Proteins and Cell Regulation 9

Fedor Berditchevski
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Editors

Tetraspanins

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PROTEINS AND CELL REGULATION

Volume 9

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Aims and Scope

Our knowledge of the ways in which a cell communicates with its environment and how it responds to information received has reached a level of almost bewildering complexity. The large diagrams of cells to be found on the walls of many a biologist's office are usually adorned with parallel and interconnecting pathways linking the multitude of components and suggest a clear logic and understanding of the role played by each protein. Of course this two-dimensional, albeit often colourful representation takes no account of the three-dimensional structure of a cell, the nature of the external and internal milieu, the dynamics of changes in protein levels and interactions, or the variations between cells in different tissues.

Each book in this series, entitled "*Proteins and Cell Regulation*", will seek to explore specific protein families or categories of proteins from the viewpoint of the general and specific functions they provide and their involvement in the dynamic behaviour of a cell. Content will range from basic protein structure and function to consideration of cell type-specific features and the consequences of diseaseassociated changes and potential therapeutic intervention. So that the books represent the most up-to-date understanding, contributors will be prominent researchers in each particular area. Although aimed at graduate, postgraduate and principal investigators, the books will also be of use to science and medical undergraduates and to those wishing to understand basic cellular processes and develop novel therapeutic interventions for specific diseases.

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Fedor Berditchevski dedicates this work to the memory of his father, Benor Berditchevski.

Foreword

Since arriving at the molecular scene more than 20 years ago tetraspanins continue to capture imagination of researchers in various fields of biology. Initially described as targets for tumour-specific mAbs (CO-029/TSPAN8, ME491/CD63), the anti-proliferative mAb (TAPA-1/CD81/TSPAN28), and the antibodies recognising "cluster differentiation" (CD) antigens (CD9/TSPAN29, CD37/TSPAN26 and CD53/TSPAN25), tetraspanins later came into prominence as regulators of membrane dynamics which play an important role in cell-cell fusion, cell adhesion and endocytic trafficking.

The first review article describing tetraspanins as a distinct family of four-transmembrane domain proteins (then called "tetraspans" or TM4SF proteins) was written in 1991 and over the years which followed a dozen of more reviews focussing on various aspects of tetraspanin function have been published. This volume represents a collection of 15 up-to-date articles covering the whole tetraspanin field.

The presence of four transmembrane domains separating two extracellular regions of unequal size, a number of conserved amino acids (including polar residues in the transmembrane domains) and a characteristic Cystein-Cystein-Glycine (CCG) triplet constitute the hallmarks of tetraspanins. High-resolution crystal structure of the large extracellular domain of CD81, and subsequent modeling indicated that despite extensive sequence divergence this domain is structurally conserved, and highly characteristic of tetraspanins. Structural features of tetraspanins are described in Chap. 1.

With completion of various genome sequencing projects it became clear that tetraspanins are present in all multicellular organisms: there are 33 identified tetraspanins in mammals, 20 in *C. elegans* and 37 in *Drosophila*. The intricacies of the evolution of the tetraspanin superfamily are discussed in Chap. 2.

What do tetraspanins do? Many researchers addressed this question through the identification of tetraspanin interacting proteins. This approach yielded nearly 60 tetraspanin-associated proteins. The interaction of many of these proteins with several tetraspanins, the interaction of tetraspanins with one another and other findings contributed to the idea that tetraspanins were organizing a network of interactions at the membrane referred to as "tetraspanin web", forming discrete

tetraspanin-enriched microdomains (TERM). Chapters 3 and 4 will discuss the organisation and molecular dynamics of tetraspanins in the light of our idea of the "web" and TERMs.

Tetraspanins directly influence the function of the molecules they associate with, including binding of the associated receptors to their ligands, receptor oligomerisation and signal transduction (Chap. 4). Tetraspanins were also shown to regulate various aspects of endocytic trafficking of the associated proteins (Chap. 5). One of the preeminent interactions is that with a subset of integrins. This interaction is discussed in details in Chap. 6, in relation with the ability of tetraspanins to modulate adhesion and migration.

The function of tetraspanins in the context of the whole organism was investigated using knock-out mice. These experiments established the role of tetraspanins in maintaining the kidney structure, platelet aggregation, retinal vascularization and pathological angiogenesis (Chap. 7). They also revealed the requirement of tetraspanins for sperm-egg fusion (Chap. 9) and normal immunity (Chap. 10). The importance of tetraspanins in the life cycle and development of invertebrates, plants and fungi is discussed in Chap. 8.

Several tetraspanins have a distribution restricted to particular organs. Uroplakins (TSPAN20 and TSPAN21), principal components of urothelial plaques that cover almost the entire apical surface of the mammalian bladder urothelium, are expressed in urothelial cells. Their functions in urothelial biology and disease are discussed in Chap. 12. Peripherin/RDS (TSPAN22) and Rom-1 (TSPAN23) are retinal specific tetraspanins. These proteins are key regulators of the photoreceptor architecture and mutations RDS and Rom-1 were linked with retinal degenerative diseases (Chap. 13).

A number of tetraspanins have been described as tumour-specific antigens whose expression is deregulated during cancer development and progression. Recent studies have shown that the role of tetraspanins in cancer is more complex than previously thought: not only do the changes in expression of tetraspanins affect growth and invasive behaviour of tumour cells, but this also modifies tumour microenvironment. Chapter 11 summarizes these studies.

Tetraspanins have been hitchhiked by several pathogens. Cell infection by several viruses and bacteria can be affected by targeting certain tetraspanins. CD81 is special because it is absolutely required for the infection by two major human pathogens, the hepatitis C virus and the malaria parasite, as described in Chap. 15. The involvement of several tetraspanins in viral life cycles, especially HIV, is reviewed in Chap. 14.

Contents

Chapter 1
Structural Bases for Tetraspanin Functions

Michel Seigneuret, Hélène Conjeaud, Hui-Tang Zhang, and Xiang-Peng Kong

Abstract The tetraspanin transmembrane glycoproteins are considered as "molecular facilitators" which simultaneously interact with, and thereby bring into close proximity specific proteins involved in cellular activation and transduction processes. Elucidation of the 3D structure of tetraspanins is an essential step in understanding of their facilitator function and of the molecular basis of their partner specificity. Although there are currently no experimental atomic resolution structures of a whole tetraspanin molecule, recent information gained from three different approaches has led to a rather comprehensive picture of the structural organization of tetraspanins. These include: (1) crystallographic structures of the main extracellular domain of the ubiquitous tetraspanin CD81; (2) a 6 Å-resolution cryo-EM structure of the tetraspanins uroplakin UPIa and UPIb in the urothelial plaque of mammalian urothelium; (3) molecular modeled-structures of the complete CD81 tetraspanin. On the basis of such structural data, a qualitative view of tetraspanin structure-function relationship is emerging, including a delineation of regions of the molecule involved in specific interactions with partners, as well as an understanding of the structural basis of the multilevel partner specificity of tetraspanins and of the tetraspanin network organization.

