Protein-Protein Interactions in the Tetraspanin Web

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Tetraspanins are evolutionarily conserved membrane proteins that tend to associate laterally with one another and to cluster dynamically with numerous partner proteins in membrane microdomains. Consequently, members of this family are involved in the coordination of intracellular and intercellular processes, including signal transduction; cell proliferation, adhesion, and migration; cell fusion; and host-parasite interactions.

Tetraspanin structure

As can be inferred from their name, tetraspanins contain four transmembrane (TM) domains. They differ from other proteins with four TM domains by a shared overall structure of the extracellular and intracellular domains and by the presence of conserved amino acid residues (19, 36) (FIGURE 1).

A review focusing on functional domains within this structure has recently been published (50); therefore, the salient features important for protein-protein interactions are briefly summarized here. The tetraspanin TM domains flank a small and a large extracellular loop (SEL and LEL, respectively). The extracellular loops vary among family members. On the other hand, the LEL contains characteristic motifs, especially the conserved cysteine-cysteine-glycine (CCG) motif, which is the focal point for the formation of two to four disulfide bridges with additional cysteine residues at fixed positions within the LEL (45). The three-dimensional structure of the homodimer of one tetraspanin LEL, CD81, has been solved (25, 26) (FIGURE 1). Most tetraspanins' LELs are glycosylated in one or more potential N-glycosylation sites. Such sites are rarely present in the SEL, and, when they are present, it is unknown whether they are indeed glycosylated. The presence of highly conserved cytoplasmic cysteine residues is an additional hallmark of this family. These cysteines have been shown to be palmitoylated and to play a central role in interactions within the tetraspanin web, as detailed below.

Tetraspanins are evolutionarily highly conserved and expressed ubiquitously

The first members of this family were identified in human (22) and in *Schistosomes* (60). Confirmation of the conservation of this family in evolution comes from recent studies that have identified members of this family in early metazoa (1), in fungi (18), and in nematodes (41). Moreover, multiple tetraspanins are expressed in each organism; for example, there are at least 37 family members expressed in *Drosophila* (55). In humans, tetraspanins are expressed in all cell types, each of which usually expresses multiple family members.

Tetraspanins are distinguished from other membrane proteins: clustering in the tetraspanin web

The most distinctive feature of the tetraspanin family is the ability of its members to form lateral associations with multiple partner proteins and with each other in a dynamic assembly, described as the tetraspanin web (44) (FIGURE 2). The tetraspanin web plays an important role in cell-cell interactions and in cell-fusion events.
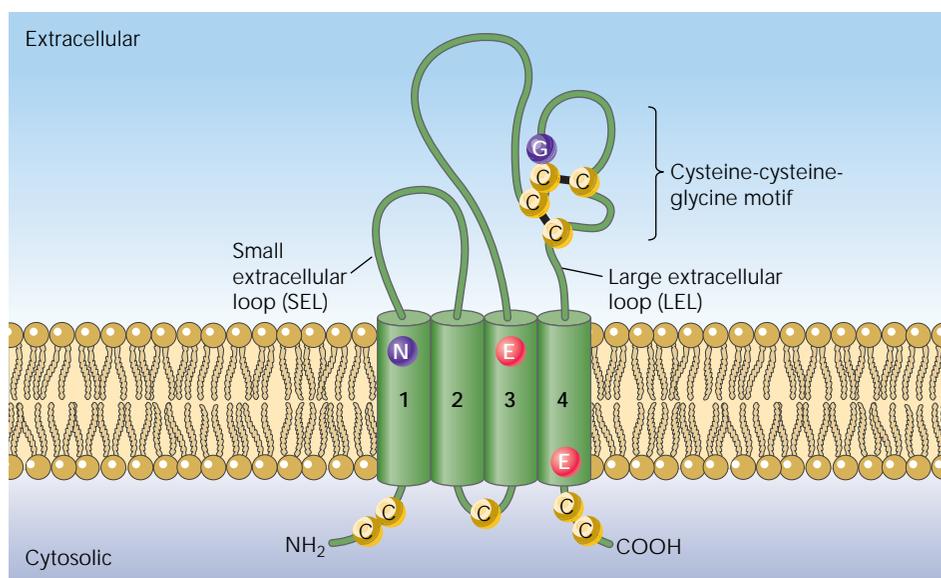

