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Review

Tetraspanins

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Abstract. The first tetraspanins were discovered on surface of human leucocytes, but it was rapidly demonstrated that they had a wider tissue expression. Twenty-six molecules display sufficient homology to belong to the same superfamily. Their function is not precisely known, but data coming from biochemical studies or knockout mice suggest that they play a major role in membrane biology. One of their outstanding properties is their ability to form a network of multimolecular complexes, the 'tetraspanin web', in which integrins are included. The structure of these complexes is under investigation, but some of the rules that govern their organization have already been unraveled. The challenge is to determine how the organization of the 'tetraspanin web' modifies the function of its constitutive molecules and consequently influences cellular behaviour. The implications may be considerable for the understanding of basic cellular processes such as migration and also of diseases related to loss or mutation of a single tetraspanin.

Key words. Tetraspanin; integrin; cancer; cell migration; reproduction; fusion.

Structure and family members

Tetraspanins are 204- (SAS) to 355 (oculospanin)-amino acid surface proteins (table 1). They are characterized by four transmembrane domains delimiting two extracytoplasmic regions of unequal sizes, a small extracellular loop (EC1) containing 20–28 amino acids and a large extracellular loop (EC2) containing 76–131 amino acids (fig. 1) [1, 2]. The crystal structure of CD81 EC2 has recently been described [3] and is detailed in the legend of figure 1. Tetraspanins are distinct from other proteins, with four transmembrane domains by the conservation of certain amino acids depicted in fig.1 [1] and probably also of certain structural features of EC2 [3]. The cytoplasmic tails contain fewer than 19 amino acids, although NET-2, NET-7, peripherin/RDS and Rom-1 have a longer C-terminal cytoplasmic domain (table 1, fig. 1). Apart from CD9, which is glycosylated in EC1 [4], and CD81 [5] and NET-2, which are not, most tetraspanins are potentially N-glycosylated in EC2 [2].

Twenty-six human tetraspanins have been identified, nearly half those resulting from follow-up of EST (expressed sequence tag) databases (fig. 2). The expression of several tetraspanins in invertebrates (*Drosophila*, *Schistosoma*, *Caenorhabditis Elegans*) [6–8] indicates that these molecules appeared early during evolution. The tetraspanin genes in human are located on different chromosomes; several are located on chromosome 11 (CD81, CD82, CD151 and NAG-2 and Rom-1) and on chromosome 12 (CD9, CD63, C0-029, SAS and NET-5). The conservation of gene structure strongly suggests that these molecules derive from a common ancestor, by duplication.

The tetraspanins include leucocyte differentiation antigens CD9, CD37, CD53, CD63, CD81/TAPA-1, CD82 and CD151; antigens first identified on tumours, TALLA-1, Co-029 or SAS [9–11] and the tetraspanins discovered in EST databases [12, 13]. Although more distinct, the uroplakins UP1a and UP1b [14], and the proteins encoded by the retinal dystrophy syndrome genes, RDS/peripherin and

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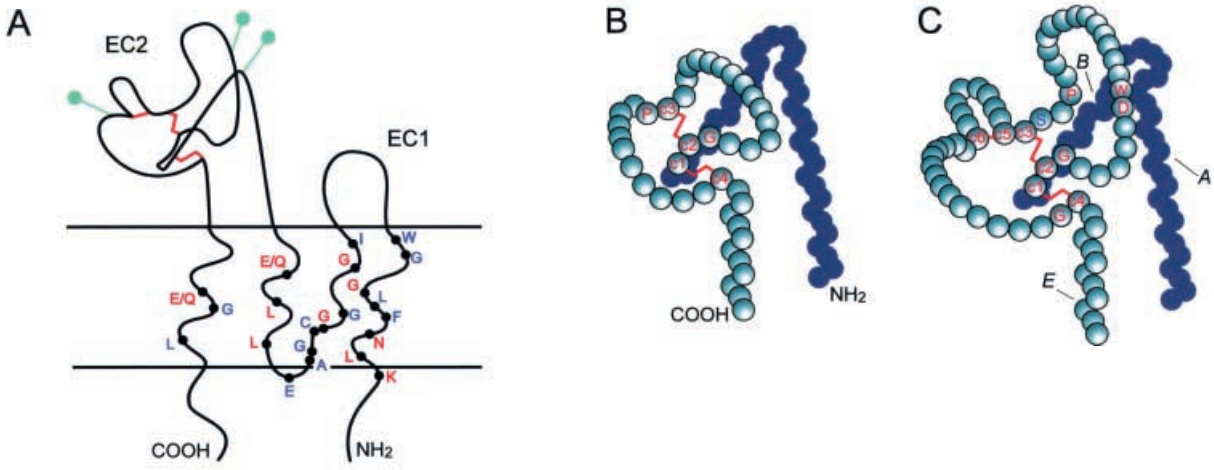