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Abbreviations

AUM	Asymmetric unit membrane (AUM)
Cryo-EM	Cryo-electron microscopy
EC1	First tetraspanin extracellular region
EC2	Second tetraspanin extracellular region
EM	Electron microscopy
IC	Intracellular
TEM	Tetraspanin-enriched microdomain
TM	Transmembrane
UP	Uroplakin
UPEC	Uropathogenic E. coli
UPIa	Uroplakin Ia
UPIb	Uroplakin Ib
UPII	Uroplakin II
UPIIIa	Uroplakin IIIa
UTI	Urinary tract infection

1.1 Introduction

1.1.1 Functions of Tetraspanins

Tetraspanins constitute a superfamily of transmembrane glycoproteins that are involved in various aspects of the regulation of cellular development, proliferation, activation, and motility. The best characterized human members include CD81, CD9, CD53, CD82, CD151, CD37 and CD63 which are expressed in various cell types, as well as the tetraspanins with more specialized function and restricted distribution such as uroplakins Ia and Ib, found in the asymmetric unit membranes of the urothelium and RDS/peripherin and ROM, located in photoreceptor outer segment discs. Many studies suggest that the role of tetraspanins is related to their ability to interact with other proteins such as adhesion molecules, receptor and co-receptor molecules, major histocompatibility complex antigens, cytoplasmic kinases as well as other tetraspanins. Current hypotheses view tetraspanins as "molecular facilitators", the function of which would be to simultaneously interact with, and thereby bring into close proximity specific proteins involved in cellular processes (for reviews see Hemler 2003; Levy and Shoham 2005). Such properties lead to the formation of large membrane complexes involved in specific activation and transduction of signaling processes. Tetraspanins themselves undergo homologous and heterologous associations, which may form the basis of a tetraspanin web (Charrin et al. 2009a). Tetraspanins have been shown to interact with lipid rafts (Delaguillaumie et al. 2004; Xu et al. 2009) and have been suggested to be involved in microdomains called tetraspanin-enriched microdomain having composition properties and detergent-solubilization different from rafts but still involving cholesterol

as a major lipid component (Berditchevski et al. 2002, for a recent review see Yanez-Mo et al. 2009).

1.1.2 Partner Specificities of Tetraspanin

A striking feature of tetraspanin associations with their partners is their multilevel specificity (for recent reviews on tetraspanin molecular interactions see Stipp et al. 2003; Charrin et al. 2009a; Yanez-Mo et al. 2009). Many interactions with partners appear to be specific to a single tetraspanin (e.g. CD19-CD81, PSG17-CD9, Pro-HB-EGF-CD9, UPIa with UPII, UPIb with UPIIIa). On the other hand, several tetraspanins have been shown to share common partners (CD9P1/EWI-F, EWI-2 or Claudin-1 with CD81 and CD9, MHC-I and MHC-II with CD81, CD82 and CD53). A particular case is constituted by integrins which appear to be privileged partners of tetraspanins. On the other hand, each specific integrin appear to interact only with one or two specific tetraspanin with variable affinity (e.g. $\alpha 3\beta 1$, $\alpha 6\beta 1$ and $\alpha 6\beta 4$ with CD151, $\alpha 4\beta 1$ with CD81, $\alpha 3\beta 1$, $\alpha 6\beta 1$ and $\alpha 1\beta 1$ with CD9, LFA-1 with CD82).

1.1.3 Structural Studies of Tetraspanins

Tetraspanins are characterized by four transmembrane segments (TM1-4) linked by one short extracellular (EC1), one short intracellular (IC) and one large extracellular (EC2) stretches (12). Tetraspanins also possess a number of conserved residues. The most conserved residues are an ubiquitous CCG motif as well as 2–6 other cysteines located on the EC2 stretch. Also significantly conserved are a number of very polar residues (Asn, Glu, Gln) and small size residues (Gly, Ala) in the transmembrane domain.

Elucidation of the 3D structure of tetraspanins is essential for understanding of their facilitator function and the molecular basis of partner specificity. A system of choice for such structural studies is naturally occurring two-dimensional crystals involving uroplakins UPIa and UPIb in the urothelial plaque of mammalian urothelium that has led to extensive investigation by cryoelectron microscopy (Min et al. 2006, see Sect. 1.5). Apart from this particular case, experimental structural studies of full-length tetraspanin molecules are lacking. This is presumably due to the usual difficulties associated with preparation of relatively large quantities of suitable samples of transmembrane proteins for X-ray crystallography, cryoelectron microscopy or high resolution NMR. However the recent reports describing high level expression systems for tetraspanins represent an encouraging step toward this goal (Jamshad et al. 2008; Takayama et al. 2008).

A usual approach to circumvent difficulties in production of full length transmembrane proteins for structural experiments is to study isolated aqueous domains of transmembrane proteins. This was done by Kitadokoro et al. (2001) who reported the crystallographic structure of a soluble form of the human tetraspanin CD81 EC2 domain (Fig. 1.1). The structure appears mushroom-shaped and consists of a

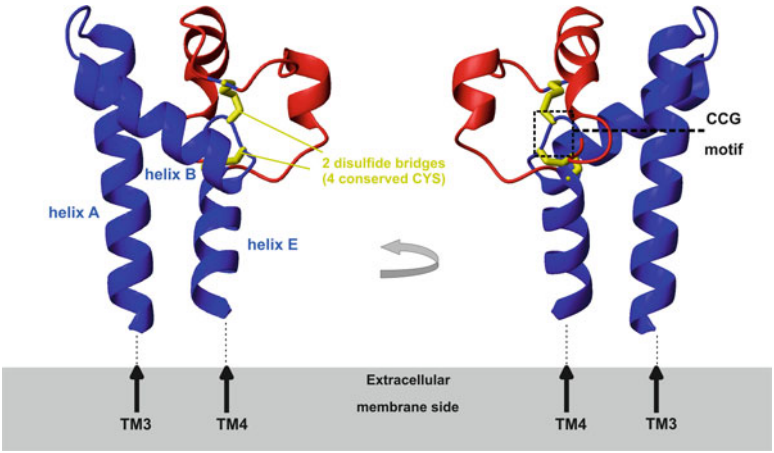

Fig. 1.1 Ribbon representation of the crystallographic structure of the human CD81 second extracellular domain EC2 according to Kitadokoro et al. (2001). The lower conserved membrane proximal subdomain is drawn in *blue* and the upper hypervariable subdomain is drawn in *red*

five-helix bundle stabilized by two disulfide bridges involving the CCG motif and two other conserved cysteines. The EC2 appears to be organized in two subdomains. The first, membrane proximal, subdomain involves two antiparallel helices (A and E), that form the stalk of the mushroom as well as a third helix (B) which is connected to helix A by a short loop. The second subdomain is sequentially inserted within the first subdomain and located on its top. It is composed of two shorter helices (C and D). The two disulfide bridges maintain the two subdomains in a defined orientation. Seigneuret et al. (2001) found that the structural features of the CD81 EC2 are conserved only partially among tetraspanins, with the membrane proximal subdomain being structurally conserved and the upper subdomain structurally hypervariable (see Sect. 1.3.3).

1.2 The CD81 Molecular Model and Its Structural Relevance for the Tetraspanin Superfamily

Considering the current shortcomings of experimental methods for structure determination of complete tetraspanins other than uroplakins, an alternate approach is structure prediction and molecular modeling. Seigneuret proposed in 2006 a molecular model of the complete structure of the human tetraspanin CD81 (Seigneuret 2006). While the crystallographic structure of the CD81 EC2 (Kitadokoro et al. 2001) was used as a starting point, the rest of the molecule was built from various prediction methods, including studies of the periodicity of sequence conservation of various properties, secondary structure prediction, protein docking and homology modeling (Fig. 1.2).

