JCB Mini-Review

Specific tetraspanin functions

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Relatively little attention has been given to the large family of abundantly expressed transmembrane proteins known as tetraspanins. Now, the importance of tetraspanins is strongly supported by emerging genetic evidence, coupled with new insights into the biochemistry and functions of tetraspanin protein complexes.

Tetraspanins are abundantly expressed transmembrane proteins (of 25–50 kD), with at least 28 distinct family members in mammals (Fig. 1), 37 members in *Drosophila*, and 20 in *C. elegans* (Todres et al., 2000). Although several types of proteins contain four transmembrane domains, they are not members of the tetraspanin family unless they contain many of the conserved residues highlighted in Fig. 2. As seen for another large family of transmembrane proteins, the integrins, several different tetraspanin family members are present on nearly all animal cell and tissue types (except on red blood cells), with individual members often present at reasonably high levels (e.g., 10–100 $\times$ 10^3/cell) (Boucheix and Rubinstein, 2001). However, in contrast to integrins, tetraspanins have received relatively little attention. This may be attributed to a lack of obvious receptor function, a dearth of functional genetic evidence, and a scarcity of key antibody reagents. There is some "generic" evidence showing that tetraspanins associate with each other, and with many other types of proteins to form large transmembrane protein networks, that regulate cell motility, trigger homotypic cell aggregation, and participate in various types of cell fusion and signaling. However, it has been difficult to sort out the detailed roles of individual tetraspanins. Highlighted here are a few important developments involving specific tetraspanins that begin to unravel the mystery of a few of these plentiful, but enigmatic, proteins.

Genetic evidence for tetraspanin function

Genetic evidence so far establishes that at least six mammalian tetraspanins (shaded in Fig. 1) are indeed functionally relevant. From this, two themes emerge: the importance of tetraspanin large extracellular loops, and the importance of tetraspanin complex formation with other proteins. Mutation of peripherin/RDS leads to several retinal diseases in humans (Kohl et al., 1998), and targeted deletion of peripherin/RDS from mice leads to disrupted photoreceptor morphogenesis (Sanyal et al., 1980). Most of the peripherin/RDS mutations that cause human disease, including the vast majority of known missense mutations, are located within the large extracellular loop (Kohl et al., 1998). The related tetraspanin protein, ROM, is less essential, but nonetheless also important for photoreceptor viability and morphogenesis in mice (Clarke et al., 2000). Disease-causing mutations prevent peripherin and ROM from forming homo- and heterotetrameric core complexes that link together into higher order structures required for photoreceptor disk formation (Loewen and Molday, 2000; Loewen et al., 2001).

The human tetraspanin TM4SF2/A15, when inactivated by a chromosomal translocation (X;2), by a premature stop codon, or by a point mutation (P172H), is associated with mental retardation (Zemni et al., 2000). Appropriately, the TM4SF2 tetraspanin is highly expressed in areas of the brain involved in learning and memory (Zemni et al., 2000). The P172H mutation demonstrates again an essential role for the large extracellular loop of a tetraspanin. Other than its association with PtdIns 4-kinase (Yauch and Hemler, 2000), little is yet known regarding the biochemistry of TM4SF2/A15.

Targeted deletion of CD81 in mice resulted in impaired B cell functions (Maecker and Levy, 1997; Miyazaki et al., 1997; Tsitsikov et al., 1997; Deng et al., 2000), and enhanced T cell proliferation (Miyazaki et al., 1997). Reduction in levels of CD19 in CD81 null mice confirms the importance of CD81-CD19 signaling complexes in B cells. Associations with molecules such as CD4 and CD8 could contribute to the signaling role of CD81 on T cells (Levy et al., 1998).

CD9 null mice produce oocytes that are deficient in sperm egg fusion (Miyado et al., 2000; Le Naour et al., 2000), indicating that oocyte CD9 plays a key role in the fusion process, or in the binding of sperm fertilin/ADAM2 protein to eggs. The occurrence of CD9-$\alpha6\beta1$ complexes is consistent with $\alpha6\beta1$ integrin also participating in fertilization (Takahashi et al., 2001). However, this is controversial because normal fertilization was observed in $\alpha6$ integrin–deficient mice (Miller et al., 2000). Besides lymphocytes and oocytes, many other cells and tissues (such as brain) show high expression of CD81 and CD9 (Schmidt et al., 1996; Sullivan and Geisert, 1998). Thus, the phenotypes of the CD9 and CD81 null mice are less dramatic than might have been expected.