Figure 1. Schematic structure of a 'typical' tetraspanin. The analysis presented in this figure is based on the 26 human tetraspanins listed in table 1. (*A*) Conserved amino acids are indicated. Red, ≥ 80%, and blue, ≥ 60% conservation. The main localisations of potential N-glycosylation sites are shown (—•). They are not exactly conserved and their number is variable. (*B*) and (*C*) An important difference between tetraspanins is the number of cysteines in EC2. Schematic representations of the possible structures of this region according to the number of cysteines are shown. The folding was sketched after the crystal structure of CD81 EC2 [3], assuming that in the other tetraspanins all cysteines are similarly engaged in disulfide bond formation. The number of amino acids represented is close to the average within each group, and the conserved amino acids are indicated. The four cysteine (numbered c1 to c4) group includes CD9, CD81, Tspan-2, and CD53 (B). Most tetraspanins have two additional cysteines (c5 and c6) usually located between c3 and c4 (*C*). With the exception of SAS, NET-6, CD37 and CD82, c5 is located immediately after c3. Three tetraspanins have eight cysteines (see fig. 2), with the additional cysteines placed between c2 and c3 (not shown). The DW, PxSc3 and Gc4 motifs are found in ≥ 80% tetraspanins of these two latter groups (70% for the S of PxSc3). Interestingly, these motifs are present in the CD53 sequence. The crystal structure of CD81 EC2 indicated that two α helices (*A* and *E*) formed the stalk of a mushroom-shaped molecule whereas the rest of EC2 formed the head [3]. An analysis of the other tetraspanins using PHD (Profile network prediction Heidelberg) [179] shows the conservation of these two α helices and thus probably of the stalk region. Another predicted feature of all human tetraspanins, demonstrated by the crystal structure of CD81, is the presence of an additional α helix (*B*) just before the CCG (c1c2G) conserved motif. The arrangement shown for this helix is that of CD81 [3]. The region between the CCG motif and the last cysteine of EC2 (c4) is the most variable in length and in structure. In CD81, this region is composed in part by two α helices, one in each of the c2–c3 and c3–c4 segments, which might not be conserved throughout the other human tetraspanins, as determined by PHD. The structure of the CD9 [180], Cd37 [181], Cd53 [182], CD53 [183], CD63 [184], Cd81 [185], CD82 [186], Cd82 [187], Cd151 [188] and NET-2 [unpublished data] genes shows a correlation between the intron/exon boundaries and the putative protein structural domains. The *A* and *B* helices are encoded by the same exon, the c2–c4 region is encoded by another exon, and a third exon encodes a segment between c4 and the E/Q polar amino acid of the fourth transmenbrane domain. Similar organisation is found in insects [6].

Rom-1 [15], can be considered as genuine tetraspanins since they have conserved some key residues, in particular those of EC2. Because they form specialized structures, these molecules will not be reviewed in detail here. The molecules L6 and il-TMP were occasionally referred to as tetraspanins. However, although these molecules have four transmembrane domains, the predicted structure appears to be different from tetraspanins (absence of consensus amino acids with tetraspanins and smaller EC2 domain). With the discovery of two closely related molecules (L6D and TM4SF5), it was suggested that they constitute a separate superfamily [16]. The inclusion of KRAG/sarcospan [17], a protein of the dystrophin/dystroglycan complex, to the tetraspanin superfamily is also unlikely.

Tissue distribution and subcellular topology

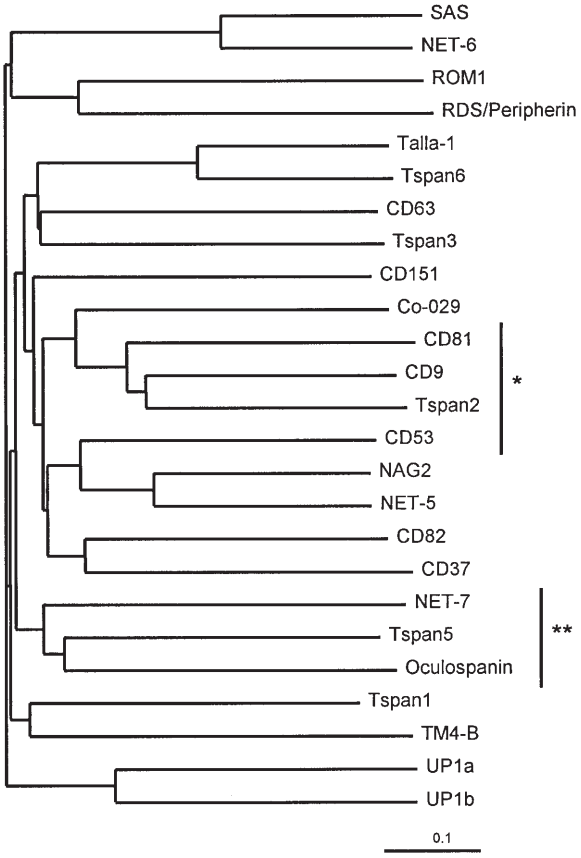
With the exception of erythrocytes, all cells seem to express several tetraspanins. [18–22]. Considering the number of tetraspanins studied, their heterogeneous expression and the various methods used to assess their presence, only some general characteristics of their tissue distribution can be given. Some tetraspanins have wide distribution, whereas other tetraspanins have a very restricted pattern of expression. This is particularly the case for tetraspanins belonging to specialized structures, the uroplakins UP1a and UP1b, which are constituents of the asymetric unit membranes of the urothelium [14], and the proteins RDS/peripherin and Rom-1, which are found in the rim of the photoreceptor outer segment disc [15]. However the expression of other tetraspanins is also limited to specific cellular subsets, for instance CD53 is mainly a leucocyte marker, whereas CD37 is found at a high expression only on lymphoid B cells [18]. A detailed tissue distribution was reported only for the tetraspanins CD9 [21-23], CD63 [22], CD82 [23] and CD151 [22], which have extensive but not ubiquitous distribution.