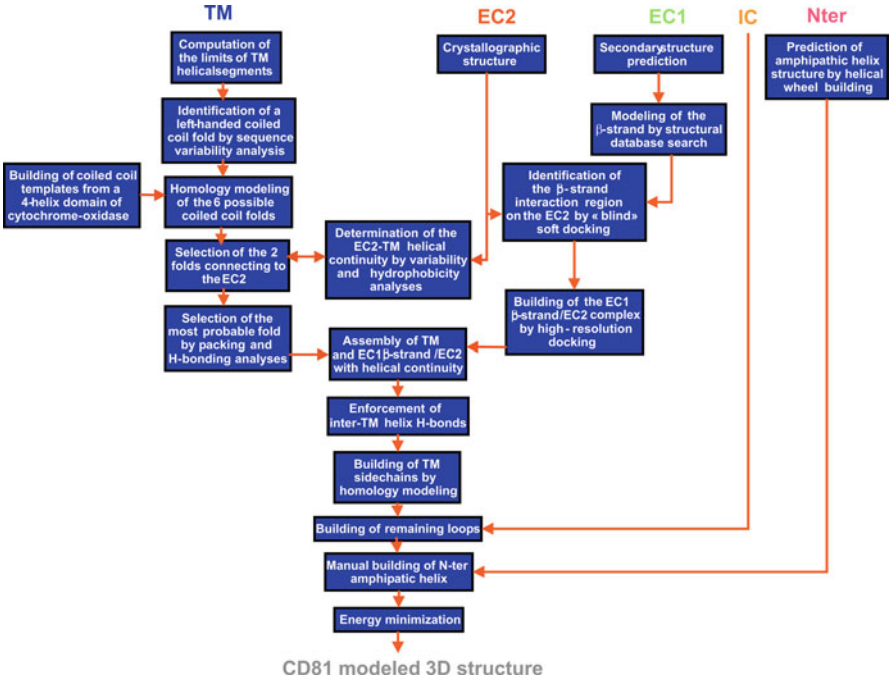

Fig. 1.2 Flowchart of the procedure used for molecular modeling of the human CD81 complete structure (Seigneuret 2006)

1.2.1 Description of Modeled Structure

The modeled structure of the complete CD81 molecule is shown in Fig. 1.3. The transmembrane domain is organized as a four-stranded left handed antiparallel coiled coil square bundle of helices. Two adjacent helices of this coiled coil directly connect to two helices of the larger extracellular domain EC2. Namely, after emerging from the bilayer, TM3 and TM4 become helices A and E of the EC2 without interruption of the helical conformation and remain packed, although departing from their coiled coil geometry due to the constraints of the EC2 tertiary structure. The smaller extracellular domain EC1 extents from the two other helices of the transmembrane coiled coil, TM1 and TM2, and packs against the conserved subdomain of the EC2 mainly on helices A, B and E. The EC1 contains a small partially hydrophobic β-strand which inserts into a conserved hydrophobic groove of the EC2 and runs roughly antiparallel to helix B. The EC2 hypervariable subdomain (Seigneuret et al. 2001), which for CD81 contains the two small helices C and D, is located on the side of the extracellular domain opposite to the EC1. It is linked to the conserved EC2 subdomain by two disulfide bridges. At the intracellular side, the N-terminal segment forms an amphipathic membrane-parallel helix when palmitoylated (palmitoyl residues are not shown on the figure). The IC loop, connecting transmembrane helices TM2 and TM3 is very short (four residues). The C-terminal segment is disordered.

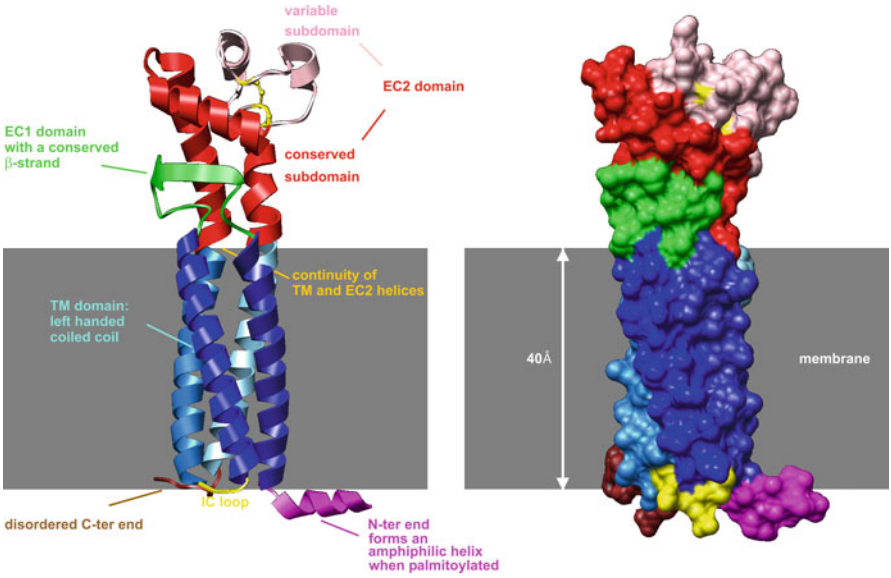

Fig. 1.3 The modeled CD81 3D structure. Ribbon (*left*) and surface (*right*) representation of the CD81 tertiary structure and topology. TM1-TM4, the conserved and variable subdomains of the EC2, the EC1, the IC loop and the N-terminal and C-terminal regions are respectively represented in *marine blue*, *blue*, *royal blue*, *light blue*, *red*, *pink*, *green yellow*, *magenta* and *brown*. Disulfide bridges are in *yellow*. The lipid bilayer (40 Å thickness) is depicted in *gray*

The structure of the modeled CD81 is very compact due to the tight packing of the left-handed coiled coil transmembrane domain and its continuity with the EC2 as well as to the packing of the EC1 on the EC2. Due to this tight packing of the EC1 in the EC2 groove, the whole extracellular domain more or less retains its mushroom shape. It protrudes out of the bilayer by 3.3 nm, i.e. about the size of an Ig domain. On the IC side, the N-terminal amphipathic helix, the IC loop and the C-terminal disordered domain emerge in the aqueous phase at similar levels, presumably interacting with the membrane surface. The modeled CD81 structure therefore assumes a cylindrical shape which is asymmetrically implanted in the bilayer, emerging much more on the extracellular than on the intracellular side.

1.2.2 Intrinsic Validity of Modeled Structure

Several arguments suggest that the above CD81 molecular model is plausible. Firstly, the model was built from predictions that, although mostly derived independently from each other, were found to constitute a self-consistent ensemble. The conclusion that the transmembrane domain of CD81 adopts a coiled coil fold was derived from analyses of the periodicity of sequence and residue size conservation

in alignments of transmembrane regions of tetraspanin. This gave rises to six possible folds corresponding to permutations of four helices within a square bundle. Several criteria were used to select one particular fold corresponding to an antisymmetric four-stranded coiled coil. Independently, it was concluded from periodicity analyses of sequence and residue hydrophobicity conservation in the regions linking TM3 to EC2 helix A and TM4 to EC2 helix B, that helical continuity occurred in both cases. It then appeared that the selected transmembrane antisymmetric four-stranded coiled coil could be connected with such helical continuity to the EC2 crystallographic structure with adequate stereochemistry and sequence continuity. Furthermore, such connectivity imposed an orientation to the EC2 in which an existing hydrophobic groove, formed within helices A, B and E, was adequately positioned for interaction with the shorter EC1 domain. It was further predicted that the CD81 EC1 contains a short β-strand enriched in hydrophobic residues. Again independently, it was demonstrated by docking simulations that the EC2 hydrophobic groove was the preferred binding site for a β strand peptide corresponding to that predicted for the EC1. In all, molecular modeling of the CD81 structure was like assembling a puzzle from independently crafted pieces that were found to fit together.

Another argument in favor of the modeled CD81 structure is that its overall structural properties are similar to those of experimental transmembrane protein structures. Its membrane spanning transmembrane domain has a hydrophobic surface devoid of polar residues (Fig. 1.4a). Besides, this transmembrane domain is flanked on each side by so-called "aromatic belts" of external tyrosines and tryptophans found in many experimental protein structures and that are thought to provide anchoring to membrane interfaces (Lee 2003). Exterior phenylalanine sidechains are also present but occupy less superficial positions, as also found for experimental protein structures (Ulmschneider and Sansom 2001) (Fig. 1.4b). Besides, interactions between transmembrane helices in the modeled CD81 structures are similar to those found in experimental protein structure. Indeed, stability of transmembrane helical bundles result mainly from two factors (Liang 2002; Schneider 2004): (1) size complementarity between adjacent transmembrane helices mainly resulting from contact between small size and bulky residues leading to efficient Van der Waals interactions; (2) hydrogen bonding between polar residues located at helix interfaces. Both types of interactions exist in the CD81 modeled structure (see Sect. 1.3.2).

