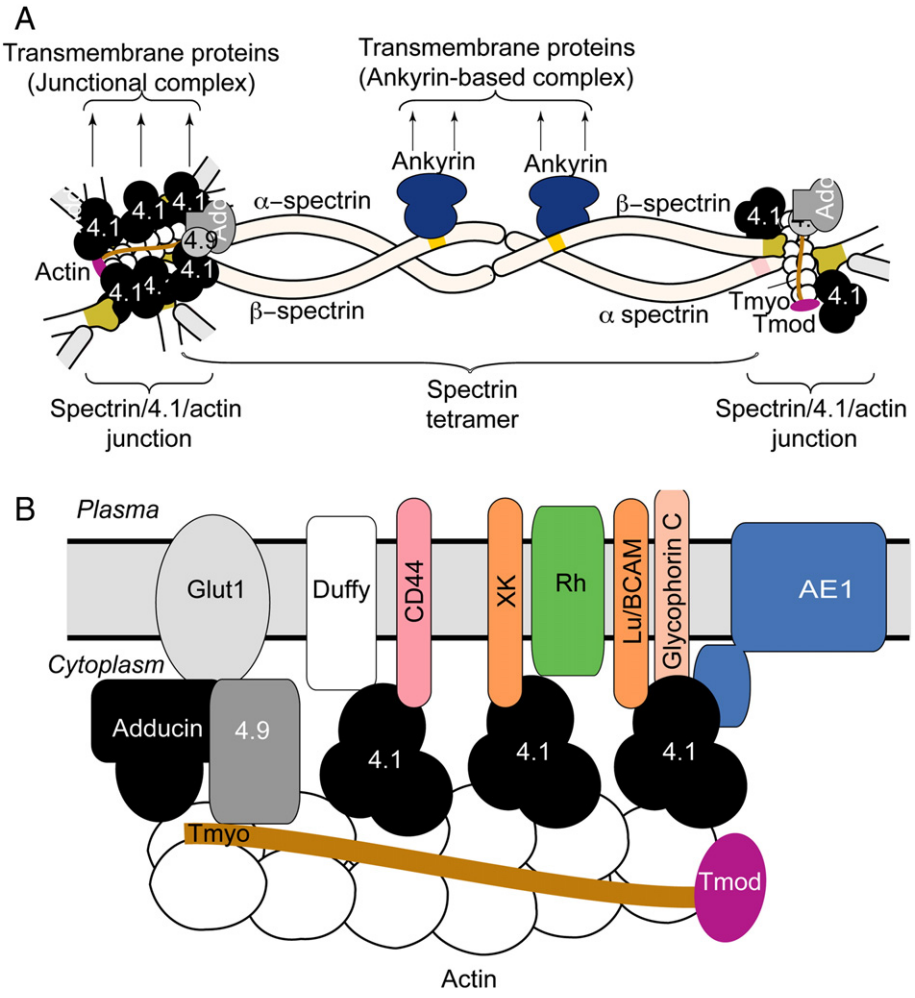

Fig. 2. 4.1 complexes in erythrocytes. (A) Organization of protein 4.1, with respect to the membrane cytoskeleton. 4.1, spectrin and actin bind together to form a junctional complex that enables 4.1 to interact with transmembrane proteins. Another transmembrane complex is associated with ankyrin. (B) Several 4.1R molecules bind to the short stretch of actin at the junctional complex allowing a number of 4.1 binding partners to be associated with it. The transmembrane proteins shown are those found in the erythrocyte (see text). For simplicity, spectrin is not shown in this diagram, but 5–7 spectrin molecules also associate with each junction point, binding to actin, 4.1 and dematin (protein 4.9). Note also that the stoichiometries of individual transmembrane proteins vary, so not all junction points will contain the full repertoire of membrane proteins shown here.

Defects in movement, coordination, balance and learning were also noted in early analyses of the 4.1R KO mice [78], although molecular correlates of this are not well established yet.

4.1.3. The cancer connection

Evidence for a connection between 4.1 proteins and cancer emerged with discovery of the DAL-1 gene (differentially expressed in adenocarcinoma of the lung) [86]. Sequencing of DAL-1 revealed that it was EPB41L3, i.e. it encoded 4.1B. Loss of 4.1B (DAL-1) was linked to metastasis of non-small cell lung carcinoma cells [87]. It was found to have a direct interaction with another tumour suppressor, Tumor Suppressor in Lung Cancer 1 (TSLC1, also known as CADM1, Igsf4, Necl2, Ra175, Syncam, SynCam1) [88], an immunoglobulin-superfamily cell adhesion molecule. The cytoplasmic domain of TSLC1 binds to the FERM domain (lobe C) [19]. CADM1 is also linked with aberrant expression of 4.1B found in invasive lesions in breast cancer, [89]. Another cell adhesion molecule CADM4 (also known as Igsf4c, Necl4, Tsll2) is lost together with 4.1B/DAL-1 inactivation in renal clear cell carcinoma (RCCC), the most frequent subpopulation of renal cell carcinoma derived from the proximal tubules [90]. The basis of loss of 4.1B expression in both cases appeared to be hypermethylation of the promotor, again consistent with its role as a tumour suppressor [91,90].