The existence of restricted epitopes linked to the masking of antigenic sites by molecular interactions may modify

Table 1. Size and genomics of human tetraspanins.

Molecule	MW (kDa)	Length a.a.	Unigene #	Chromosome localization
CD9	24–26	228	Hs.1244	12p13
CD37	40–50	281	Hs.153053	19q13
CD53	32–40	219	Hs.82212	1p12-p31, 15q24 (seq)
CD63 (ME491)	30–60	237	Hs.76294	12q13
CD81 (TAPA-1)	26	236	Hs.54457	11p15.5
CD82 (R2/IA4)	50–80	267	Hs.25409	11p12
CD151 (PETA-3)	27	253	Hs.75564	11p15
C0-029	27–34	237	Hs.84072	12q14
NAG-2	28–35	238	Hs.26518	11
Oculospanin	unk	355	AAG42857*	unk
CD231/TM4SF-2/TALLA-1	38–45	244	Hs.82749	Xq11
SAS	unk	210	Hs.50984	12q13
Uroplakin Ib	28 (bov)	260	Hs.271580	3q13.3-q21
Uroplakin Ia	27 (bov)	258	Hs.159309	19q12 (seq)
Peripherin/RDS	39	345	Hs.281564	6p21
Rom-1	33	351	Hs.1943	11q12
Tspan-1	unk	241	Hs.38972	1p32
Tspan-2	25 (rat)	224	Hs.122540	1q12 (seq)
Tspan-3	unk	253	Hs.100090	unk
Tspan-5	unk	268	Hs.20709	4
Tspan-6	unk	245	Hs.121068	Xq21.1
NET-2	unk	305	Hs.16529	7q31
NET-5	unk	239	Hs.129826	12p13 (seq)
NET-6	unk	204	Hs.284243	7p21
NET-7	unk	294	Hs.95583	10q21
TM4-B	unk	245	Hs.271943	9q37

Most gene localisations resulted from genetic or cytogenetic analysis; (seq) indicates that the localization was derived from the draft assembly of the human genome sequence (http://genome.ucsc.edu/goldenPath/hgTracks.html). Usually the genome sequence confirmed the cytogenetic localization. For CD53, the two localisations are different. The GenBank accession number is given for oculospanin (*).



the pattern or intensity of expression. For instance, the epitope recognized by TS151r monoclonal antibody (mAb) is not accessible when CD151 interacts with the integrin $\alpha 3\beta 1$ [24]. It remains to be determined if this observation applies to other anti-tetraspanin antibodies.

If tetraspanins are found on the cell surface, certain tetraspanins are also found in intracellular compartments. For example, CD63, which has a lysosome-targeting signal [25], is strongly expressed in platelet lysosomes [26], in Weibel-Palade bodies of endothelial cells [27] and in the azurophil granules of neutrophil granulocytes [28]. Its expression on the cell surface is induced or strongly increased after activation of platelets, neutrophils and basophils, following mobilisation of the intracellular pool. It was also shown that CD151, but not CD9, was strongly expressed by endothelial cells endosomes [20]. A study of the expression of tetraspanins on the megakaryoblastic

Figure 2. Distance tree of human tetraspanins. The distance between human tetraspanins is indicated by the relative branch length (0.1 = 10% amino acids differences). Comparisons were based on the whole aminoacid length of each tetraspanin. * indicates tetraspanins with four cysteines in EC2; ** indicates tetraspanins with eight cysteines in EC2, the other tetraspanins have six cysteines in EC2. RDS and Rom-1 belong to the group with six cysteines but they have an additional cysteine in EC2 for interchain bond formation. NET, New EST tetraspans; RDS, retinal dystrophy syndrome; UP, uroplakin.

cell line Mo7e showed that the intracellular localization of tetraspanins CD63 and CD151 was predominant as compared with tetraspanins CD9 and CD81 [29]. Moreover, tetraspanins CD37, CD53, CD63, CD81 and CD82 were found in major histocompatiblity (MHC) class II-enriched compartments (MIIC) of B lymphocytes [30], particularly on their internal membranes [31], and consistent with this, on multivesicular MIIC-derived exosomes [32]. In these compartments, CD63 and CD82 are associated with human leukocyte antigen (HLA) class II molecules DR, DM and DO [31]. The tetraspanins CD63, CD82 and CD9 are also present on the exosomes of dendritic cells [33].

Molecular interactions

One of the most striking features of tetraspanins is their ability to form (multi)molecular complexes with each other and other surface proteins. The description of these complexes stems from research orginating in different fields of biology whose studies converged toward tetraspanins. Early studies on the B-lymphoid surface molecule CD19 and the diphtheria toxin receptor (proHB-EGF) showed their association with CD81 and CD9, respectively. The reports of tetraspanin/integrin associations came from groups working on either tetraspanins or integrins and looking for associated molecules.

Associated molecules are not limited to these three but include a presently undefined number of other molecules. For clarity, the CD19/CD21/CD81/Leu 13, pro-HB-EGF and tetraspanin complexes will be described separately, but we will see how they can be grouped within an unifying concept, the 'tetraspanin web'.