1.2.3 Comparison with Experimental Data

A first way of evaluating the accuracy of the CD81 model is to validate that residues subjected to post-translational modification in CD81 and other tetraspanin modifications are accessible and not buried in the molecule.

The structure was found to be in agreement with these data in that all such residues have a significant exposed surface area (see details in Seigneuret 2006). CD81 contains six palmitoylatable cysteines which are all found to be accessible in the

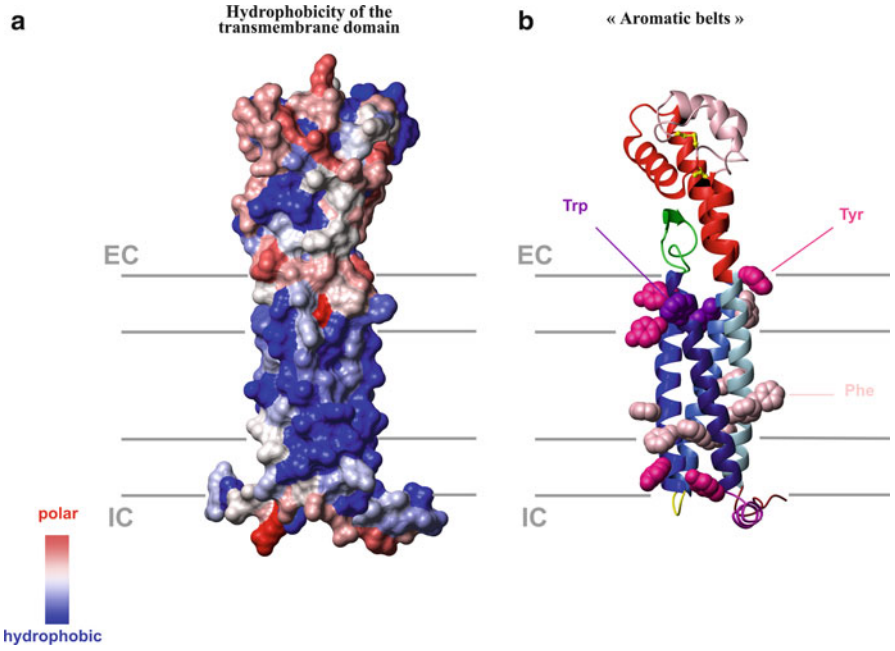

Fig. 1.4 Polarity and organization of aromatic residues in the modeled CD81 3D structure. (**a**) Surface representation of residue polarity calculated according to Eisenberg et al. (1984). The surface color ranges linearly from *red* to *blue*, corresponding respectively to polar and hydrophobic. (**b**) Mixed CPK/ribbon representation of the modeled CD81 structure highlighting exterior aromatic residues of the transmembrane domain. Phe, Tyr and Trp residues which are respectively colored *pink*, *magenta* and *purple*. The rest of the molecule is color-coded as in Fig. 1.3. The expected limits of the lipid bilayer (40 Å thickness) and of the hydrophobic region (30 Å thickness) are indicated as *thin gray lines*

CD81 structural model and located in regions of the modeled protein close to the intracellular lipid headgroup regions of the membrane. CD81 residues in the position similar to the positions of cysteines in CD151 are also accessible. Besides, unlike CD81, most tetraspanins are also glycosylated. In particular CD9 is glycosylated at two positions located in the EC1 and the corresponding CD81 residues are largely solvent accessible in the modeled structure. Finally, in the modeled CD81 structure, no EC2 residue known to be accessible either in CD81 itself or in other tetraspanins was found to be masked by the EC1. These include CD81 residues known to be involved in the interaction with HCV glycoprotein E2, as well as residues corresponding to glycosylation sites in CD53 or CD63 and a residue corresponding to a RDS/peripherin cysteine involved in disulfide-mediated dimer formation.

A stronger confirmation of the overall validity of the CD81 modeled structure appeared when a cryo-EM structure of the uroplakin complexes at 6 Å resolution was published (Min et al. 2006; see also Sect. 1.5). The experimental structures of uroplakin tetraspanins UPIa and UPIb were found to share the following features

with the CD81 modeled structure: (1) cylindrical rod shape of the whole molecule; (2) arrangement of the four transmembrane helices as a square bundle with a left handed tilt; (3) similar arrangement and contacts of the four helices TM1-TM4 within the transmembrane bundle; (4) continuous extension of the transmembrane helices into the extracellular domains EC1 and EC2 yielding a similar orientation of the extracellular region relative to the transmembrane region; (5) close packing of the EC1 on an hydrophobic region of the EC2. While there are however a few differences between the two structures, these can be straightforwardly explained. In particular, Min et al. (2006) found that the cross angles between adjacent helix pairs are ~10° and ~25°. In the modeled CD81 structure, all such cross angles have an identical value of ~20° (unpublished). While this later value is in between the experimental values for UPIa/UPb, this difference likely represents an approximation related to the modeling procedure for which a very regular left-handed antisymmetric coiled coil was used as a template for the CD81 transmembrane domains. Indeed coiled coil arrangements of transmembrane helices have been reported to be less regular than for soluble coiled coils (Langosch and Heringa 1998; Walshaw and Woolfson 2001). On the other hand, both the UPIa/b experimental structure and the CD81 modeled structure have similar interaxis distances between adjacent transmembrane helices (~10 Å). Another difference is that the UPIa/b EC1, although closely packed to the EC2 hydrophobic surface as in the CD81 model, does not appear to contain an extended β strand. However, Seigneuret (2006) found that, according to secondary structure prediction methods, only ~70% of tetraspanins contain a β strand within the EC1. UPIa and UPIb were among those for which no β strand was found. It is probable that for several tetraspanins, insertion of the EC1 into the specific geometry of the hydrophobic groove of the EC2 is associated with distortion from the β strand geometry. In all, it appears that there is a good agreement between the UPIa and UPIb cryo-EM structure and the CD81 modeled structure.

1.3 General Consequences of the CD81 Model for Tetraspanin Structure

1.3.1 Overall Structure

The availability of the CD81 modeled structure raises the question as to which aspects of the structure are common to all tetraspanins. The striking resemblance found with the cryo-EM structure of UPIa/UPIb (Min et al. 2006), although these are relatively distant members (only 12–13% sequence identity), suggests that several features are ubiquitous. Those probably include the arrangement of the transmembrane domains as a relatively square bundle with a left handed tilt, the relative arrangement of transmembrane helices TM1-TM4 within the bundle, the continuity of transmembrane helices with the extracellular regions and the tight packing of the EC1 with the EC2.