Loss of 4.1B (DAL-1) appeared to be an early event in pathogenesis of meningiomas, and it had the characteristics of a tumour suppressor [92–94]. It is important in prostate cancer progression and metastasis [95].

4.1B knockout mice also reveal a role for this protein in regulation of cell proliferation [96]. During pregnancy, 4.1B is greatly upregulated in mammary epithelium, a period when epithelial cells undergo rapid proliferation. Loss of 4.1 B in the knockout mice was associated with increased proliferation; the presence of 4.1 B was associated with cell cycle arrest at G1. These data point to a further functional link between loss of 4.1B function and the pathogenesis of mammary carcinomas.

Other 4.1 proteins also appear to be tumour suppressors. 4.1R is another meningioma suppressor [97]. It is worth noting here that CD44, a hyaluronan receptor, which requires 4.1R for correct cell surface accumulation in red cells (Section 5.1) is also broadly expressed, and linked to cancer progression [98]. Protein 4.1N is also reported to be involved in the invasiveness of breast cancers [99].

There is a broader connection here to other tumour suppressors. NF2 and merlin, two well-characterised tumour suppressors, have, like 4.1 proteins, a FERM domain [100]. Loss of interaction of FERM domains with important cell adhesion molecules appears to be a common mechanism in tumour pathogenesis.

4.1.4. Relation to tissue organization: cell–cell connections

Since the interactions of cell adhesion molecules are revealed in analysis of cancers, is there a broader role for 4.1 proteins in normal

functioning of cell adhesion molecules, i.e., in tissue biogenesis and cell–cell and cell–matrix interaction? Immunofluorescence in many cell/tissue types shows that all of the four vertebrate 4.1 proteins are associated with membrane structures, most especially close to regions of cell–cell and cell–matrix contact [e.g. 40,83,101]. Analyses of knockout mice reveal extensive links to cell adhesion and tissue organization.

In the case of 4.1R, knockout mice have impaired cell–cell contact in stomach epithelium, and the gastric glands are disorganised [102]. This probably arises from defects in the E-cadherin/β-catenin mediated adhesion since 4.1R has been shown to bind to β-catenin [102]. However, 4.1R may not interact with all β-catenin complexes. Indeed, β-catenin and 4.1R do not coincide in cardiac intercalated discs, so presumably N-cadherin/β-catenin complexes do not bind 4.1R in this tissue [83].

4.1G homozygous knockout male mice have loss of fertility depending on strain background. These mice indicate that 4.1G plays a role in spermatogenesis by mediating cell–cell adhesion between spermatogenic and Sertoli cells via a cell adhesion molecule nectin-like protein 4 [103].

A different nectin-like protein, NECL1, interacts with 4.1N in brain [104], and we note in Table 3 a group of cell adhesion molecules such as CASPR2, in the nervous system that interact functionally with 4.1B [105–108].

In the case of 4.1B, again knockout mice are viable, but numerous morphological defects are clear in various tissues. For example, in concert with β8-integrin, 4.1B regulates morphogenesis in embryonic heart [109]. Major roles are also revealed in the nervous system, especially in relation to myelinated axons. The knockout mice display mildly impaired gait and motility [106]. Morphological defects are observed in the paranodal and juxtaparanodal regions of myelinated axons, and destabilization of septate-like junctions in paranodes [105,106].

The interaction of 4.1 proteins with neurexin family proteins in the formation of septate or septate-like junctions seems to be an ancient function since coracle, the fruitfly 4.1, interacts with neurexin and this is essential in epithelial integrity mediated by septate junctions [110]. We may note at this point, too, that a complex of coracle, neurexin, gliotactin (another transmembrane protein, related to vertebrate neuroligins) and the Na^+/K^+-ATPase form an interdependent complex required for septate junction function [111]: the relationship between 4.1 proteins and Na^+-linked processes may be conserved between fly and mammals.

In summary then, there is abundant evidence for the 4.1 proteins of vertebrates controlling cell physiology via functional interactions with a wide variety of class of transmembrane proteins including ion channels and exchangers, and cell adhesion molecules. In the following sections, we discuss the molecular analysis of interactions of the 4.1 proteins with a wide variety of membrane proteins (see Table 3): collectively, these analyses suggest that physiological analysis of animal models has much yet to reveal about the contribution of these proteins to animal life.

Table 3
Some examples of transmembrane proteins that interact with 4.1 proteins in mammals.