The B lymphoid activation complex CD19/CD21/CD81/Leu 13

At the surface of B lymphocytes, CD81 belongs to a multimolecular complex called CD19/CD21/CD81/Leu-13 [34]. CD19 is a protein of 95 kDa, a member of the immunoglobulin superfamily for which no ligand has been identified. CD21 (CR2) is the receptor for the C3d fraction of complement and for the Epstein-Barr virus [35]. The Leu-13 antigen is the product of a gene inducible by interferon.

The coligation of CD19 with the B cell receptor (receptor for antigen) decreases the threshold of response to the antigen by two orders of magnitude. Thus, during a primary immunising response, B lymphocytes can respond to low antigen concentrations despite the weak affinity of the antigen receptors. At the molecular level, CD19 acts by recruiting PI3K (phosphatidyl-inositol-3 kinase) and the guanine exchange factor Vav [36]. Mice defective in CD19 have a decreased circulating level of immunoglobulins and a defective response to T-dependent antigens [34]. Physiologically, CD19 is likely to be engaged by binding of complement-opsonized antigen with both the antigen receptor and CD21. Thus, the CD19/CD21 complex links natural immunity to acquired immunity [37].

The monoclonal antibodies directed against components of the CD19/CD21/CD81/Leu-13 complex share similar effects of costimulation on peripheral B lymphocytes. Costimulation of Ca^{2+} mobilisation is also observed in B-lymphoid cell lines. Antibodies to these molecules have an inhibiting effect on the proliferation of B-lymphoid cell lines and induce homotypic aggregation [34]. These effects probably derive partly from engagement of CD19 and its downstream signalling pathways. CD19 chimeras which do complex with CD81 do not induce homotypic aggregation and inhibition of proliferation, which suggests that CD81 relays or amplifies part of the signal triggered by CD19.

The CD9/proHB-EGF complex

CD9 is associated with the membrane precursor of heparin-binding-epidermal growth factor (EGF) (pro-HB-EGF), a member of the EGF/transforming growth factor -α (TGFα) growth factor family, which is also the diphtheria toxin receptor. The expression of CD9 on the surface of cells expressing proHB-EGF increases the number of sites for diphtheria toxin with no change in affinity, whereas the number of HB-EGF molecules remains constant [38]. It is likely that CD9 modifies the conformation of proHB-EGF and thus uncovers new binding sites for the toxin. In addition, the overexpression of CD9 increases the juxtacrine activity of proHB-EGF [39]. A similar observation was reported for proamphiregulin, which is structurally related to proHB-EGF [40].

Recently, CD9 was found to associate with the membrane precursor of TGFα and to increase the juxtacrine activity of this molecule. In contrast to HB-EGF, transfection of CD9 decreased the proteolytic conversion of the precursor to soluble TGFα [41].

Tetraspanin complexes

In parallel to the work on the CD19/CD21/CD81/leu13 and CD9/proHB-EGF complexes, other molecular complexes containing tetraspanins were reported. It appeared gradually that the tetraspanins were associated together and with numerous other surface molecules [42–46]. The technique of immunoprecipitation used for many of these studies gave surprising results, since many molecules are coimmunoprecipitated by tetraspanins, and the profiles of co-immunoprecipitated molecules are identical whatever the tetraspanin immunoprecipitated. Among these molecules are β1 integrins ($\alpha3\beta1$, $\alpha4\beta1$ and $\alpha6\beta1$ in particular) [43–46], HLA-DR [47], CD4, CD8 [48], CD19

antigens [49, 50] and γ-glutamyl-transpeptidase [51] (see table 2 for a detailed list). If coimmunoprecipitation was the technique of choice to reveal these complexes, their existence on the cellular surface was confirmed by other techniques, such as fluorescence resonance energy transfer (FRET), cocapping or chemical cross-linking.

These results suggest the existence on the cell surface of tetraspanin-enriched multimolecular complexes. The structure of these complexes is becoming clearer: it is possible, by varying the detergents used to extract proteins, to show the existence of small primary complexes containing only one tetraspanin and a limited number of associated molecular partners. Among these primary complexes are the complexes formed by the tetraspanin CD151 and the integrins α3β1 or α6β1, the tetraspanin CD81 and the integrin α4β1, and the tetraspanin CD9 and CD81 with CD9P-1/KIAA1436/FPRP [20, 24, 52–54]. The association of the other tetraspanins with these molecules is observed only by lysing the cells with the detergents (CHAPS, Brij) which maintain the majority of the molecular interactions involving the tetraspanins, thus suggesting an indirect association. Since under conditions allowing the observation of primary complexes, for instance in digitonin extracts, the interactions between

Table 2. Association of tetraspanins with other cell surface molecules.