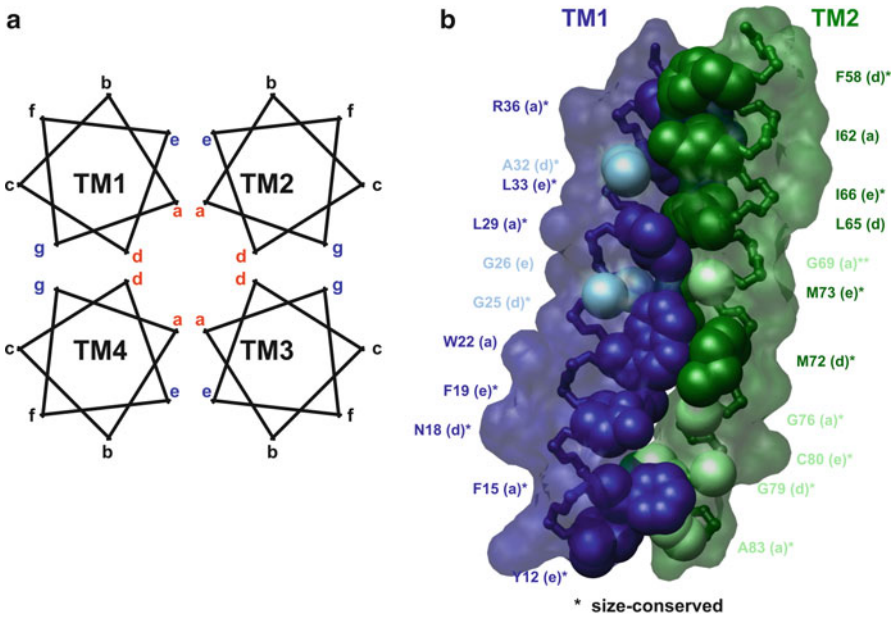

Fig. 1.5 Transmembrane helix interactions in the modeled CD81 3D structure: (**a**) Helical wheel representation of the four stranded antisymmetric coiled coil organisation of the TM domain of CD81. Residues at positions *a* and *d* are buried and contribute mainly to helix packing. Residues at positions *e* and *g* are semi-exposed and contribute to both helix packing and the molecular surface. (**b**) Interhelix packing with complementarity of size-conserved bulky and small interior sidechains for the TM1-TM2 interface. The peptide backbone of both helices and the C_{α}s plus side chains of residues contributing to the helix-helix interface (coiled coil residues *a*, *d* and *e*) are shown respectively as bond and atom representation. Small and large residues are colored in *light* and *dark blue* for TM1 and in *light* and *dark green* for TM2, respectively. Size-conserved residues among tetraspanins with more than 30% sequence homology with CD81 are indicated by an *asterisk*

1.3.2 Coiled Coil Interactions and Hydrogen Bonding in the Transmembrane Domain

It appears likely that the arrangement of the transmembrane domain as a four-stranded antisymmetric left-handed coiled coil is common to all tetraspanins. Due to a more regular pitch of individual helices (3.5 residues per helix turn), the packing of coiled coils (Fig. 1.5a) involves specific core residues positions termed *a* and *d* for the more internal and *e* and *g* for the more external with repetitive spacing in the sequences (heptad repeats) (Lupas 1996). Helix interactions in soluble (Lupas 1996) or transmembrane (Langosch and Heringa 1998; Javadpour et al. 1999; Liu et al. 2004) coiled coils are known as "knobs-into-holes" type of interactions between sidechains of residues located at the critical *a*/*d* and *e*/*g* helix positions. For transmembrane coiled coils, the importance of residue sidechain type or volume conservation among homologous proteins in such packing has been emphasized

Table 1.1 Interhelix proximities between small and bulky core residue pairs in the transmembrane domain of the modeled CD81 structure

TM1-TM2	TM2-TM3	TM3-TM4	TM4-TM1
Y12(*e*)*-A83(*a*)*	G61(*g*)-W111(*d*)	L91(*a*)*-C227(*e*)	A208(*g*)-L35(*g*)
Y12(*e*)*-C80 (*e*)	I62(*a*)-A108(*d*)	F94(*d*)*-S223(*a*)*	I215(*g*)*-G25(*g*)*
F15(*a*)*-A83(*a*)*	L65(*d*)-A108(*d*)	F95(*e*)-C227(*e*)	V212(*d*)*-A32(*d*)*
F15(*a*)*-C80 (*e*)	L65(*d*)-C104(*g*)	E105(*a*)*-A213(*e*)*	S223(*a*)*-N18(*d*)*
F15(*a*)*-G79(*d*)*	G69(*a*)*-L101(*d*)*	G112(*a*)-I205(*d*)*	
F19(*e*)-C80 (*e*)	M72(*d*)*-C91(*a*)		
W22(*a*)-G69 (*a*)*	G76(*a*)*-F94(*d*)*		
G26(*e*)-M73(*e*)*	G79(*d*)*-L90(*g*)*		
L29(*a*)*-G69(*a*)*			

Coiled coil positions of the residues are indicated in *italic*. Proximities were measured using a 1 Å distance cutoff between sidechains (or C_α for glycines) to account for the uncertainties of the modeling. Residues that are size-conserved are indicated by an *asterisk*

(Eilers et al. 2002). Seigneuret (2006) analyzed sequences alignments of each transmembrane helix of tetraspanins with at least ~30% homology with CD81 and found definite conserved heptad repeats for both residue types and residue size (although not as regular as for soluble coiled coils). Indeed, many transmembrane left-handed coiled coils are known to depart locally from a regular knobs-into-holes arrangement (Langosch and Heringa 1998; Walshaw and Woolfson 2001). For tetraspanins with lower homology, some deviations from size-conserved heptad repeats occur for a limited number of positions. This likely arises from correlated residue substitutions (Gobel et al. 1994; Kovalenko et al. 2005) in which concerted residue size variations occur. These data suggest a left-handed antisymmetric coiled coil organization for TM1-4 of all tetraspanins. On the other hand, actual tetraspanin transmembrane coiled coils are probably less regular than in the CD81 model, as suggested by the cryo-EM UPIa/UPIb structure (Min et al. 2006).

Such coiled coil organization gives rise to contacts of small and bulky core residues at the interface between adjacent helices that promote shape complementarity and efficient Van der Waals interactions. In Fig. 1.5b, as an example, the interface between TM1 and TM2 is depicted. Although modeling of the sidechain conformation and packing is likely to be approximate (so that atomic contacts are not always effective), there is a visible size complementarity of several small and bulky core residues of the two helices, most of which are size-conserved. In particular, near the intracellular end of TM2, there is a cluster of small residues (G69, G76, G79, C80, G82 and A83). These residues mainly interact with another cluster of very bulky residues of TM1 (Y12, F15, N18, F19 and W22) in a size-complementary manner. Table 1.1 lists proximities between small and bulky core residues in all adjacent transmembrane helix pairs. More than half of such proximities occur between pairs of size-conserved residues, suggesting that size complementarity may indeed play a role in the stability of the CD81 transmembrane domain. This stabilizing role of residue size complementarity provides an explanation for the conservation of many small and bulky residues in the transmembrane domain of tetraspanins.

Kovalenko et al. (2005) also evidenced the role of contact between small and bulky residues in the TM1-TM2 interaction using an original Monte Carlo modeling procedure taking into account correlated substitutions. These authors, using alignments of 28 tetraspanin sequences, reported the occurrence of heptad repeats only for TM1, TM2 and TM3. It must be noted that their TM4 alignment of CD81 and CD9 with other sequences is different from that of Seigneuret (2006). Kovalenko et al. (2005) also found that mutations of conserved TM1 and TM2 interior glycines in human CD9 caused aggregation of mutant proteins inside the cell suggesting misfolding of the transmembrane domain.

The CD81 model also suggests that hydrogen bonds can be formed between interior polar residues of different transmembrane helices involving conserved very polar residues such as asparagine, glutamic acid and glutamine. Three hydrogen bonds were proposed for CD81: N18 (TM1)—S223 (TM4); W22 (TM1)—E219 (TM4) and E109 (TM3)—A209 peptide carbonyl (TM4) (Seigneuret 2006). The hydrogen bonding network may be in part different for other tetraspanins, particularly since the position of the E219 residue is specific to CD81. While there is also a very polar (usually Q or E) residue in TM4 in many other tetraspanins, it is located farther from the cytoplasmic side. In a recent modeling study, Bari et al. (2009) suggested the following interhelix hydrogen bonds for human CD82: N17 (TM1)—S249 (TM4), as in CD81, and Q99 (TM3)—E242 (TM4). This represents probably the most common situation since the corresponding very polar (Q, E, D or N) interior residue positions are shared by the majority of tetraspanins, including CD151, CD53 and CD37. On the other hand, other tetraspanins lack some of these very polar residues such as CD63 (in TM1) or CD9 (in TM4). Here other hydrogen bonding schemes involving interior serine or threonine residues or peptide carbonyls must occur. It is interesting to emphasize that, in almost all tetraspanins, TM2 is devoid of interior polar residue and, therefore, it is likely to interact with adjacent helices only through Van der Waals contact interactions. On the other hand, while the exact interhelix hydrogen-bonding network may be variable in tetraspanins, it always involves TM1-TM4 and TM3-TM4 interaction.