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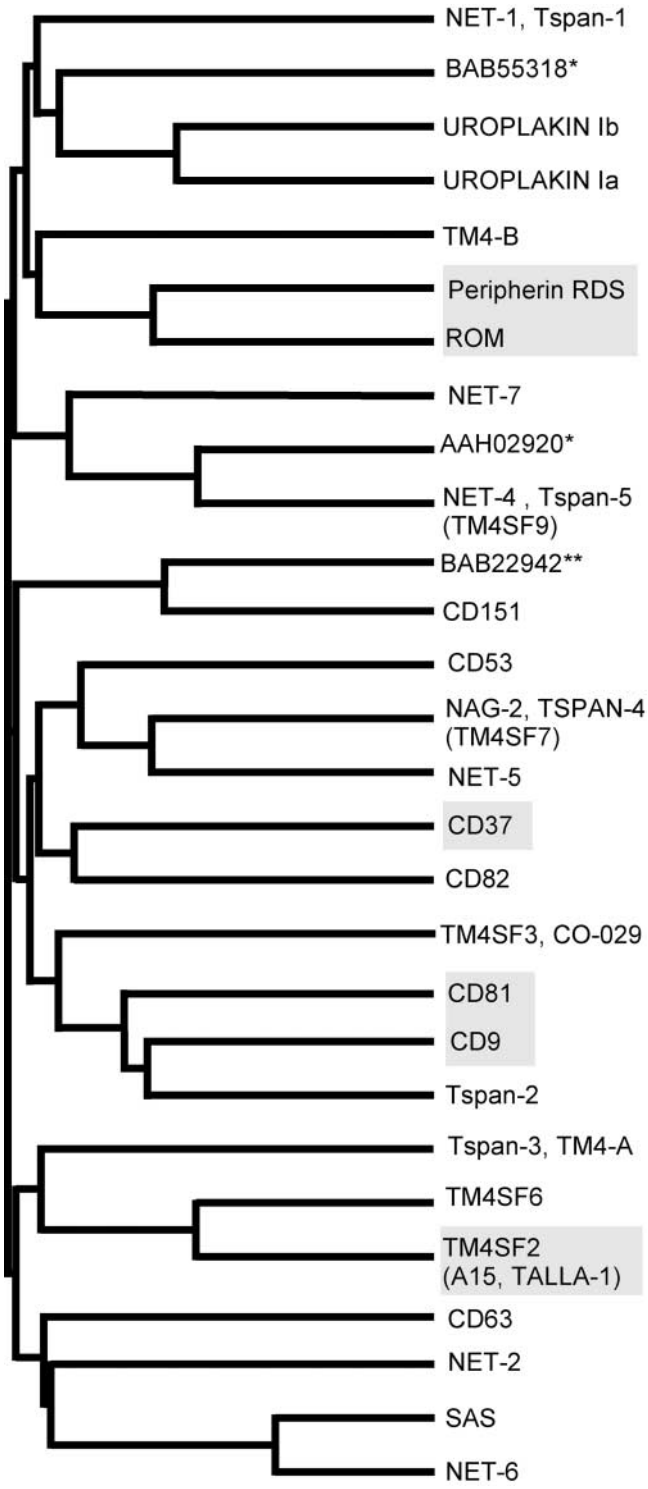

Figure 1. **The mammalian tetraspanin family.** 27 human and 1 murine protein sequences were clustered using the CLUSTALW program. *Accession number; no other names are available. **Murine sequence. Shaded tetraspanins are those that are mutated in humans, and/or deleted in mice (see text for details).

Disruption of CD37 in mice yielded a relatively subtle alteration in B cell IgG production, and a T cell–dependent immune response deficiency that was especially obvious under suboptimal stimulation conditions. Thus, CD37 regulates B cell humoral responses as well as T cell–B cell interactions (Knobeloch et al., 2000). The biochemical basis for CD37 functions on B cells is unclear, but could involve CD37 associations with CD19, CD21, MHC class II molecules, or other tetraspanins (Angelisova et al., 1994).

Other genetic evidence for tetraspanin functions comes from flies and fungi. Among the 37 *Drosophila* tetraspanins, the only one so far identified in a genetic screen is late bloomer, a facilitator of synapse formation (Kopczynski et al., 1996). Complementation and functional overlap among the many *Drosophila* tetraspanins likely accounts for the absence of additional genetic evidence. In another example, the rice blast fungus *M. grisea* contains a tetraspanin-like protein Pls1p (with 10/26 key residues shown in Fig. 1) that is essential for penetration of the fungus into host leaves (Clergeot et al., 2001). *Drosophila* and *M. grisea* tetraspanins display functions (synapse formation and fungal penetration) resembling the membrane organizing and cell migration and invasion functions of mammalian tetraspanins (Boucheix and Rubinstein, 2001).