Membrane protein	4.1 protein	Reference
Ligand-gated channel		
Glutamate receptor GluR1	4.1N	[34,123]
Glutamate receptor GluR3	(4.1N)	[35]
$InsP_3$ receptor	4.1N	[129]
α7 acetylcholine receptor	4.1N	[180]
Glutamate (NMDA) receptor	4.1B	[124]
Voltage-gated channel		
NaV1.5	4.1R	[80]
Store-operated channel		
TRPC4	4.1R	[132,181]
7 helix receptor		
D2 and D3	4.1N	[119]
mGluR1α	4.1G	[120]
mGlur8	4.1B (+R,G,N)	[182]
Adenosine A1	4.1G	[121]
Parathyroid hormone (PTH)/PTH-related protein receptor (PTHR)	4.1G	[122]
Duffy	4.1R	[24]
Solute/gas transporter		
Erythrocyte/kidney anion exchanger AE1 (Band 3)	4.1R	[183]
Rh (CO_2/NH_3) channel	4.1R	[24]
Na^+/Ca^{2+} exchanger NCX1	4.1R	[80]
Na^+/H^+ exchanger NHE1	4.1R	[82]
Membrane transport protein XK	4.1R	[24]
Plasma Membrane Calcium ATPase 1b (PMCA1b)	4.1R	[37]
Sarcoplasmic reticulum Ca^{2+}-ATPase SERCA2	4.1G	[83]
K-Cl co-transporter KCC2	4.1N	[133]
Na^+ bicarbonate cotransporter1 (NBC1)	4.1B	[141]
Cell adhesion molecules		
CD44	4.1R	[162]
Lutheran (Lu)/BCAM	4.1R	[184]
βI-integrin	4.1R	[85]
β8-integrin	4.1B	[185]
E-cadherin/β-catenin complex	4.1R	[102]
Nectin-like molecule 1	4.1N	[104]
CADM4/Nectin-Like 4	4.1G and 4.1B	[90,103]
PTA-1 (CD226)	4.1G	[148]
SynCAM1/TSLC1/CADM1/NECL2	4.1N and 4.1B	[19,89,90,124]
Neurexin/CASPR/Paranodin	4.1B	[108,186]
Other		
Linker of activated T cells (LAT)	4.1R	[84]
Fc γ RI	4.1G	[187]

5. Functional interaction of 4.1 with membrane proteins

5.1. Erythrocytes

Analyses of 4.1R-deficient human and mouse erythrocytes reveal the complex array of membrane proteins that can link to 4.1R (Fig. 2B). One of the hallmarks of 4.1R-interactive membrane proteins in erythrocytes is that they are lost from 4.1R-null cells [24,73-75,76,112]. This is because in the process of erythropoiesis, during the final asymmetric cell division that releases a reticulocyte from the nuclear remnant of the erythroblast, the interactive proteins are mis-sorted to the nuclear remnant and lost from the erythrocyte precursor [113]. In these 4.1R deficient cells nearly all the FERM domain binding proteins are lost including the cytokine receptor Duffy, the XK/Kell transport complex, CD44, the rhesus component Rh and glycophorin C [24]. An exception to this is the anion exchanger, band 3. 4.1R knockout mouse red blood cells do not lack band 3, although access of a conformation-sensitive antibody to it is changed [24]; the implication is that 4.1R is required for the normal conformation of this protein. Furthermore, the proteins adducin and dematin which provide a further route for interaction with the anion exchanger [114] and, in addition, interact with the glucose transporter, Glut 1 [115], are found at a lower stoichiometry on the F-actin junctional complexes in the 4.1R deficient erythrocytes. CD47 is also reported lost from human, but not mouse, 4.1-deficient erythrocytes [75].

It appears then that a major function of 4.1R is to capture membrane proteins during cell differentiation and retain them at sites specified by short actin filaments at the spectrin–actin junction point.

5.2. Cardiomyocytes

Although mRNAs encoding all four 4.1 proteins are detectable in mouse and human heart, only 4.1R, 4.1N and 4.1G are detectable in mouse ventricular cardiomyocytes [55,80,83]. They show discreet subcellular locations.

4.1R locates at the plasma membrane of cardiomyocytes, both along the lateral edge and at intercalated discs, and in cross-striations coincident with transverse tubules [55,80,83]. At intercalated discs, there is significant colocalization with components of the Ca^{2+}-clearance system: the Na^+/Ca^{2+}-exchanger NCX1 and the α-subunit of the Na^+, K^+-ATPase [83]. At these sites, 4.1R seems to define a structural compartment distinct from known cell adhesion regions. It does not localise with the major points of cell adhesion, i.e. the cadherin-based structures or gap junctions [83]. Since there is also no evidence from electron microscopy of 4.1R-deficient cardiomyocytes having any form of cell adhesion defect [80], the localisation of 4.1R is consistent with a role in organizing signalling systems, rather than cell adhesion.

Given the phenotype of the 4.1R knockout mouse (Section 4.1.2 – elongated action potential and QT interval as well as alterations in Na^+/Ca^{2+} exchange current and persistent sodium current) the discrete location of 4.1R with both the sodium pump and the Na^+/Ca^{2+} exchanger potentially indicates the existence of a membrane domain in intercalated discs in which 4.1R participates in coordinating Na^+-based signalling systems (summarized in Fig. 3). Such a domain would probably contain spectrin since spectrin is present at these points [116]. Furthermore, it is almost certainly similar to regions identified by Mohler and co-workers as containing ankyrinG and the voltage gated sodium channel, NaV1.5 [117]. In cardiomyocytes from the 4.1R knockout mice approximately 40% of NaV1.5 is lost [80]. It is likely that the remainder is linked to proteins such as ankyrin [118], and so remain captured at the cell surface. The overlapping spectrum of 4.1 and ankyrin binding proteins is discussed in Section 9.