ASSOCIATED MOLECULE	Tetraspanins[a]	Confirmed by or comment	Reference
β1 integrins			
α1β1	CD9		149
α2β1	CD9, CD151[b]		20, 73, 150
α3β1	CD9, CD63, CD81, CD82, CD151, NAG-2, Co-029	cross-linking (most evident for CD151); masked epitope (CD151)	20, 24, 44, 46, 52, 68, 73, 76, 128, 136, 149–151
α4β1	CD9, CD53, CD81, CD82, CD151		24, 29, 43, 45, 57
α5β1	CD9, CD151[2]		20, 29, 43, 150, 152
α6β1	CD9, CD63, CD81, CD82, CD151, NAG-2, Co-029		20, 24, 29, 44–46, 128, 149, 151
Other integrins			
α6β4	CD9, CD151[b]		20, 63, 73, 149
Integrin $\alpha_{IIb}\beta_3$	CD9, CD151	cross-linking (CD9)	29, 153–156
Integrin $\alpha_L\beta_2$	CD63, CD82[b]		157, 158
Other adhesive receptors			
CD44	CD9		73
Syndecan	CD9		73
CD42 (GpIb/V/IX)	CD9	cross-linking	153, 156
CD47	CD9		156
Molecules of the immune system			
HLA-DR	CD9, CD37, CD53, CD81, CD82	FRET, cocapping (CD81)	42, 45, 88, 158
MHC class I	CD82	FRET	160
	CD53, CD81	FRET only	159
CD4 (human/mouse)	CD9, CD81, CD82,	cocapping	89, 95, 158, 161
CD8	CD81, CD82		89, 95
CD3 (human/mouse)	CD9, CD81		89, 161
CD19	CD9, CD81, CD82	cocapping; functional effect	56, 58, 162
CD21	CD81	indirect via CD19	56, 162
Leu-13	CD81		56, 162, 163
CD46	CD9, CD81, CD82, CD151	indirect via integrins	149
CD5 (mouse)	CD9	cocapping	161
CD2 (rat)	CD53		164
Others			
ProHB-EGF	CD9, CD63, CD81, CD82	functional effect (CD9); cross-linking (CD9)	38, 61, 62
proTGFα	CD9	functional effect	41
CD9P-1	CD9, CD63, CD81, CD82, CD151[b]	cross-linking (CD9)	53, 54
γ-glutamyl transpeptidase	CD37, CD81, CD53, CD82	cocapping	51
EGF receptor	CD82	functional effect	86
UPII, UPIII	UPIa, UPIb	cross-linking	65

[a] Except when indicated by a footnote, only the tetraspanins cited were tested.

[b] Some other tetraspanins were found not to associate or contradictory results were published. These differences may be due, at least in part, to cell lysis by different detergents. Only reports showing an association are cited.

tetraspanins are broken, it is probable that the other tetraspanins interact with the four molecules mentioned above only through CD9, CD81 or CD151 [24].

The first studies determined that the cytoplamic regions of CD19, $\alpha 3\beta 1$ and $\alpha 4\beta 1$ integrins were not involved in the interaction with tetraspanins [44, 55–57]. In contrast, the cytoplasmic region of CD4 was found to be required for association with CD81 or CD82 [48]. The determination of specific tetraspanin/partner pairs allowed a preliminary characterization of the tetraspanin regions involved in the formation of these primary complexes. These studies, based on swapping of particular segments, have pointed out the role of the second half of tetraspanins, and/or EC2, for the interaction of CD81 with CD19 [58], of CD9 with CD9P-1 [53] and of CD151 with the integrin $\alpha 3\beta 1$ [59]. The latter study revealed the possible implication of the C-ter region (after the PxSCC site) of CD151 EC2 and the importance of a segment within the 'stalk-like' region of the integrin $\alpha 3$ subunit . Similarly, CD9 EC2 was found to be required for the upregulation of proHB-EGF activity as a receptor for diphtheria toxin [60, 61].

The tetraspanin web, a dynamic network of molecular interactions

These data suggest a model in which the tetraspanins would be 'organizers' of multimolecular complexes on the cell surface, each tetraspanin recruiting specifically one or more molecular partners in these complexes (fig. 3). These multimolecular complexes are based on the assembly of primary complexes (tetraspanin/partner-specific pairs) which would interact through tetraspanins, thus building larger complexes (fig. 3).

CD81/CD19 and CD9/HB-EGF are likely to represent two of these primary complexes. Indeed, CD19 was shown to associate with CD9 and CD82, under conditions maintaining tetraspanin/tetraspanin associations, and to be tyrosine phosphorylated upon CD9 engagement [48, 58]. Tetraspanins CD63, CD81 and CD82 do not increase the capacity of proHB-EGF to bind diphtheria toxin [60, 61] even if proHB-EGF is included in the tetraspanin web as shown by its association with these tetraspanins and the integrin $\alpha 3\beta 1$ [62]. The restricted effect of CD9 may be due to the fact that within the tetraspanin web, only CD9 binds directly to proHB-EGF.