In fact, the framework of transmembrane helix interactions in tetraspanins seems to be similar to that of GPCRs. Liu et al. (2004) have emphasized the importance of the size conservation of interior small residues often located at *a* and *d* coiled coil positions in the packing of GPCR transmembrane helices. They found that while a specific position may be size-conserved among the whole GPCR superfamily, each subfamily may have a preference for a specific residue type. A similar situation is indeed found for tetraspanins. Also, while the transmembrane helices of GPCR are linked by hydrogen bonds, the exact pattern of such bonds depends on the subfamily (Lomize et al. 1999), as is also likely for tetraspanins.

Interestingly, Kovalenko et al. (2005) found that mutations of interior small residues involved in coiled coil interhelix interactions diminished intermolecular CD9 interactions. Bari et al. (2009) found that mutation of all three very polar interior residues of CD82 involved in interhelix hydrogen bonds affected its interaction with CD9 and CD151 (Bari et al. 2009). This suggests that interactions with other transmembrane partners are critically dependent upon intramolecular interactions within transmembrane helices of tetraspanins.

1.3.3 Organization of the Extracellular Domain

In the human CD81 EC2 crystal structure, a surface hydrophobic patch contributed by residues from helices A, B and E is apparent and corresponds to crystallographic contact between adjacent EC2 molecules. The conservation of some of these residues among tetraspanins led to the suggestion that the hydrophobic patch might be involved in tetraspanin interactions with themselves (Kitadokoro et al. 2001). Here, in the modeled CD81 structure, these residues are in part masked by their interaction with the EC1, a fact that is also found in the UPIa/UPIb cryo-EM structure. This suggests that such masked residues are actually conserved internal hydrophobic residues of the extracellular domain that become unmasked in the soluble EC2. The idea that the EC2 hydrophobic patch is not involved in tetraspanin-tetraspanin interaction is consistent with the report (Berditchevski et al. 2001) that the removal of the EC2 by mutagenesis of CD151 does not affect its association with itself and other tetraspanins (CD9, CD81, CD63). Moreover, a study by Drummer et al. (2005) reports that mutations of two residues involved in the CD81 EC2 hydrophobic patch (F150 and V146), although decreasing EC2 oligomerization in solution, has no detectable effect on CD81 homodimerization in situ (Drummer et al. 2005). This suggests that other structural factors are involved in tetraspanin-tetraspanin interactions.

Secondary structure prediction (with ~75% accuracy) suggests that ~70% of tetraspanins possess a β-strand region in the EC1. These include among others CD81, CD82, CD9, CD151, CD37 and CD63. As stated above for UPIa/b, this may not be the case for other tetraspanins for which the corresponding EC1 region while still packed in the EC2 groove may deviate significantly from the β-strand conformation. In all cases, this EC1 region is enriched in hydrophobic residues, which interact with conserved hydrophobic residues of the EC2 groove. In a previous study, the structural conservation and variability pattern of the EC2 was characterized among the tetraspanin superfamily. It was found that, in spite of limited sequence similarity, helices A, B and E of the EC2 form a structurally conserved subdomain among tetraspanins (Seigneuret et al. 2001). The present data suggests that, for ~70% of tetraspanins, the EC1 shares similar features, i.e. it has a largely conserved structure in spite of significant sequence divergence. Since the conserved EC2 subdomain and the EC1 are packed together, these appear to constitute a structurally-conserved extracellular subdomain. This conserved subdomain is topped by a smaller structurally hypervariable subdomain from the EC2 (Seigneuret et al. 2001, see Sect. 1.4.1).

1.3.4 Organization of the Tetraspanin Intracellular Region

Structural conservation on the intracellular side of tetraspanin appears to be more contrasted. The small IC loop connecting TM2 and TM3 is four residues long in the CD81 structural model. It corresponds to a sequence pattern which is found in

~60% of tetraspanins. It is therefore likely that the loop adopts a conformation similar to that of CD81 in these species. The N-terminal intracellular stretch of CD81, which contains two palmitoylatable cysteines, has been modeled as a membrane-parallel amphipathic helix and corresponding regions of ~40% tetraspanins are also predicted to have a comparable amphipathic pattern. Although many tetraspanins contain a single cysteine in this region, the missing cysteine is often replaced by a hydrophobic residue so that the amphipathic character is retained. The idea that this amphipathic helix is formed only upon palmitoylation suggests a possible mechanism for regulation of tetraspanin interactions. The heterologous interactions between tetraspanins have been shown to depend upon palmitoylation (Charrin et al. 2002). It is tempting to propose that interactions between the amphipathic N-terminal helices are involved in such interactions. Finally, the intracellular C-terminal stretch is among the most divergent regions in tetraspanins. While it is suggested to be disordered in CD81, it may adopt specific conformations in other members, especially since it is often involved in very specific functions (for review see Stipp et al. 2003; Charrin et al. 2009a).

1.4 Conservation and Variability in Human Tetraspanins and Specificity of Partner Associations

The multilevel specificity of interactions of tetraspanins with their partners raises two complementary questions: (1) what is the structural origin of the high diversity of tetraspanins partners specific for each member? (2) what is the structural origin of the occurrence of partners common to several tetraspanins? The results described above suggest that a large part of the tetraspanin molecule is overall structurally conserved for a majority of the family members and at least for the more common human members (CD81, CD82, CD9, CD151, CD37 and CD63). This corresponds to the transmembrane domains and the region of the extracellular domain directly connected to the transmembrane domains, namely the conserved part of the EC2 (including helices A, B and E) and the EC1 (including its β strand). For CD81 the conserved regions amount to ~75% of the molecule. In the large extracellular domain (hypervariable region of the EC2) and both intracellular extremities of the tetraspanin molecule, one finds regions that are structurally non-conserved (or less conserved) among the family members. This suggests that specificity for a partner in tetraspanins is dictated by two distinct types of variability at the molecular level: the occurrence of such structurally variable regions and variability of surface residues in structurally conserved regions.

1.4.1 Structural Variability in Tetraspanins

The best documented occurrence of structural variability in tetraspanins concerns the hypervariable subdomain of the EC2. Seigneuret and colleagues (2001) performed multiple sequence alignments and secondary structure predictions the EC2 of 43