FIGURE 1. **Structural features of tetraspanins**
Tetraspanins are composed of 4 transmembrane domains (TM; green cylinders) containing conserved residues. TM1 and TM2 flank the SEL, and TM3 and TM4 flank the LEL. Tetraspanins contain several conserved amino acid residues, as detailed in previous reviews (19, 36). Shown here is the conserved CCG motif in the LEL, which forms disulfide bridges (black lines) with additional conserved cysteines (peach circles). The example shown is that of CD81 LEL, whose 3-dimensional structure has been solved (25, 26) and which contains 2 disulfide bridges. The number of disulfide bridges varies from 2 to 4 among family members (45). The CD81 LEL does not contain potential glycosylation sites, unlike most tetraspanins. Highlighted here are conserved cysteines , which are potential palmitoylation sites in the amino and carboxy terminals and the inner loop of the intracellular portion of the molecules.

Thus CD81 was shown to localize in immune synapses of both B and T cells (38). Both CD9 and CD81 were shown to be involved in egg-sperm fusion (21, 23), and mice that are deficient for CD9 and for CD81 spontaneously develop multinucleated giant cells in the lung (52). The expression of CD81 is required for the infection of hepatocytes by *Plasmodium yoelii* in mice, and an anti-CD81 MAb inhibits the infection of human hepatocytes by *Plasmodium falciparum* (48). The tetraspanin *PSL1*, expressed by the fungus *Magnaporthe grisea*, is necessary for its binding and penetration of rice leaves (12).

The associations within the web of tetraspanins with their nontetraspanin partner molecules have been referred to as primary (4). A distinct aspect of primary interactions is that a single tetraspanin molecule can form different partnerships in different cell types. Thus the same tetraspanin molecule that forms a partnership with protein X in one cell type can form a partnership with protein Y in a second cell type. For example, CD81 associates in B cells with a B-lineage-specific molecule, CD19 (6), whereas in T cells it associates with CD4 and CD8 (54). The most common partners of tetraspanins are specific members of the integrin (2) and the immunoglobulin superfamilies (6, 49). Finally, partnerships can be formed with the extracellular or the intracellular domains of the interacting molecules. Thus CD81 associates with the extracellular domain of CD19 (5, 37) and with the intracellular domain of another partner, EWI-2 (28, 49). Both CD19 and EWI-2 are members of the immunoglobulin superfamily (FIGURE 3).