Like 4.1R, 4.1N is present in intercalated discs, but unlike 4.1R, it is not localised at the lateral plasma membrane. Both 4.1R and 4.1N are in internal structures that are most probably T-tubules. 4.1G is also in intracellular structures, some of which are coincident with sarcoplasmic reticulum. 4.1G exists in an immunoprecipitable complex with spectrin and SERCA2 (Fig. 3) [83].

5.3. Other non-erythroid cells

Wider evidence for a role of 4.1R in membrane trafficking comes from studies on embryonic fibroblasts in the 4.1R KO mouse [85]. In these, the trafficking of βI-integrin is compromised, resulting in lower integrin cell-surface expression and correspondingly reduced motility of the cells into a wound [85]. It is also worth noting here that 4.1R has been identified in high throughput interaction screens as interacting with numerous components of trafficking complexes (Fig. 4).

The theme of 4.1 proteins being required for efficient cell surface expression of membrane proteins is recapitulated in the other three paralogues.

5.3.1. GPCRs

In the case of G-protein coupled receptors (GPCR or 7 helical receptors), several are known to have their cell surface localisation modulated by one or other 4.1 protein. We have already mentioned Duffy in relation to 4.1R. D2 and D3 dopamine receptors bind to 4.1N [119]; mGluR1α [120], Adenosine A1 receptor [121] and Parathyroid hormone (PTH)/PTH-related protein receptor (PTHR) [122] bind to 4.1G. In general, the interaction promotes cell surface expression and ligand binding/signalling activity. However in the case of Adenosine A1 receptor, the interaction gave a negative modulation with a higher proportion of the receptor being retained internally [121].

5.3.2. Ligand-gated channels and formation of targeted complexes

Several ligand-gated receptors also bind to 4.1 paralogues. GluR1 and GluR3 bind 4.1N, and this increases their cell-surface expression [34,35]. 4.1N is required for GluR1 activity dependent on insertion into the plasma membrane [123]. Similarly, the NMDA receptor binds 4.1B [124].

The synaptic cell adhesion molecule 1 (SynCAM 1) interacts with both 4.1N and 4.1B, and this provides an example of the cross-linking function of 4.1 proteins [124]. 4.1B specifically interacts with both SynCAM1 and NR1/NR2b NMDA-type glutamate receptors. In transfected cell systems, 4.1B specifically recruits NMDA receptors to sites of cell adhesion specified by SynCAM1. It also enhances synaptogenic properties of SynCAM1 in in vitro culture systems, and this requires the FERM domain. 4.1N interacts with AMPA-type GluR1, as well as SynCAM1, and likewise targets GluR1 to SynCAM1-mediated sites of cell adhesion. However, the results from these in vitro cell culture experiments have been challenged by results from mice with severe knockdown of 4.1N and 4.1G [125]. Although they retain only very minor amounts of 4.1N and 4.1G they show no major alteration to the function of glutamatergic synapses. Whether the residual expression of 4.1N is sufficient to allow function, or whether the remaining 4.1 proteins (4.1R and 4.1B) can compensate for the loss of 4.1G and 4.1N remains to be determined.

Inositol tris phosphate(IP_3) receptors seem to be tissue-specific in their interaction with 4.1N. Several accounts demonstrate interaction in nervous tissue [126–129]. In epithelia, the situation is more complex: WIF-B cells epithelial IP_3 receptors apparently do not interact [130], but 4.1N is required for translocation of type 1 IP_3 receptors to the basolateral membrane domain in polarized MDCK cells [131].

5.3.3. Other channels

5.3.3.1. Store-operated channel. In endothelia, TRPC4 has been reported to interact with 4.1 [132]. Deletion of part of the C-terminal cytoplasmic domain that contains a sequence resembling a 4.1-binding motif leads to alterations in the inactivation characteristics of this channel.

5.3.3.2. Ion co-transporter. KCC2 mediates electroneutral K^+-Cl^- co-transport in mature neurons. KCC2 has also been reported to function in correct maturation of spiny excitatory synapses on cortical pyramidal neurons, independent of its transport activity [133]. This function is linked to binding of its C-terminal domain to the FERM domain of 4.1N. Intriguingly, since the CTD of 4.1N also binds AMPA receptors, the potential exists for 4.1N to cross-link KCC2 and AMPA receptor in a single complex during maturation of postsynaptic mechanisms (see [134] for further discussion).

5.4. Three-way complexes with PDZ/membrane-associated guanylate kinases

Analysis of protein 4.1R deficient human and mouse red cells revealed that it was not just transmembrane proteins that were lost; a palmitoylated protein known as p55 (MPP1) was also lost from the cells [76]. p55 is a member of a family of proteins known as membrane-associated guanylate kinases (MAGUK). These are also adapter proteins, and they are characterised by PDZ, SH3 and guanylate kinase-like domains. 4.1R binds to at least some of these via a differentially spliced region known as I3 [135,136].

p55 also binds to the C terminus of glycophorin C via its PDZ domain; thus 4.1R, p55 and glycophorin C form a three-way (ternary) complex [76,137–139]. The precise significance of this interaction is not yet clear in erythrocytes, but presumably serves to control the dynamics of the retention of glycophorin C (and potentially other proteins) at the plasma membrane.