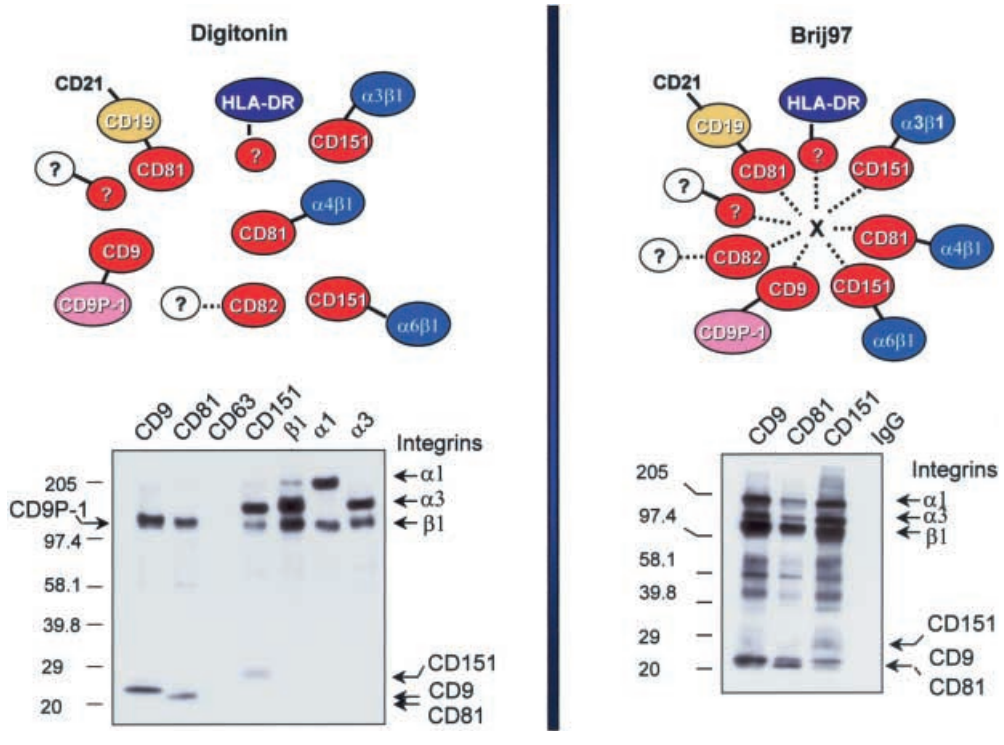


Figure 3. The tetraspanins complexes. The pattern of coprecipitation observed in Hela cells after biotin surface labelling and extraction with digitonin or the mild detergent Brij97 (lower part of the figure) suggests that tetraspanins may exist in two different types of molecular complexes [24]. First order complexes revealed by digitonin extraction (*left panel*) contain only a single tetraspanin and its specific molecular partner(s), whereas second-order complexes coprecipitated following Brij97 extraction (*right panel*) include several tetraspanins and most of the other partners. In second-order complexes, it is hypothesised that the assembly of the first-order complexes is mediated by tetraspanin/tetraspanin interactions (*x*). The arrangement of molecules is arbitrary; red and green symbols indicate respectively tetraspanins and $\beta 1$ integrins. Question marks account for complexes of unknown composition, since specific partners were not found for all tetraspanins studied and mAbs for biochemical studies are not available for all tetraspanins. Complexes formed between tetraspanins and CD19, HLA-DR or $\alpha 4\beta 1$ are observed in cells expressing these molecules like the B lymphoid cell line Raji [24, 58].

It appears clearly that partner molecules may be in different states according to the tetraspanin web, either associated to it or not. Reciprocally, the example of CD151, which locates with integrin $\alpha 6\beta 4$ to hemidesmosomes in keratinocytes, opposited to CD9, CD81 and the integrin $\alpha 3\beta 1$ which are not associated with these structures, shows that tetraspanins may lose their link to the tetraspanin web [63]. Because Rom-1 associates with peripherin/RDS in the rim of rod outer segments [64], and uroplakins associate with each other [65] in vesical plaques, they may represent extreme situations in which tetraspanins have completely lost their relation with the tetraspanin web in order to serve tissue-specialized functions. Also, large variations in the level of tetraspanins may profoundly modify the organisation and composition of the tetraspanin web. Such variations are observed during differentiation of B-lymphoid cells or megakaryocytes [66] or can be induced by exogeneous stimuli, for instance CD9 increase in kidney epithelial cells following osmotic shock [67] or CD63 translocation to the cell surface in platelet [68] and leukocyte activation.

The functional significance of these associations remains to be determined. How a tetraspanin modifies the functional properties of an associated molecule will be discussed in the following section.

In vitro analysis of functional effects

The effects induced by anti-tetraspanin antibodies are multiple and often spectacular [2]. They include homotypic adhesion [69], inhibition of cell migration [70], antiproliferative effect [5] or costimulation. There are some indications that the presence of a tetraspanin at a sufficient level on the cell surface is enough to trigger some of these effects by the corresponding antibodies [60]. The fact that these effects are produced by different tetraspanin antibodies suggests a similar triggering of the tetraspanin web.

The induction of human platelet activation/aggregation by anti-CD9 mouse immunoglobulin (Ig)G1 is particular, since it is initiated by the cross-linking of the platelet FcγRII [71, 72]. This mechanism does not seem to be involved in other major effects of tetraspanin antibodies.

The functional consequences of proHB-EGF/CD9 and CD19/CD81 associations have already been described, and this section will focus on migration, costimulation and membrane-remodelling events.

Cell migration

From experiments aiming at the identification of surface molecules controlling migration of cultured tumor cells came the first evidence for the involvement of a tetraspanin, CD9, in cell migration. Among 3000 hybridomas, antibodies produced against the lung adenocarcinoma cells MAC8, the strongest inhibition of cell motility was found in an antibody which was shown to recognize CD9 [70]. This was repeatedly confirmed for CD9 and other tetraspanins in various cellular models [43, 60, 73–76]. A relation between the level of expression of the tetraspanin CD63 in transfected melanoma cells and the inhibition of migration, by anti-CD63 mAbs has been reported [75]. Particular experimental conditions may lead to stimulation of cell migration as for Schwann cells on axons in the presence of a CD9 mAb [77] or MDA-MB231 cells on matrigel by anti-tetraspanin mAbs [78]. Interestingly, the transfection of various tetraspanins has been shown to reduce spontaneous migration when no extracellular matrix components were added [75, 79, 80], whereas the motility was increased on some $\beta 1$ integrin substrates [75, 81, 82]. This indicates that results of in vitro experiments must be interpreted cautiously regarding their in vivo relevance.