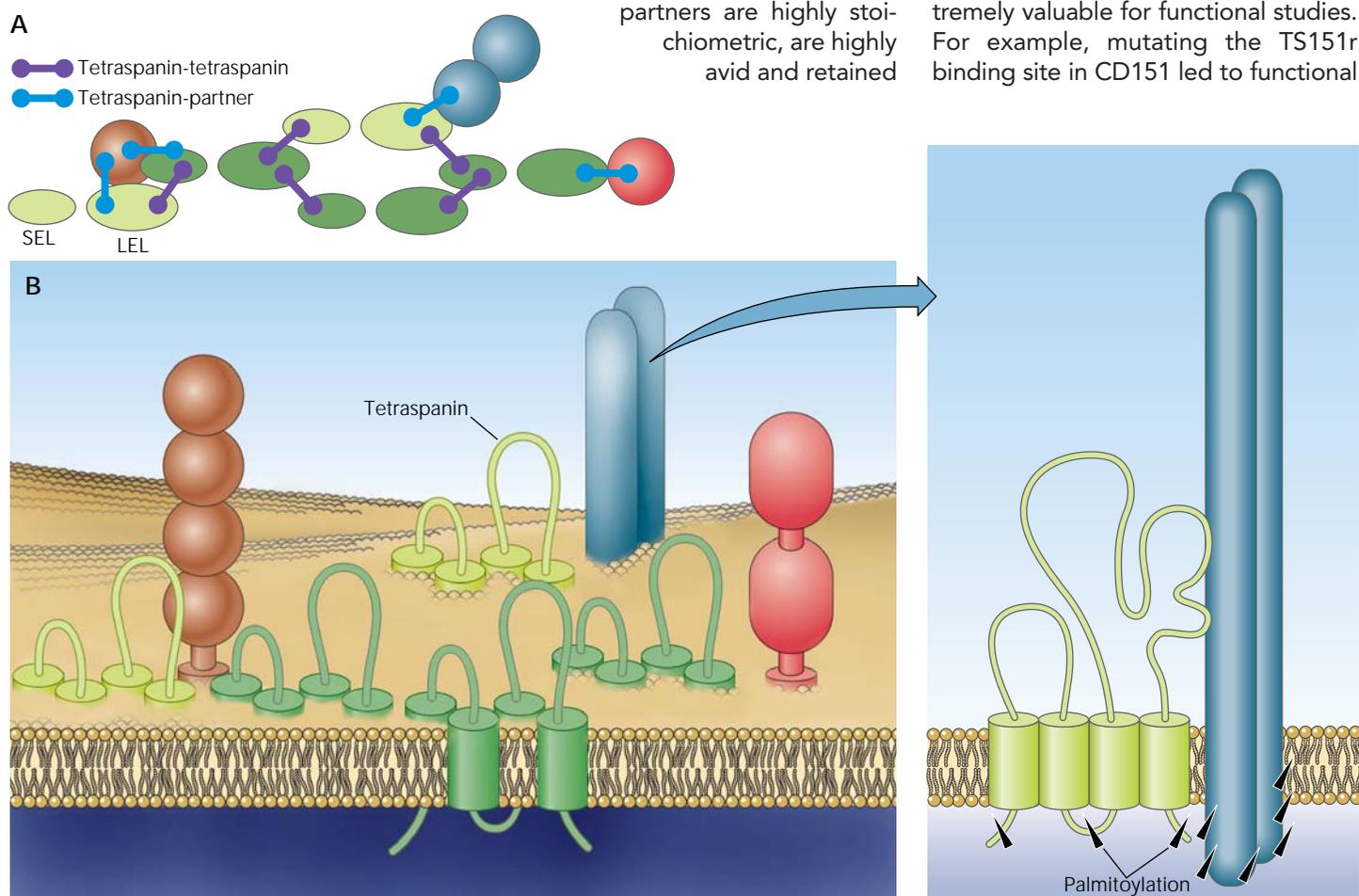Some associations with partners are highly stoichiometric, are highly avid and retained after lysis in harsh detergents (such as 1% NP-40), and are direct, as demonstrated by chemical crosslinking (61, 66). Examples of such avid partnerships are the association of CD151 with $\alpha3\beta1$-integrin and the pairing of uroplakins (57, 65). Reported additional associations between tetraspanins and partner proteins have been documented by nonquantitative coimmunoprecipitation in milder detergent conditions, such as 1% Brij 96 or 1% Brij 97, or by fluorescent energy-transfer studies (51). However, several detailed studies have demonstrated that specific MAbs, e.g., the anti-CD151 MAbs, TS151r (46), and 8C3 (42), can disrupt these associations (24, 42, 46). Therefore, coimmunoprecipitation studies with antibodies that disrupt associations may underestimate the stoichiometry of interactions. Nevertheless, such MAbs are extremely valuable for functional studies. For example, mutating the TS151r binding site in CD151 led to functional