4.1R has been reported to interact with other MAGUK proteins such as ZO-2, a tight junction protein [140] and HLDG/SAP-97 [135], interactions that seem to mediate targeting to lateral membranes of epithelial cells [136].

Examples exist of all four vertebrate 4.1 proteins forming analogous ternary complexes with a MAGUK protein and a transmembrane protein.

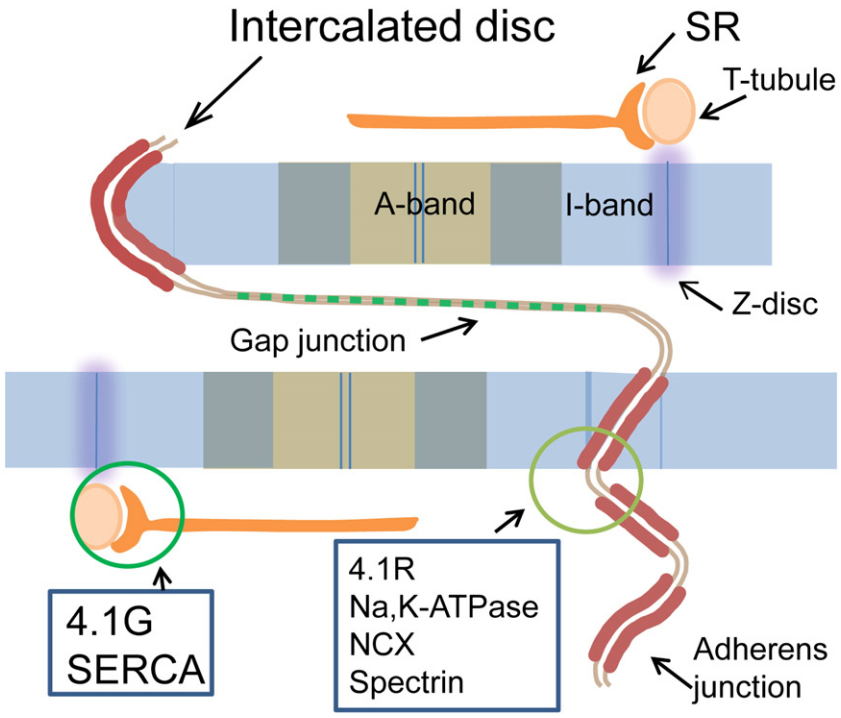

Fig. 3. Diagram of part of an intercalated disc (ID) at the junction between two cardiomyocytes showing two different types of domains associated with 4.1 proteins. The ID membrane is heavily folded. Along part of the fold is the heavy plaque of the adherens junctions to which the thin filaments coming from the myofibrils are associated. In plaque free regions of the membrane, particularly at the tops of the folds, protein 4.1R [83] and spectrin [116] are found along with a number of signalling proteins such as Na^+,K^+-ATPase and NCX1 [83]. Away from the ID where the SR meets the Z-disc, a domain containing 4.1G and SERCA is found [83].

p55 also interacts with 4.1B, and has been reported to form a ternary complex with the sodium bicarbonate exchanger NBC1 [141].

4.1N forms a ternary complex with AMPA-type glutamate receptors and SAP-97/HDLG, at least in certain sites in the nervous system [142,143]. This seems to be the descendant of an ancient animal complex, since the fruit fly glutamate receptor GLURIIA is clustered by the *Drosophila* 4.1 coracle [36]. Since, as noted above, 4.1N and AMPA receptors also interact with SynCAM1, it seems highly likely that this actually is a quaternary 4.1N-AMPAR-SAP97-SynCAM1 complex [124]. Similarly, there are suggestions that the corresponding complex for NMDA receptors may be 4.1B-NMDA-CASK-SynCAM1 [124]. Additionally, the two MAGUKs CASK and SAP-97/HDLG interact with each other [144] and appear to mediate trafficking of at least one voltage-gated ion channel [145] as well as sorting of NMDA receptors [146]; the potential for cross-talk and targeting between different 4.1-MAGUK complexes becomes very large.

Protein 4.1G is required for the MAGUK MPP6 interactions in axons [147]. In T-cells 4.1G and HDLG form a three-way complex with PTA-1 [148]. PTA-1 is a raft-associated lymphocyte type 1 transmembrane protein which binds the 4.1G CTD. Upon T cell stimulation with phorbol ester or PTA-1 cross-linking, PTA-1 can bind to the FERM domain of 4.1G, and together with HDLG becomes tightly associated with the cytoskeleton. PTA-1 is also associated with the integrin LFA-1, and it is possible that the three-way 4.1G/HDLG/PTA-1 complex provides a regulated mechanism for controlling the dynamics of LFA-1.