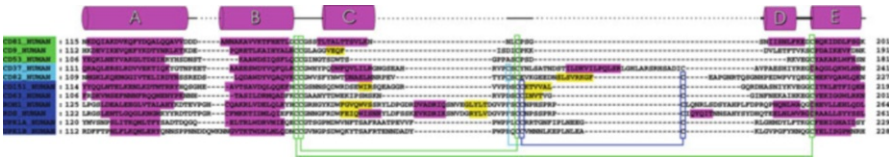

Fig. 1.6 Secondary structure prediction of the tetraspanin EC2 of common human tetraspanins. Sequences regions highlighted in *magenta* and *yellow* correspond, respectively, to helices and strands. The background colors of sequence names correspond to the different tetraspanin groups: *green*: group1, *light blue*: group 2a, *blue*: group 2b. The positions of the experimental helical regions of the CD81 EC2 structure are indicated as *magenta* tubes at the *top* of the figure. The *lines* connecting conserved cysteine residues indicate disulfide bridges with color coding corresponding to the tetraspanin groups. Secondary structure prediction was performed using the Jpred method (Cuff et al. 1998)

different tetraspanins types and compared the results to the crystallographic structure of the soluble CD81 EC2 domain (Kitadokoro et al. 2001). They found that the three helices A, B and E of the membrane proximal subdomain could be adequately predicted for all tetraspanins and possess similar lengths. On the other hand, the region located between the CCG motif and the last conserved cysteine, corresponding to helices C and D in CD81, is extremely variable in size and is composed of stretches separated by a variable number of cysteines and each yielding a predicted secondary structure variable from one tetraspanin to another (helix, strand, or loop). A multiple sequence alignment with predicted secondary structure is shown in Fig. 1.6 for the most common human tetraspanins. It was inferred from this study that the tetraspanin EC2 contains both conserved and hypervariable subdomains. The conserved subdomain retains a three helix bundle organization similar to that found in CD81 with helices A, B and E, the former and the later linking the EC2 to the transmembrane domain. The hypervariable subdomain is maintained in a defined orientation and topology with regards to the conserved subdomain by the two canonical disulfide bridges involving the CCG motif and the two other cysteines conserved in all tetraspanins. This variable subdomain is made of peptide stretches having secondary structure specific to each tetraspanin and substituting for helices C and D of CD81. In addition, this variable domain may contain one or two additional disulfide bridges. This led to a classification of tetraspanin according to the number of EC2 disulfide bridges (Fig. 1.6): group 1 (e.g. CD81, CD9, CD53) contains only the two canonical disulfide bridges; group 2 contain three disulfide bridges, it is further divided in group 2a (e.g. CD82, CD37) and group 2b (e.g. CD151, CD63, RDS/peripherin, ROM1, UPIa/UPIb) depending on the location of the third disulfide; group 3 contains four disulfide bridges and includes five tetraspanins (Tspan5, Tspan10, Tspan14, Tspan15, Tspan17 and Tspan33). Figure 1.7 shows, together with the experimental CD81 EC2 structure, homology models of two tetraspanins EC2 in which the variable stretches are non regular loops. The constant orientation and topology of the hypervariable subdomain is due not only to the two canonical disulfides (the relative orientation of which is due to the fact that each one involves successive cysteines in the CCG motif) but also to the conserved

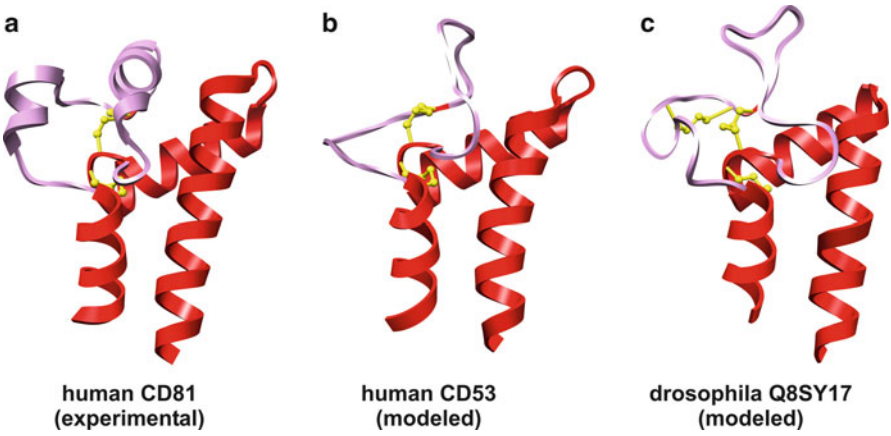

Fig. 1.7 Homology modeling of the tetraspanin EC2. A, ribbon representation of the experimental structure (Kitadokoro et al. 2001) of the human CD81 EC2 (**a**) and the predicted structures of the human CD53 (**b**) and *Drosophila Melanogaster* Q8SY17 (**c**) EC2. Conserved and variable subdomains are colored in *red* and *pink*, respectively. Disulfide bridges are in *yellow*

glycine and a conserved proline that impose specific conformational constraints to the main chain. A recent modeling study appears to confirm the occurrence of conserved and variable subdomains in peripherin/RDS (Vos et al. 2010). Using circular dichroism, secondary structure prediction and a "threading" molecular modeling approach, these authors predicted a structure with three helices at positions similar to helices A, B and E of CD81 and a region between helices B and E containing helices, strands and coils, structurally different from that of CD81.

The hypervariable subdomain in various tetraspanins is known to play an important role in their interaction with specific partners or ligands as well as in cellular processes for which tetraspanin partners still have to be identified (reviewed in (Stipp et al. 2003; Charrin et al. 2009a; Yanez-Mo et al. 2009)). Association of CD151 with α3 and α6 integrin was found to be critically dependant on a 194–196 QRD sequence located in this subdomain. Recently, the 185–192 region located upstream was also found important for the interaction with α3 integrins (Yamada et al. 2008). For CD9, known to interact laterally with proHB-EGF, residues G158, V159 and 175, also located in the variable subdomain appear to have essential roles in the upregulation of this receptor for binding of diphteria toxin (Hasuwa et al. 2001). Again for CD9, a 173–175 SFQ sequence appears essential for oocyte-sperm fusion (Higginbottom et al. 2003). Interestingly, one of these later CD9 residues is required for PSG17 binding. Also for CD81, almost all residues important for HCV E2 glycoprotein binding have been mapped in the hypervariable region (Kitadokoro et al. 2001; Drummer et al. 2002).

The hypervariable region of the EC2 represents the most salient example of how structural variability in tetraspanins may promote specificity for interaction with partners. However, structural variability at the cytoplasmic side may also contribute to such specificity. The amphipathic helix conformation of the palmitoylated N-terminal region proposed for CD81, as well as the sequence pattern responsible for

the conformation of the IC loop appear to occur in ~40% and ~60% of tetraspanins, respectively. Therefore, other tetraspanins may possess different structures in these regions that may contribute to selectivity for partners. Furthermore, the C-terminal stretch is the most heterogenous region in tetraspanins both in sequence and length. Although disordered in CD81, it may adopt specific structures in other tetraspanins and seem to be associated with very specific interactions (Stipp et al. 2003). A likely role for such cytoplasmic regions is the binding of cytoplasmic signaling enzymes such as PKCs as suggested for CD9 (Zhang et al. 2001).

1.4.2 Surface Residue Variability and Conservation in the Structurally Conserved Regions of Tetraspanins

Apart from the occurrence of structurally variable and hypervariable domains, a likely origin for the partner specificity of tetraspanins lies in the variability of surface residues in the overall structurally conserved part common to most tetraspanins. This corresponds to a large median region corresponding to the whole transmembrane domain, and the structurally conserved part of the extracellular domain proximal to the transmembrane domain (i.e. the structurally conserved subdomain of the EC2 and the EC1). Recently, Conjeaud and Seigneuret (to be published) have used the CD81 modeled structure to delineate surface and buried residues in this structurally conserved region of the molecule. Multiple sequence alignments were then used to assign surface residues in the structurally conserved regions for the best characterized human tetraspanins (i.e. the CD's, RDS/peripherin, ROM1 and UPIa/b). Pairwise sequence conservation between such surface residues was then computed for each couple of tetraspanins, separately for the transmembrane domain and the structurally conserved part of the extracellular domain. The results are displayed in Fig. 1.8.