The mechanism underlying the effect of tetraspanins on cell migration is unknown but must be considered in view of the existence of tetraspanin/integrin molecular complexes. Integrins are major extracellular matrix receptors that form a bridge between the extracellular matrix and the cellular cytoskeleton. They are also involved in cell-cell adhesion and are part of signalling pathways in relation to basic cellular functions such as proliferation, survival or cell migration. They act in a coordinated way with other surface adhesion molecules in order to sustain and control cell movement. It has not been clearly demonstrated that tetraspanins can directly modify integrin function. Anti-tetraspanin antibodies or ectopic expression of tetraspanins generally do not modify cell adhesion to integrin substrates [43, 57, 73, 83]. Moreover, tetraspanins are not localized in focal adhesions, which are the sites at which the interaction between cell and extracellular matrix is the strongest [83, 84] (although further studies would be required to determine the exact location of the tetraspanin CD151, which associates at a high stoichiometry with integrin $\alpha 3\beta 1$). On the other hand, the phosphorylation state of focal adhesion kinase (FAK), one of the major kinases activated in integrin signalling, is modulated by anti-tetraspanin mAb binding, but in opposite ways, depending on experimental conditions. Plating serum-starved cells on anti-tetraspanin mAbs induced dephosphorylation of FAK. In contrast, plating cells on anti-tetraspanin mAbs in addition to collagen potentiated FAK phosphorylation. If ectopic expression of CD9 in fibrosarcoma cells did not modify the initial phosphorylation of FAK following adhesion to laminin-coated plastic, this phosphorylation was more stable in time. A concommitant reorganization of cortical actin cytoskeleton was also observed [83]. Tetraspanin/integrin complexes are also associated with phosphatidylinositol 4-kinase [85], an enzyme involved in the production of 4,5-PIP2, a regulator of cytoskeletal architecture. Finally, anti-tetraspanin anti-

bodies, like anti-α3-integrin antibodies, induce a PI3K-dependent production of matrix metalloproteinase 2 (MMP-2) in MDA-MB-231 cells [78].

Another mechanism by which tetraspanins may control cell migration involves the EGF receptor. After ectopic expression of CD82/KAI-1, signalling by EGF is attenuated, and reduced migration in wound closure assay is observed in response to EGF. This is associated with morphological differences, since CD82-expressing HB2 human mammary epithelial cells show less lamellipodial formation and cytoskeleton reorganisation in response to EGF. This attenuation of the signal could be due to an accelerated endocytosis of the EGFR-EGF complex [86].

Costimulation and signal transduction

Binding of mAbs to CD53 on monocytes and B cells was shown to induce Ca^{2+} mobilization [87]. Triggering of B-lymphoid cell lines by tetraspanin mAbs induced protein tyrosine phosphorylation [58, 88, and unpublished data]. CD9 antibodies in Schwann cells were shown to induce both effects [77].

Costimulatory effects of anti-tetraspanin antibodies have been reported in humans and mice. CD81 has a costimulatory activity with CD3 for inducing interleukin (IL-2)-dependent proliferation of human thymocytes [89]. Co-immobilization of CD82 mAbs with CD3 mAbs stimulates CD4 T cell proliferation [90]. This treatment inhibits the proliferation of Jurkat T cells but stimulates the production of IL-2 and interferon γ (IFNγ). Potentialisation and stabilisation of tyrosine phosphorylation as well as major morphological changes were observed [91]. By comparing the functional properties of four tetraspanins, CD9, CD53, CD81 and CD82, it was shown that mAbs to any of these molecules were able to deliver a costimulatory signal for CD3-mediated activation of the T cell line Jurkat [60]. A costimulatory effect of CD9 ligation on murine naive lymph node T cells was also reported [92]. It was found to be independent of CD28 and to operate synergistically with this molecule [93]. Interestingly, in contrast to CD28 costimulation, initial activation induced by the CD9 mAb is followed by apoptosis, which can be prevented by addition of IL-2 [94]. The costimulation of spleen T lymphocytes by CD81 mAbs is similar to that of CD9 in its independence from CD28 and lack of IL-2 production, but the proliferation is comparable in strength and duration to CD28 costimulation [94a]. The mechanism of T cell costimulation is unknown, but by analogy with CD19 in lymphoid B cells, it would be interesting to determine whether a signal-transducing molecule is associated with tetraspanin complexes in T cells.

It should be noted that in various cell lines, tetraspanins were found to be associated with enzymatic activities (table 3). It is not known whether these activities associate directly with tetraspanins or with non-tetraspanin-associated molecules.

Tetraspanins and membrane-remodelling processes

Initial observations of the involvement of tetraspanins in membrane fusion events came from virus biology. Thus, anti-tetraspanin antibodies to CD81 and CD82 inhibit the

Table 3. Signal transduction and signaling molecules.