In summary, then, the 4.1-MAGUK proteins interact to form three- or four-way complexes with membrane proteins that have the potential to target diverse membrane proteins to sites specified by cell adhesion molecules. Since the MAGUKs themselves can interact with each other, the potential exists for 4.1-MAGUK complexes to assemble large complexes in dynamically regulated arrays.

6. 4.1 and modulation of phosphorylation signalling

There are two primary examples where altered phosphorylation results from changes in 4.1 expression.

In the 4.1R knockout mouse, the linker of activated T cells (LAT) is hyperphosphorylated [142]. Regulation of phosphorylation of LAT seems to require direct interaction of 4.1R with LAT, 4.1R apparently acting as a negative regulator of this process.

In the analyses of the dynamics of PTHR, it was discovered that 4.1G modulates phosphorylation in response to forskolin. 4.1G was found to suppress adenylyl cyclase-mediated cAMP production [149]. The consequences of this in relation to modulation of signalling pathways remain an interesting prospect of the future. For example, in the 4.1R knockout mouse, 4.1G expression in heart is increased [80]. Are there consequently alterations in phosphorylation events in 4.1R knockout mice? What are the functional consequences in 4.1G knockouts?

7. The question of specificity. Binding motifs on membrane protein partners

In each of the cases of the major erythrocyte binding partners, the cytoplasmic domain of the relevant membrane protein binds to one of the three lobes of the FERM domain (see text above, Section 3). In all cases, the interaction appears to be very specific by normal biochemical criteria.

4.1R binds to the cytoplasmic domain of the erythrocyte anion exchanger, band 3. The interaction is via a short sequence in lobe A of the FERM domain, LEEDY [150] to a sequence I/LRRY in Band 3. The interaction appears to be essential for erythropoeisis in zebrafish since mutation in the Band 3 sequence results in binuclearity and failure of chromosome segregation and cytokinesis [151].