The first observation is that the pairwise homology between surface residues of the structurally conserved part of the extracellular domain is low, sometimes close to zero and rarely reaching above 20%. In addition, when mapped onto the CD81 modeled structure, the conserved residues are scattered over the molecular surface. This suggests that although this portion of tetraspanins is structurally conserved, it retains a potential for tetraspanin-specific interactions. Therefore aside the occurrence of structurally variable subdomains, a second possible origin for the diversity and specificity of tetraspanin partners is the high variability of the surface residues of the structurally conserved part of the extracellular domain. Whilst this idea will require further experimental support in the future, recent findings from Yalaoui and colleagues indicate that a 22 residue stretch encompassing the end of EC2 helix A, the loop connecting helices A and B and helix B are all important for the ability of CD81 to support infection of hepatocytes by Plasmodium yoeli (Yalaoui et al. 2008). It was inferred that this CD81 region is mandatory for its interaction with an unidentified partner that could function as a sporozoite receptor.

A second observation drawn from Fig. 1.8 is that the pairwise homology between surface residues of the transmembrane domain can reach much higher values, (from

	Pairwise homology of transmembrane domain surface residues (%)											
	CD81	CD9	CD82	CD151	CD53	CD37	CD63	UP1a	UP1b	ROM1	RDS	
CD81		54	39	34	38	29	27	20	20	18	13	CD81
CD9	20		43	44	29	29	28	23	22	10	15	CD9
CD82	8	5		29	35	44	28	15	11	16	10	CD82
CD151	10	10	21		30	25	27	23	18	14	11	CD151
CD53	15	3	23	23		32	31	10	17	16	10	CD53
CD37	10	15	22	10	7		19	14	15	14	11	CD37
CD63	7	5	18	8	13	13		15	18	12	12	CD63
UP1a	5	10	12	7	10	7	17		48	10	9	UP1a
UP1b	5	10	12	7	10	7	22	27		10	7	UP1b
ROM1	5	5	14	11	9	2	7	11	11		55	ROM1
RDS	9	9	11	7	9	7	7	20	9	25		RDS
	CD81	CD9	CD82	CD151	CD53	CD37	CD63	UP1a	UP1b	ROM1	RDS	
Pairwise homology of EC2 domain structurally conserved part surface residues (%)												

Fig. 1.8 Pairwise homology between surface-exposed residues of the structurally conserved portion of tetraspanins. Portions framed in *red* and *blue* correspond respectively to the surface of the transmembrane domain and to the surface of the EC2 domain structurally conserved portion. Pairwise homologies are indicated in % and also background color-coded in a linear *grayscale* (*white*: 0%, *black* 100%). Surface residues were selected as those having a side chain molecular surface exposure of more that 15% as measured with the Naccess program (http://www.bioinf.manchester.ac.uk/naccess/)

30% to more than 50%). However, such high values occur only for tetraspanin pairs involved in similar functional processes (e.g. peripherin/RDS and ROM1, UPIa/b) or for some of the more ubiquitous CD tetraspanins. The fact that tetraspanin pairs without similar functional involvement do not yield such a high homology indicate that the result is not due to a bias associated with the majoritary occurrence of hydrophobic residues on transmembrane surfaces (i.e. the average random residue homology at the surface of transmembrane domains is not higher than at the surface of soluble domains). This indicates a high conservation of the transmembrane domain molecular surface for some specific tetraspanin pairs. To study this property in more details, a planar representation of tetraspanin molecular surfaces was developed (Fig. 1.9) allowing one their direct comparison. Figure 1.10a shows the result of such comparison for CD81 and CD9 for which the pairwise identity between transmembrane surface residues reaches 54%. The mapping of conserved residues on the surface of the CD81 modeled structure is shown in Fig. 1.10b. It appears that the conserved residues are organized as continuous patches of variable sizes on the tetraspanin transmembrane domain molecular surface. This leads to the proposal that such conserved patches are interaction motifs for common partners of tetraspanins. Interestingly, these observations appear to be consistent with results concerning the regions implicated in binding of EWI-2 and CD9-P1/EWI-F to tetraspanins (common partners for both CD9 and CD81). The interaction of EWI-2 with CD9 was mapped to two regions: the EC2 and the part encompassing the second half of

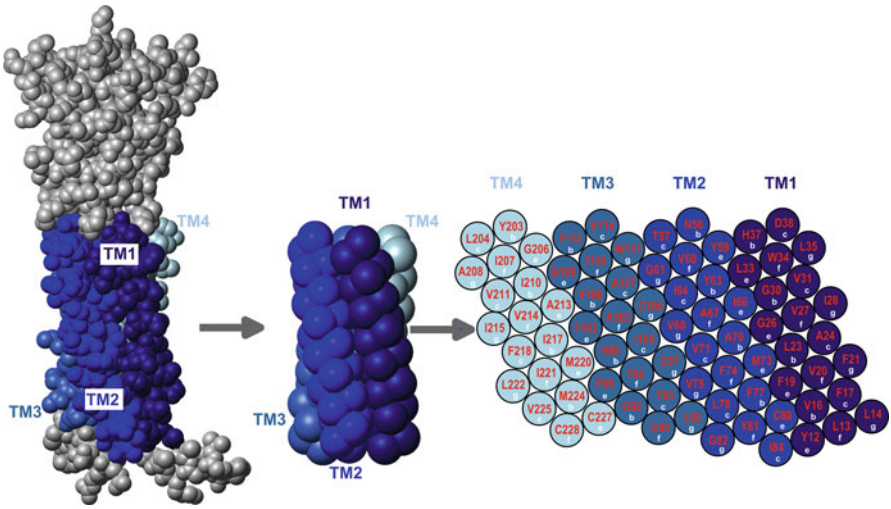

Fig. 1.9 Principle of the planar representation of tetraspanin molecular surfaces. Each residue contributing to the molecular surface of the transmembrane domain of the modeled CD81 structure (i.e. positions *b,c,f* and *g* in Fig. 1.5) was replaced by a sphere centered on its C_α. The rest of the molecule was discarded. The resulting cylinder was "cut" between TM4 and TM1, unfolded and projected onto a planar surface. A similar representation was then performed for each tetraspanin by matching the surface residues with those of CD81 using sequence alignments of the transmembrane domains. TM1-TM4 are respectively represented in *marine blue*, *blue*, *royal blue* and *light blue*

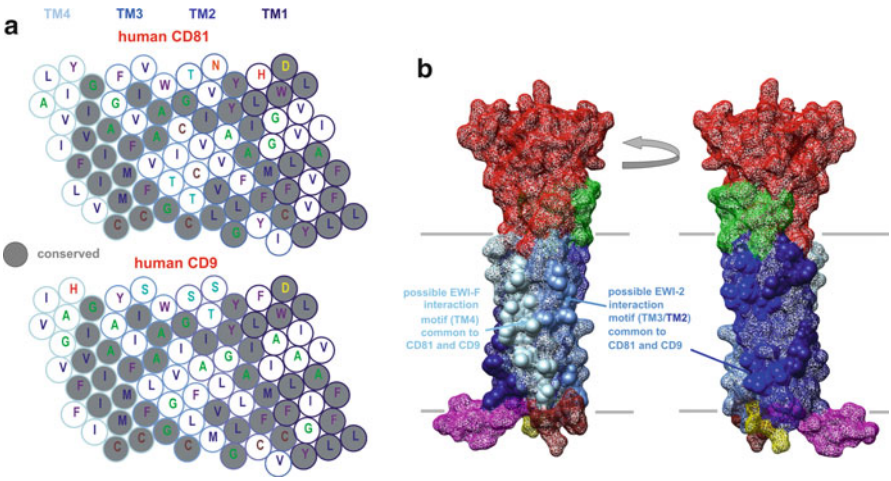

Fig. 1.10 (**a**) Detection of homologies between residues of the molecular surfaces of the transmembrane domains of CD81 and CD9. Conserved residues are on *gray* background. (**b**) Mapping of the conserved transmembrane domain surface residues between CD81 and CD9 on the molecular surface of the CD81 modeled structure. Contributions of the conserved residues are in solid surface, the rest of the molecule is in mesh surface. Colors are as in Fig. 1.3