Specific tetraspanin complexes

Early studies pointed to the potential of tetraspanins to associate with a wide variety of partner proteins in a tetraspanin web. Not only do tetraspanins associate with each other, but they also associate with many Ig superfamily proteins, proteoglycans, complement regulatory proteins, integrins, growth factors, growth factor receptors, and signaling enzymes (for review see Boucheix and Rubinstein, 2001). Assembly of the tetraspanin web is based on multiple levels of interactions (Serru et al., 1999; Stipp et al., 2001a,b; and references therein). The first level includes primary interactions between specific tetraspanins and other proteins. These interactions are direct, and resist disruption by detergents such as Brij96, digitonin, and/or Triton X-100 (see examples below). Second level interactions are indirect, more numerous, and much more sensitive to disruption by Brij96, digitonin, and/or Triton X-100. Soluble second level complexes (maintained in detergents such as Brij56, Brij99, and CHAPS) arise as tetraspanins associate with each other, and thereby link together multiple primary complexes. Tetraspanin CD151 palmitoylation does not markedly alter tetraspanin complex density or decrease detergent solubility but nonetheless does plays a role during the assembly of level 2 complexes (Yang et al., 2002). A third level of tetraspanin complex assembly occurs in the context of insoluble complexes, resistant to detergents such as CHAPS, Brij-99, or Brij-58. For example, in 1% Brij58, Brij99, or CHAPS, tetraspanin complexes become large (excluded from Sepharose 4B or 6B gel filtration columns) and light (appearing in the "light membrane" 5–25% sucrose fractions of gradients) (Skubitz et al., 2000; Claas et al., 2001). Resistance of tetraspanin complexes to detergent solubilization is consistent with their partial lipid raft-like properties (Yashiro-Ohtani et al., 2000; Claas et al., 2001). Direct tetraspanin–ganglioside association, as seen by covalent cross-linking of GM3 to CD9 (Ono et al., 2001), may also contribute to stabilization of detergent-resistant level 3 tetraspanin complexes.

Primary tetraspanin interactions within the tetraspanin web are characteristically specific (limited to only a few types of tetraspanins), proximal (captured by covalent cross-linking), soluble (included within gel filtration columns, present