FIGURE 2. **Membrane clustering of tetraspanin-enriched microdomains**
A: aerial view of tetraspanin-partner and tetraspanin-tetraspanin interactions. *B*: side view of lateral associations of tetraspanins with partner molecules. Clustering is facilitated by palmitoylation of conserved cysteines in the intracellular domains of the interacting proteins (*right*). Tetraspanins are shown in shades of green; partners of the immunoglobulin superfamily are shown in red and brown, and those of the integrin are in blue.
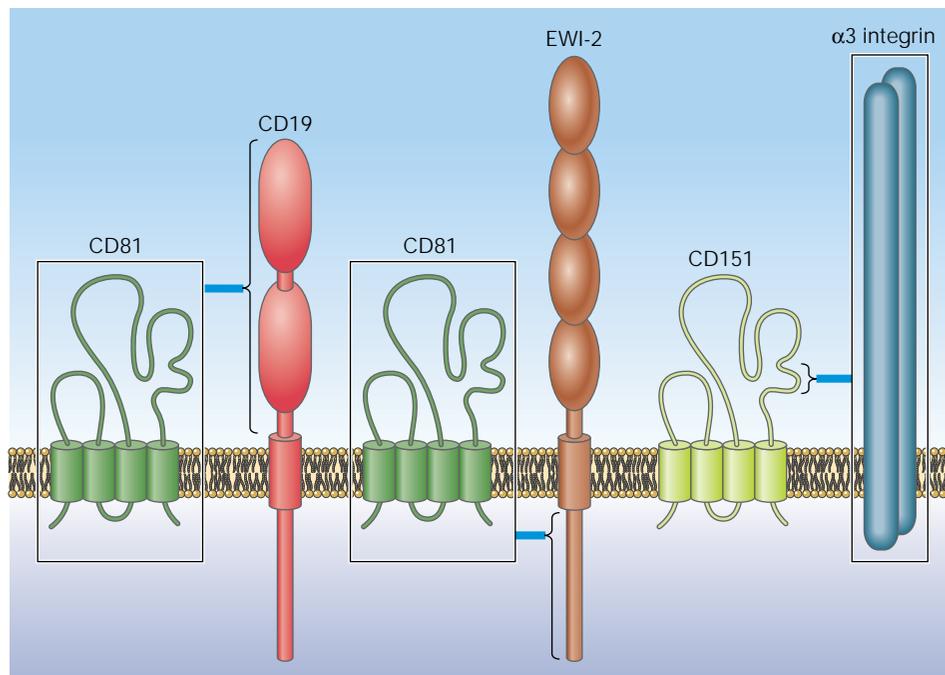
FIGURE 3. Interactions of tetraspanins with partner proteins can be formed via different structural domains

The site of interaction between CD151 and the α3-integrin has been mapped to the QRD sequence in the CD1515 LEL, which is the binding site of the TS151r MAb. The interaction between CD81 and EWI-2 depends on the cytoplasmic portion of EWI-2, whereas the interaction of CD81 with CD19 requires the extracellular domain of CD19. Brackets indicate mapped interacting domains. See text for references.

changes in integrin-dependent spreading (24), and the removal of CD151 from α3β1-integrin by the MAb 8C3 led to reduced binding of the stripped integrin to laminin (42).

Tetraspanin-tetraspanin associations, formed between members of the family, have been referred to as secondary (4). These secondary associations are not disrupted by mild detergents (1% Brij 96 or 1% Brij 97) and can be augmented by the presence of divalent cations (7). Tetraspanin-tetraspanin interactions are not stoichiometric, and it is still unknown whether all tetraspanins expressed in a certain cell are associated with each other. Recent studies have demonstrated that palmitoylation is necessary for the maintenance of tetraspanin-tetraspanin interactions. Site-directed mutagenesis of all of the juxtamembrane cysteines of CD9 and of CD151 resulted in the disruption of these secondary interactions (7, 63).

Importantly, tetraspanins associate indirectly with additional proteins, which have been referred to as class 3 interactions. These tertiary interactions are not disrupted in milder detergents, such as 1% CHAPS. Functionally, these interactions cluster in tetraspanin-enriched microdomains, enabling lateral dynamic organization in the membrane and the cross-talk with intracellular signaling and cytoskeletal structures.

Tetraspanin web proteins cluster in the membrane in tetraspanin-enriched microdomains

The fact that several tetraspanin molecules form primary, secondary, and tertiary biochemical interactions and that the engagement of individual tetraspanins could lead to identical functional responses in single cell types led to the concept of the tetraspanin web (4). Additional studies documented the clustering of tetraspanins and partner proteins with cholesterol (8) in tetraspanin-enriched microdomain membrane (TEM) (3).

Functional lateral associations of tetraspanins and partner proteins in TEM have been documented in the immune system. For example, antigen-presenting cells (APC) secrete exosomes (17), which are enriched in tetraspanins and were shown to be extremely effective in antigen presentation (70). These exosomes are the secreted fusion products of the plasma membrane with major histocompatability complex (MHC) class II compartments (MIIC). MIIC consist of multivesicular bodies that are enriched in MHC class II and in the MHC class II-like molecules, which function in the intracellular processing and loading of peptide antigens. In addition, MIIC are enriched in the tetraspanins CD37, CD53, CD63, CD81, and CD82. Tetraspanins were also shown to cluster on the cell surface of APC with a selected subset of MHC class II molecules. In humans, such clusters are identifiable by a specific MAb that interacts only with clustered MHC class II epitopes (CDw78). These clusters were shown to preferentially function in the actual presentation of selected peptide antigens (30).