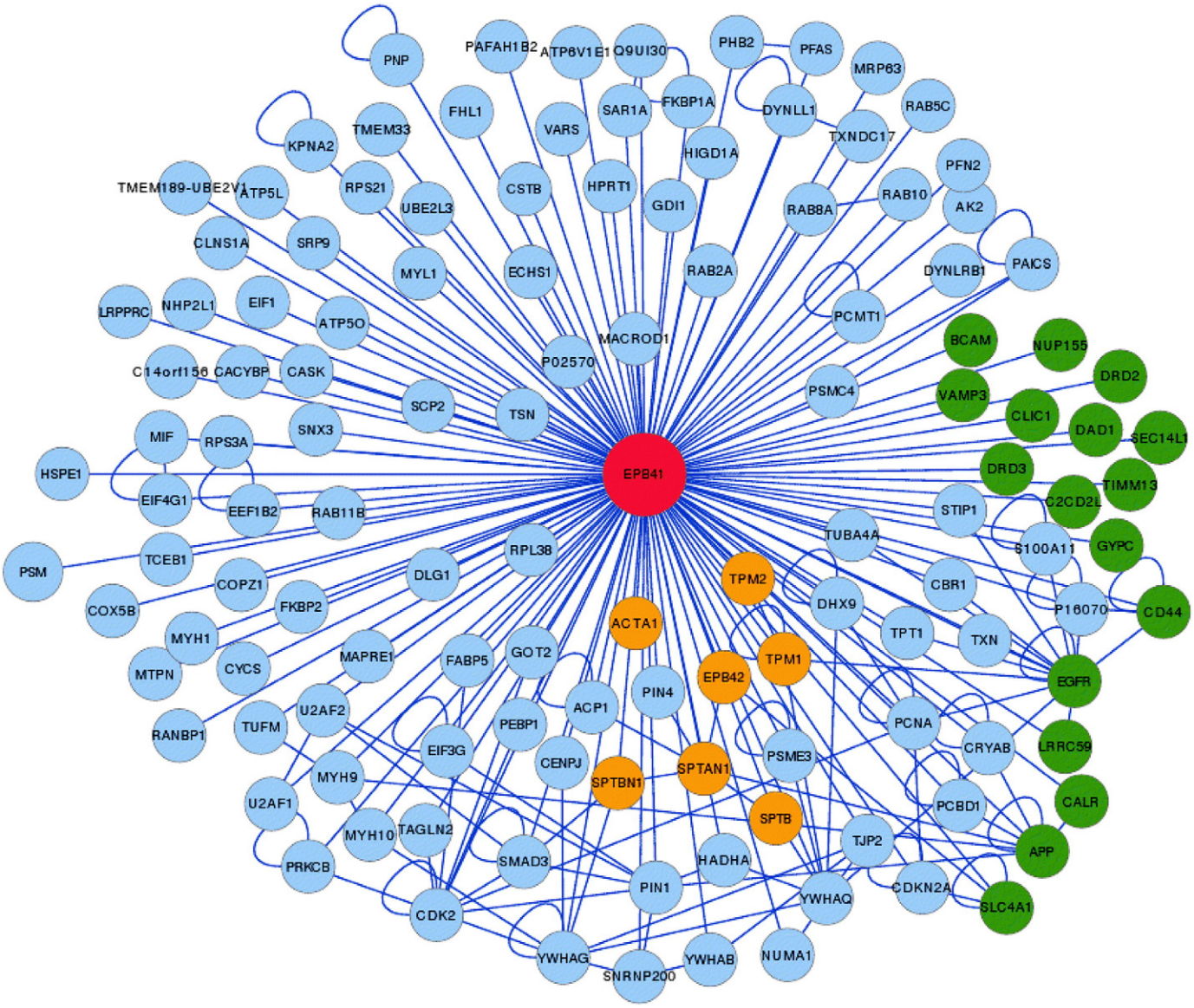

Fig. 4. EBP41 protein interaction network. The human EBP41 protein interaction dataset was extracted from databases, including IntAct [159], MINT [188] and DIP [189], and visualised using Cytoscape [190]. Each node in the network represents a first-neighbour interaction partner protein of EBP41 (red-coloured node). Different isoforms of a gene are shown as one node. The proteins, which are annotated with the GO term [191] GO:0016021 "Integral to membrane", are coloured in green, whereas the proteins with orange colour-code are cytoskeletal partners of 4.1R.

FERM domain lobe B, binds to the cytoplasmic domain of glycophorin C, a type I membrane protein [10,22]. Comparison of the sequences of a number of type 1 membrane proteins led to the suggestion of a common motif $RXKX_{0-4}GXYX_3E$ recognised by lobe B of the FERM domain that is present in glycophorin C and the Neurexin family of cell adhesion molecules [152]. NMR has revealed the pattern of interactive residues on lobe B that stretch across the lobe (Fig. 1B,C) [22].

Further analysis revealed that there was a three-way interaction with the MAGUK protein p55. In this complex, the PDZ domain of p55 also interacts with the C terminus of glycophorin C [139,153,154], and the FERM domain binds to a differentially spliced I3 region in p 55 [137,139]. There are similar interactions with the domain in other MAGUK proteins (for example, mammalian SAP97/hDLG or discs large in fruit flies) [136,155].

The interaction of lobe C with CD44 reveals a requirement for a pattern of basic juxtamembrane residues [23]. Similar residues are required for the interaction of lobe C of 4.1B with TSLC1 as shown by x-ray crystallography [19].

8. 4.1 proteins as hubs for protein and phospholipid interaction

Many biological processes are modulated or organized via direct protein–protein interactions. A common analogy for the organization of interacting networks is that they are like wheels with many interacting partners on the rim, making interacting spokes that link to a hub. Since the wheel will cease to function if the hub is lost (unlike the loss of a single spoke) it is often thought that proteins that represent hubs would be essential [see e.g. 156–158].

In general terms, hub proteins are often thought of as having large numbers of interactions, they are essential in genetic analyses of model organisms, are co-expressed with their partners, and are frequently the target of regulation, especially by post-translational modification.

A further conceptual consideration is that hub proteins may or may not interact with all their partners simultaneously. In this sense, hubs can be divided into "party hubs" and "date hubs" [156]. A party hub would be able to interact with multiple partners simultaneously; date

hubs would interact with their partners in distinct temporal or spatial organization.

Can the 4.1 proteins be considered as hubs? The fruitfly 4.1 protein, coracle, is an essential gene. It is recessive embryonic lethal with defects in dorsal closure. By comparison, no loss of a single vertebrate 4.1 protein seems to be embryonic lethal. However, since the four vertebrate paralogues evolved from a single invertebrate ancestor, it is likely that the functions of the ancestral protein were distributed among its vertebrate descendants. As yet, there is no published account of a quadruple knockout of all four mouse 4.1 genes: given the range of phenotypes the individual knockouts display, it might be predicted that the quadruple knockout would be lethal.

Assessing the range of interactions of the 4.1 proteins in vertebrates is still limited. As indicated in both Table 3 and Fig. 4, 4.1R interacts with multiple transmembrane proteins, as well as the cytoskeletal proteins spectrin, actin and tropomyosin simultaneously; the phospholipids PIP_2 and phosphatidylserine also interact simultaneously and at distinct binding sites within 4.1R. In this sense, it fulfils the concept of a 'party' hub.

4.1R also fulfils a prediction that hubs are tightly regulated: as we note above, the FERM domain is the target of regulation via phosphorylation [28,29], binding of the lipid PIP_2 [27], differential splicing of the headpiece [31] and binding of calmodulin [23].

4.1R is not expressed only in erythrocytes: in the case of the heart, the existence of a Na^+-linked signalling complex in the intercalated disc as we describe in Section 5.2 and Fig. 3 suggests that there is a separate, although mechanistically related, 4.1R hub there that would connect the cytoskeleton to a different range of transmembrane proteins.

Beyond these interactions, interaction databases such as IntAct [159] document results from high throughput protein interaction screens. Remarkably, the interaction databases document more than 100 further interactions for 4.1R (Fig. 4). Given the range of tissues that express 4.1R, one might conceptualise it as a multi-party hub.

Since the study of 4.1N, 4.1G and 4.1B is still not as advanced as that of 4.1R, we may anticipate the discovery of numerous further interactions of these proteins. Nevertheless, in considering 4.1N and 4.1B in the nervous system, it is notable that AMPA and NMDA receptor organization in relation to sites of cell adhesion seems to revolve around complexes of 4.1N and 4.1B (see Section 5.3).