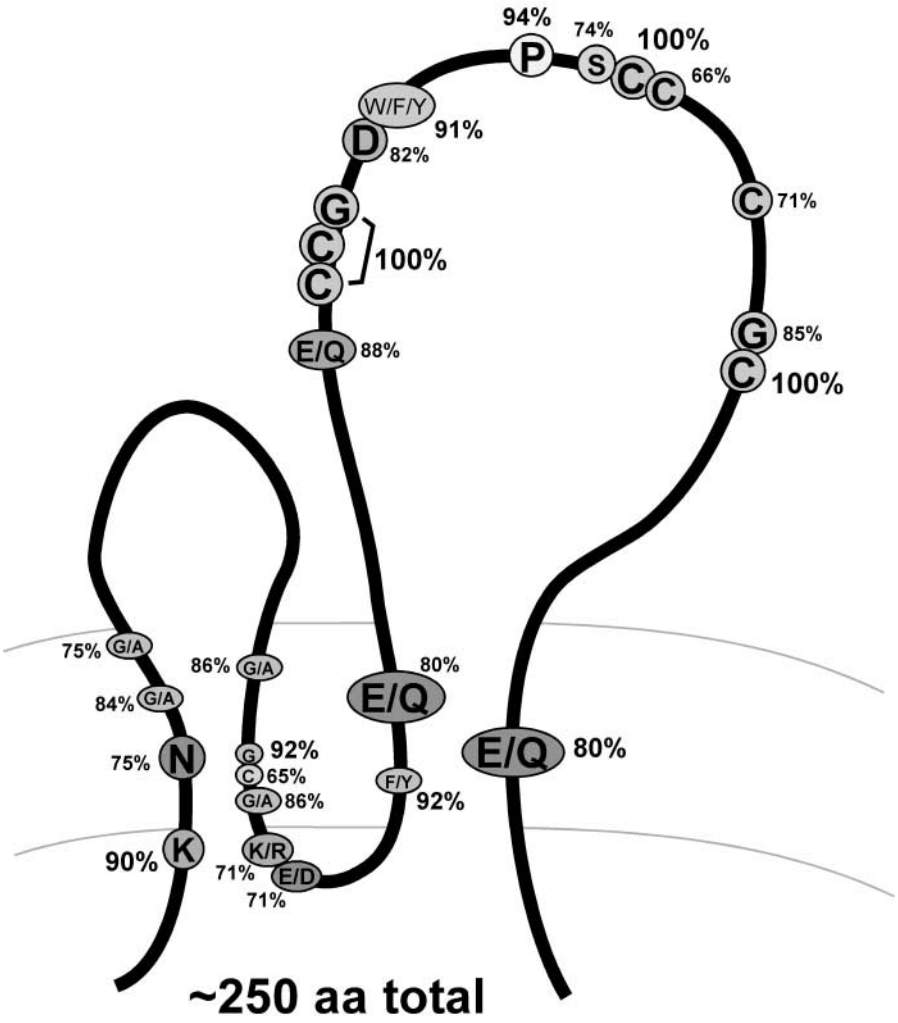

Figure 2. **Key residues present in most tetraspanins.** The percentage conservation at each of the indicated 26 amino acid positions is derived from an alignment of 28 distinct human tetraspanins (Fig. 1), together with 37 *Drosophila* tetraspanins (Todres et al., 2000). Notably, uroplakins 1a and 1b, peripherin, and ROM contain as many of these conserved residues (15–18 amino acids) as do the so-called "true" tetraspanins CD9 and CD81. Thus they also should be considered as "true" tetraspanins. Many proteins (such as connexins, CD20, sarcospan, and claudins) contain four transmembrane domains, but are not tetraspanins because they lack all or nearly all of the conserved tetraspanin residues indicated here. Despite their names, TM4SF1, TM4SF4, and TM4SF5 also lack all or nearly all of the conserved residues indicated here, and should not be considered as members of the tetraspanin family (Wright et al., 2000).

in high density sucrose gradient fractions), and of reasonably high stoichiometry (i.e., >50% in the complex). Also, it is reassuring to have evidence that the partner molecules are working together functionally. A few examples of level one interactions are highlighted here. In the urinary tract, two tetraspanins (uroplakin Ia and Ib) each associates directly with nontetraspanin type I transmembrane proteins (uroplakins II and III, respectively), as confirmed in covalent cross-linking experiments (Wu et al., 1995). Supporting the relevance of the uroplakin Ib-III complex, production of mice deficient in uroplakin III resulted in global anomalies in the urinary tract, coupled with markedly altered expression, processing, and targeting of the tightly associated tetraspanin uroplakin Ib (Hu et al., 2000). Although the uroplakin Ia-II and uroplakin Ib-III complexes (like the peripherin–ROM complexes mentioned above) each acts as a discrete primary structural unit, all four components form a tightly packed structure, essential for urothelial plaque formation (Liang et al., 2001).

For the CD9 and CD81 tetraspanins, the most robust protein partners yet found are type 1 transmembrane proteins named EWI-F and EWI-2 (also called PGLR, CD9P-1, and FPRP). EWI-2 and EWI-F, together with EWI-3 and EWI-101 (CD101), comprise a novel subfamily of structurally related immunoglobulin superfamily proteins that share a specific Glu-Trp-Ile (EWI) motif in their second Ig domain (Clark et al., 2001; Charrin et al., 2001; Stipp et al., 2001a,b). Tetraspanins CD9 and CD81 each can associate directly (as captured by covalent cross-linking) with either EWI-2 or EWI-F in distinct complexes, and with high stoichiometry (70–100%). Also, associations occurred in the soluble phase, were well maintained in detergents such as digitonin, Brij 96, and Triton X-100, and were not sensitive to cholesterol depletion (Charrin et al., 2001; Clark et al., 2001; Stipp et al., 2001a,b). Further studies are now needed to evaluate how EWI proteins might influence various CD81 and CD9 functions in brain, liver, developing muscle, nervous system, oocytes, lymphocytes, or elsewhere. For example, EWI proteins could potentially modulate CD9 effects on sperm egg fusion (Le Naour et al., 2000; Miyado et al., 2000), myoblast fusion (Tachibana and Hemler, 1999), diphtheria toxin binding (Cha et al., 2000), or the mitogenic activities of membrane bound forms of HB-EGF (Nakamura et al., 2000), and TGFα (Shi et al., 2000). Likewise, EWI proteins need to be examined for modulation of CD81 functions in the brain (Kelic et al., 2001), and during hepatitis C virus binding (Pileri et al., 1998; Higginbottom et al., 2000), myoblast fusion (Tachibana and Hemler, 1999), and lymphocyte signaling (Levy et al., 1998). In this regard, strong association of EWI-2 with CD81 on B cell lines

(Clark et al., 2001) would seem likely to influence the well-characterized CD81-CD19-CD21 signaling complex on B cells (Levy et al., 1998).