Tetraspanins also cluster in specialized structural units. For example, there are specialized family members that are present in the apical surface of mammalian bladder epithelium (urothelium) in crystalline structures called urothelial plaques. These plaques are highly enriched in four proteins, two of which, uroplakins Ia and Ib (UPIa and UPIb), are tetraspanins that associate with their nontetraspanin partners uroplakin II (UPII) and uroplakin III (UPIII) (67). Biochemical studies have shown that the four uroplakins interact in pairs of one tetraspanin and one nontetraspanin: UPIa/II and UPIb/III (33). Uroplakins serve as an excellent model to illustrate how primary interactions are formed because of the reduced complexity of their interactions in the urothelium, as detailed below.

Certain tetraspanins are enriched in specific subcellular organelles. The best example of this is CD63, which contains the lysosomal targeting motif GYEVM. Interestingly, the *Drosophila* sunglasses (*sun*) tetraspanin, which is most closely related to CD63 and is accumulated in lysosomes, does not contain this motif (62).

Tetraspanin-partner associations during biosynthesis: lessons from knockout mice

The fundamental question of whether tetraspanin-partner associations occur during biosynthesis has recently begun to be addressed. The most stringent dependency on tetraspanin-partner coexpression during biosynthesis has been shown for uroplakins. UPIa and UPIb form partnerships with UPII and UPIII, respectively, that are highly stoichiometric. Transfection of uroplakin molecules into nonurothelial cells demonstrated that these specific pairings are initiated in the endoplasmic reticulum (ER) and are required for exit of the interacting molecules from this compartment (57). This conclusion was strengthened by the subsequent generation of UPII- and UPIII-deficient mice, which have impaired urothelial plaques because of lack of heterodimer formation (15, 29). These studies in knockout mice establish the role of the nontetraspanin uroplakins (UPII and UPIII) in the exit of their associated tetraspanin uroplakins (UPIa and UPIb) from the ER/pre-Golgi to the cell surface.

It was therefore expected that CD151, which is strongly associated with the α3-integrin (46, 65) would be required for the cell-surface expression of this integrin. This expectation was strengthened by a study in a human cell line that indicated that CD151 and α3-integrin associate during biosynthesis (24). However, CD151-deficient mice express normal levels of α3-integrin and of other integrins (59). This study demonstrates that neither a very strong association on the cell surface nor even an association during biosynthesis necessarily implies a regulatory role in the trafficking and cell-surface expression of a partner.

Unlike CD151, CD81 is necessary for cell-surface expression of its B cell partner, CD19 (47). The partnership of CD81 with CD19 was first demonstrated in human B cells (6). Subsequently, three independent lines of CD81-deficient mice showed reduced CD19 cell-surface expression (35, 40, 56). However, some CD19 molecules are expressed on the cell surface in the absence of CD81, possibly implicating additional proteins in the regulation of CD19 expression. The possibility of redundancy of tetraspanins to support CD19 expression was investigated by analyzing mice deficient in CD9 (47), CD37 (27), Tssc6 (53), and Tspan3 (unpublished observations). All of these express normal levels of CD19. It is also important to note that the CD81/CD19 partnership differs from that of the paired uroplakins in that the relationships are not reciprocal; CD19-deficient mice have normal expression of CD81 (47).

Therefore, web assembly may occur in multiple steps in conjunction with trafficking within the different cellular compartments in which web subunits are formed (FIGURE 4). Tetraspanin-partner interactions may be initiated early, in the ER, as seen for the pairing of uroplakins or of CD151 with the α3-integrin. However, these early interactions are not necessarily required for cell-surface expression of the associated tetraspanins and their partner proteins, as seen for CD151 and the α3-integrin. Tetraspanin-tetraspanin associations are more likely to occur in the Golgi compartment because they depend on palmitoylation, as detailed below.

Maintenance and dynamics of interactions in the tetraspanin web

Palmitoylation of the intracellular cysteine residues has been shown to play an important role in the maintenance of tetraspanin-tetraspanin interactions. The replacement of these cysteines by other amino acids, which abolished the palmitoylation of CD9 (7) and of CD151 (3, 63), reduced their association with other tetraspanins.