9. 4.1 and ankyrin: common and distinct transmembrane partners

Among the transmembrane proteins that bind to 4.1, several also bind to another cytoskeletal adaptor, ankyrin. Like 4.1, ankyrin binds spectrin, and it too emerged as an adaptation of early eumetazoa [52,160]. A separate spectrin-linked complex exists based around ankyrin (Fig. 1A). This has been conceptualized as a metabolon for CO_2 transport [161]. It contains (among other proteins) the Rh/RhAG complex, small molecule transporters that appear to mediate part of the CO_2 transport capacity of red cells. It also contains the anion exchanger, band 3, which catalyses electroneutral exchange of $HCO3^-$ ions (i.e. dissolved CO_2) for Cl^-. Both the Rh and Band 3 bind 4.1R and ankyrin, as does CD44. In general, it seems likely that there are discrete binding sites for ankyrin and 4.1. This has been demonstrated for both band 3 and CD44 [162,163].

An emerging topic of interest is the relationship of 4.1 to Na^+-linked signalling. Fruitfly 4.1 (coracle), as we note in Section 4.1.4, has a functional relationship with the Na^+, K^+-ATPase [111]. In mammalian heart, 4.1R overlaps in distribution with the Na^+, K^+-ATPase and NCX1 [83]. AnkyrinB, too, overlaps with and binds both these proteins, as well as IP_3 receptor, another 4.1 binding protein [164–166].

Why would it be advantageous for individual proteins to bind both 4.1 and ankyrin? One possibility is that 4.1 proteins and ankyrins are differentially regulated. 4.1R binds PIP_2 in the FERM domain, and this regulates the interaction with transmembrane partners [27], but apparently not ankyrin. In this case, PIP_2, acting via 4.1, would be able to exert an effect on the regulatory properties of a channel/transporter, while ankyrin would remain unchanged as a cytoskeletal tether. An alternative hypothesis might be that ankyrin and 4.1R recognize different states of the target proteins. For example, ankyrin binds band 3 tetramers in red cells, and stoichiometrically, this seems to account for binding of all the tetramers [167]. The remaining band 3 population is presumably dimeric, so it seems likely that ankyrin and 4.1 discriminate between self-association states of the same protein [24]. Finally, both proteins have the potential to be cross-linkers, and bind more than one protein simultaneously. Ankyrin and 4.1 could assemble discrete mixtures containing some common proteins into functionally distinct complexes which could be targeted to different sites of cell adhesion. For example, ankyrin binds neurofascin family cell adhesion molecules [168], while 4.1 binds others such as neurexin family and SynCAM1.

10. Conclusions and perspectives

The 4.1 proteins are a remarkable group of paralogous proteins in vertebrates, whose functions are a long way from being fully understood. They emerged with eumetazoa, and, since then, conserved FERM and CTD regions appear to have retained interactions with various cell adhesion and transporter proteins giving them the potential to position transmembrane signalling complexes at sites specified by activated cell adhesion molecules.

The shear breadth of the interactions of these proteins means that characterization of knockout mice is far from straightforward, with different phenotypes being revealed in different tissues and at different developmental stages. Complicating factors include the overlapping spectrum of interactions of each 4.1 protein, meaning that phenotypes resulting from any individual protein 4.1 gene knockout may be masked by compensation by a different 4.1 protein gene.

We also highlight two emerging areas of future interest. A major question arises as to the relationship of 4.1 proteins to the ion channel signalling systems in heart. Energy captured in the Na^+ gradient across cardiomyocyte plasma membranes drives the channels and exchangers that underpin the signalling that controls the regular beating of the heart. As yet, although there are functional and spatial correlations between 4.1R and some of the key cardiac proteins (Na^+, K^+-ATPase, NaV1.5 and NCX1 among others) no molecular basis for this has been published. Likewise, ankyrin is known to bind each of these. Defining the full role of 4.1 proteins in a "4.1 Na^+ hub" and its relationship to a hypothetical "ankyrin Na^+ hub" is a challenge for the future.

The other issue is that additional aspects of 4.1 function are hinted at from the interactions databases. Fig. 4 shows the 4.1 interactions identified in a combination of high throughput interactome analyses as well as text mining from the peer-reviewed literature. The high throughput data (with all its limitations) reveal numerous interactions not properly developed yet in the literature, but which fit with known aspects of function. There are, for example trafficking defects in 4.1R knockout fibroblasts that reduce βI-integrin at the cell surface [85]. Similarly, 4.1B and 4.1G regulate integrin-dependent spreading of astrocytes [169], and an isoform of 4.1B functions within the Golgi [170]. Numerous trafficking/sorting proteins are noted in the interaction analysis (Fig. 4). Spreading and motility, as well as metastatic processes (most closely linked to 4.1B), all require small G proteins of the ras family (rho/rac and others): elements of these pathways too are found in the interaction analysis (Fig. 4). The relationship between 4.1 proteins and rho/rac signalling remains to be investigated.

Disclosures

None.

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