Among the tetraspanins, CD151 stands out because of its stable association with ~100% of cellular α3β1 integrin in several cell lines, and the majority of α6 integrins on a lymphoid cell line (Serru et al., 1999; Yauch et al., 2000). The association of CD151 with the α3β1 integrin can be captured by covalent cross-linking and requires the CD151 large extracellular loop, as well as the integrin α3 subunit extracellular stalk region (Yauch et al., 2000; Berditchevski et al., 2001). Supporting functional relevance of the CD151–α3β1 complex, anti-CD151 antibody inhibited neurite outgrowth when α3 integrin was engaged with laminin-5 ligand, but not when other integrin ligands were used and α3β1 was unengaged (Stipp and Hemler, 2000). As part of a CD151–α6β1 complex, CD151 exerts a major influence on α6β1 integrin–dependent morphogenesis when fibroblast and endothelial cells are grown on basement membrane matrigel (Zhang et al., 2002). In particular, mutation of the short COOH-terminal tail of CD151 completely abolished α6β1-dependent mobilization of fibroblasts into a meshwork of cellular cables. The functional relevance of specific CD151 association with α6β4 integrin in hemidesmosomes remains to be established (Sterk et al., 2000). However, in a kidney epithelial cell line that lacks α3 integrin, CD151 associated predominantly with α6β4 and markedly influenced cellular morphology. In each of the above-mentioned studies, the CD151 molecule had minimal effects on α3 or α6 integrin–mediated cell adhesion, but rather modulated the subsequent outside-in functions of the integrins. Although molecular details are still sketchy, tetraspanins are reported to alter integrin-dependent signaling through focal adhesion kinase, in parallel with rearrangement of the cortical actin cytoskeleton (Berditchevski and Odintsova, 1999).

Although the extracellular domain of CD151 is required for integrin association, it is the intracellular and/or transmembrane domains that regulate cell morphology (Zhang et al., 2002) and associate with signaling enzymes such as conventional PKC isoforms (Zhang et al., 2001). Thus, CD151 may function as a "transmembrane linker" (Hemler, 1998). Such a function helps to explain how the extracellular domain of the integrin α3 subunit would be involved in the recruitment of intracellular PKC and intracellular phosphorylation of the α3 cytoplasmic tail (Zhang et al., 2001). Because other tetraspanins also use extracellular domains to associate with partner proteins, and cytoplasmic or transmembrane domains to associate with intracellular signaling enzymes (Boucheix and Rubinstein, 2001), the transmembrane linker theme could extend well beyond the CD151–α3β1 complex.

Concluding comments

This review highlights specific details regarding a few tetraspanins, which may represent emerging trends, that could be extrapolated to many other tetraspanins. However, many questions remain. For example, it is not clear why several tetraspanins have been associated either positively (C0-O29 and CD151) or negatively (CD9, CD63, and CD82) with tumor cell metastasis (Boucheix et al., 2001). Possibly, the tendency of multiple tetraspanins to localize into endothelial and epithelial cell–cell junctions (Yánez-Mó et al., 1998, 2001) will provide a context in which to influence tumor cell behavior.

Recent technical advances are now helping to guide tetraspanin research. For example, detailed brain analyses and DNA array screens are turning up tetraspanins with increasing frequency. In addition, advances in protein identification by mass spectrometry have accelerated the rate of discovery of tetraspanin-associated proteins. As more of the specific level 1 and 2 tetraspanin partners are identified, and the rules for level 3 membrane microdomain assembly are clarified, the current tetraspanin morass should gradually be revealed as a coherent and intricate tetraspanin network. A general working hypothesis is that tetraspanins act as molecular facilitators or transmembrane linkers, using intracellular and/or transmembrane domains to recruit key signaling enzymes, whereas large extracellular loops engage other transmembrane proteins in specific lateral associations (Hemler, 1998; Zhang et al., 2001). In this manner, tetraspanins may assemble a novel type of protein/lipid microdomain with considerable functional potential.

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