Significantly, the palmitoylation of partner proteins also contributes to their incorporation into TEM, as demonstrated in a recent study of the specific associations of CD151 with the laminin-binding integrins α3β1, α6β1, and α6β4 (64). This study demonstrated that not only CD151 but also its coprecipitated, laminin-binding α3-, α6-, and β4-integrins, but not α2-integrin, were palmitoylat-ed. Moreover, the replacement of all seven cysteines by serine residues in the β4-integrin abolished the palmitoylation of this integrin. This, in turn, impaired its inclusion in TEM with other laminin-binding integrins, with CD151, and with the additional tetraspanins CD9, CD63, and CD81. Functionally, this depalmitoylation had an effect on cell spreading.

The palmitoylation of CD81 was also shown to affect its association with a member of the serine/threonine-binding signaling protein family called 14-3-3s. Interestingly, this association occurred only under oxidative stress, which inhibited the palmitoylation of CD81. This was confirmed by substituting all five cysteines in CD81 with alanines, which induced the association of CD81 with the 14-3-3 protein (11).

Most importantly, palmitoylation has been shown to play a key role in the dynamic reorganization of TEM in B cells responding to stimulation. An earlier study demonstrated that coengagement of two complexes on the surface of B cells, the B cell receptor (BCR) complex and the CD19/C21/CD81 complex, reduced the threshold of B cell activation (14). Subsequently, it was demonstrated that the coengagement of the two molecular complexes on the surface of B cells induced membrane reorganization in a CD81-dependent manner, enabling enhanced signaling (10). The most recent study (9) demonstrated that the coengagement of both complexes was accompanied by an increase in the incorporation of labeled palmitic acid into CD81. Moreover, the newly palmitoylated molecules were associated with detergent-resistant microdomains (DRM) and were chased from the low-density sucrose fractions containing the DRM, suggesting that this modification is dynamic.

Tetraspanins as cell-surface receptors

An endogenous soluble ligand, PSG17, has been identified for mouse CD9 (58). The tetraspanin CD9 is required for egg-sperm fusion, and females of CD9-deficient mice have severe fertility impairment (23, 31, 39). Interestingly, the PSG17 ligand
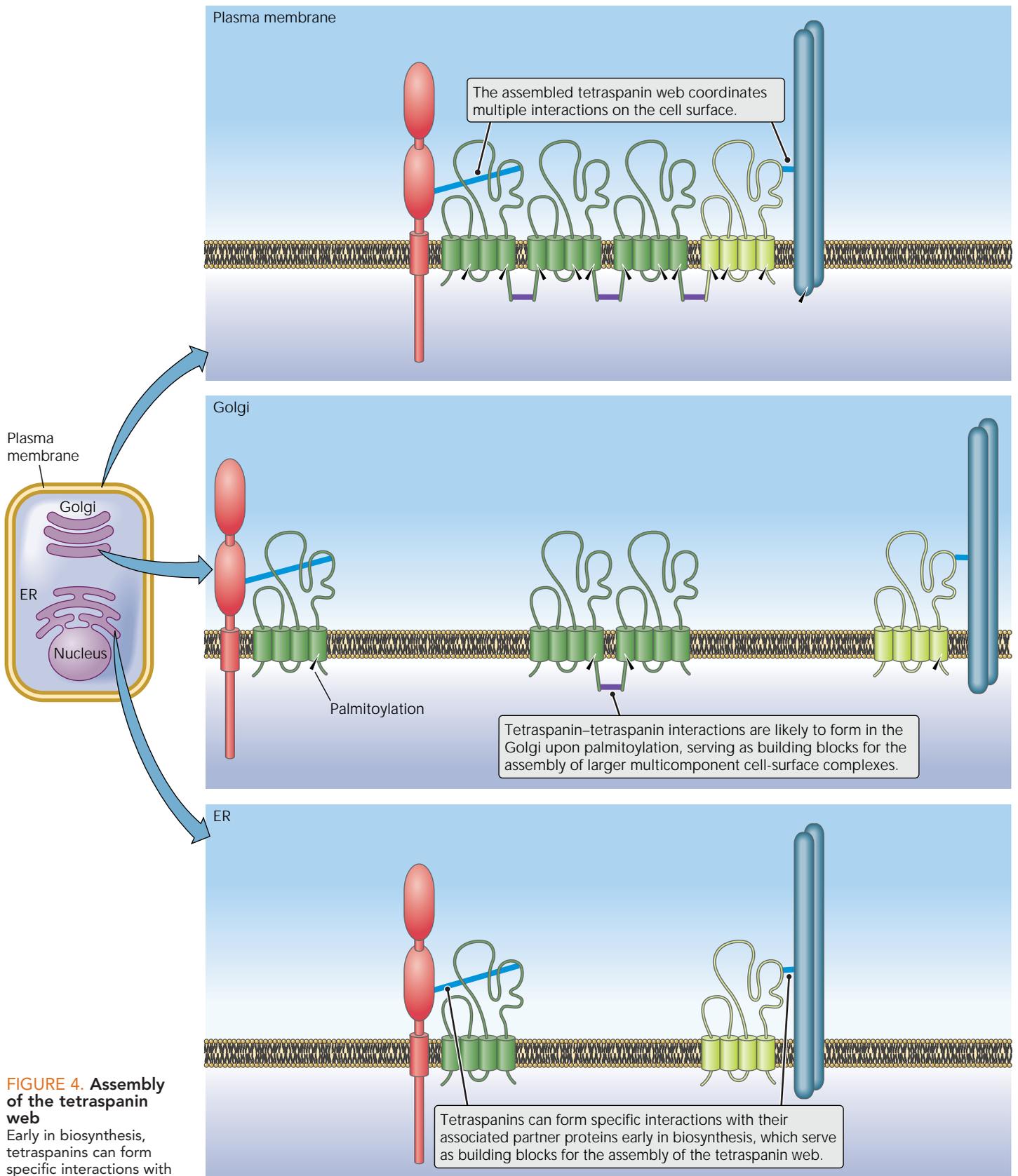
Plasma membrane

The assembled tetraspanin web coordinates multiple interactions on the cell surface.

Golgi

Palmitoylation

Tetraspanin–tetraspanin interactions are likely to form in the Golgi upon palmitoylation, serving as building blocks for the assembly of larger multicomponent cell-surface complexes.

ER

Tetraspanins can form specific interactions with their associated partner proteins early in biosynthesis, which serve as building blocks for the assembly of the tetraspanin web.

FIGURE 4. Assembly of the tetraspanin web
Early in biosynthesis, tetraspanins can form specific interactions with their associated partner proteins in the ER, as demonstrated for the pairing of uroplakins and for the association of CD151 with the α3-integrin. Tetraspanin-tetraspanin interactions are likely to form in the Golgi upon palmitoylation, leading to the assembly of larger multicomponent complexes. Palmitoylation of specific partners, such as laminin-binding integrins, may also contribute to the clustering in the tetraspanin web. Tetraspanins (shades of green) partner immunoglobulin (red) or integrin (blue) family members. Tetraspanin-partner interactions (blue ties), tetraspanin-tetraspanin interactions (purple ties), and palmitoylation (black triangled arrows) are also shown.

was shown to partially block sperm-egg fusion (16); however, the biological relevance of this finding is unclear, because the pregnancy-specific protein is not likely to regulate gamete fusion. An additional tetraspanin, CD81, has been identified as the receptor for the hepatitis C virus envelope protein E2 (43). The binding site for E2 on CD81 has been mapped to an epitope located within the inner helices, which are in close proximity to the disulfide bridges within the LEL (FIGURE 1) (20). Interestingly, the homologous region in CD9 is important for egg-sperm fusion (69) and the same region in CD151 is required for its lateral interaction with the α3-integrin (24). Currently, CD9 and CD81 are the only known tetraspanins that were identified as cell-surface receptors for soluble ligands.

Tetraspanin signaling ports

Additional studies have implicated tetraspanins in the coordination of intracellular signaling pathways with the cytoskeleton, as detailed in a recent review (32). Briefly, Rho-GTPase was shown to link the cytoskeleton with CD82 (13); G protein-coupled receptors were demonstrated to be dynamically regulated by CD9 and CD81 in human cells (34) and by *sun* in *Drosophila melanogaster* (62); and phosphatidylinositol 4-kinase and protein kinase C, the signaling components that interact with integrins, were also shown to associate with several tetraspanins (68). In light of these facts, we suggest that TEM are microenvironments analogous to transportation ports. However, unlike seaports and airports, which function as hubs of activity but occupy fixed positions, tetraspanin-signaling ports are dynamic and quick to respond and to coordinate intracellular and inter-cellular activities. ■

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