Tetraspanin	Cell type	Enzyme	Ref.
Association with enzymatic activities			
CD9	carcinoma cell lines	Ser/Thr kinase	165
CD53	thymoma	Tyrosine phosphatase	166
CD63	rat basophilic cell line		
CD63	neutrophils	tyrosine kinase (Lyn, Hck)	157
CD9, CD63, CD81 CD151, TALLA-1 [a]	various	PI4K	52, 85, 167
CD82	HB2 mammary epithelial cells, primary keratinocytes A549; small-cell lung carcinoma	EGF-R	86
Signal transduction			
CD9	schwann cells	Ca++, tyrosine phosphorylation	77
CD9, CD151	platelets	Fc receptor recruitment by mAbs	71, 72, 168
CD82	U937	Fc receptor recruitment by mAbs	169
CD53	monocytes, B cells	Ca++	87
CD9, CD63, CD81 CD82, CD151	MDA-MB-231	FAK phosphorylation/ dephosphorylation	83
CD81, CD53	B cells	tyrosine phosphorylation	88, and unpublished data
CD9	transfected B cells	tyrosine phosphorylation	58

[a] (CD37, CD53, CD82, association not detected).

formation of syncytium induced by human T-lymphotropic virus (HTLV) [95]. It was shown that the coexpression of CD82 with HTLV1 envelope glycoproteins resulted in marked inhibition of syncytium formation and association of these molecules was demonstrated [96]. CD9 antibodies also inhibited infection by FIV (feline immunodeficiency virus, a cat-specific retrovirus which serves as model for human immunodeficiency virus (HIV) [97] and CDV (canine distemper virus) [98]. Expression of CD9 was shown to increase syncytium formation and CDV production [98], and to increase infection with FIV [99]. In both cases, the early events following virus entry were not modified.

The relation between cell fusion and tetraspanins was further studied in muscular differentiation, where cell fusion is part of myotube formation. The addition of CD9 or CD81 antibodies inhibited conversion of murine myoblasts (C2C12 cells) to elongated myotubes despite normal expression of muscle-specific proteins. In contrast, ectopic expression of CD9 increased myotube formation [100].

The role of CD9 in cell fusion has been clearly established by the study of CD9 null mutation which induces an isolated fertility defect of the female [101–103]. In the absence of CD9, the ability of the oocyte to fuse with sperm is considerably reduced (> 95%). CD9 is strongly expressed on normal oocytes [104], and antibodies to mouse CD9 block the in vitro fertilization of normal oocytes. The mechanism of the sperm/egg fusion defect is not elucidated, and the initial hypothesis that CD9 could play a role in the fusion process by regulating the interaction between the sperm ADAM protein fertilin and the oocyte integrin $\alpha 6\beta 1$ (found in the tetraspanin web) has been challenged by the recent finding that integrin $\alpha 6\beta 1$ is not required for sperm/egg fusion [105].

Perturbation of membrane dynamics by CD9 mAb was also observed in differentiating human megakaryocytes (MK). The CD9 mAb did not prevent normal maturation of a normal multilobed nucleus but deeply modified the demarcation membrane system and prevented the formation of proplatelet territories and platelets [66].

Finally, an anti-rat CD81 mAb has also been reported to inhibit FcγRI-mediated degranulation of mast cells, without inhibition of proximal signalling [106]. Considering the preceding data, it would be of interest to determine whether this observation is related to a defect of granule fusion to plasma membrane.

It is to early to determine whether the effects of tetraspanins or tetraspanin antibodies on these membrane dynamic events are related, i.e. supported by a common molecular mechanism. Apart from the fact that fusion peptides, included in the sequence of viral proteins but also of some mammalian proteins like fertilin, are lipophilic and prone to insertion in foreign cell membranes, the mechanism by which the fusion occurs is not completely understood. Tetraspanins are clearly good candidates for studies on molecular mechanisms of cell fusion.

Table 4. Tetraspanins and cancer: correlations.

Tetraspanin	Cell type	Ref.
High level of tetraspanin expression is a good prognosis factor or associated with low grade histology [a]		
CD9	melanoma	170
	breast cancer	113, 117
	lung carcinoma	118
	colon carcinoma	120
CD63	melanoma	112
CD82	prostate	114, 171
	pancreas	115
	lung carcinoma	119
	colon	121
Tetraspanin expression is inversely related to the metastatic potential		
CD9	breast cancer	113, 117
	lung carcinoma	118
	colon carcinoma	120
	pancreas	123
	oesophageal carcinoma	172
CD82	prostate	114, 171, 173
	lung carcinoma	119
	colon	124
	hepatoma	116, 174
	breast	175
	lung (non-small-cell carcinoma)	125
	bladder cancer	176

[a] Simultaneous reduction of CD9 and CD82 has an additive effect on metastatic potential and is a bad prognosis factor [121, 125].

Tetraspanins in organ functions and diseases

Tetraspanins and the immune system

The consequence of the absence of CD81 and CD37 in the immune system was investigated using knockout mice. CD81 knockout leads to a reduction of expression

Table 5. Tetraspanins and cancer: experimental data.

Tetraspanin	Cell type	Cell line transfected	Ref.
Transfection of tetraspanin reduces metastatic potential			
CD9	melanoma	B16	79
CD63	melanoma	KM3	127
CD82	melanoma	B16	177
	prostate carcinoma	AT6.1, AT6.3	114
	breast	MDA-MB-435	178
CD151	epidermoid carcinoma	HEp-3	82
Transfection of tetraspanin increases metastatic potential			
Co-029	colon carcinoma	AS-D6.1A$_I$	128

of the B cell antigen CD19 associated with a decrease of calcium mobilisation following CD19 engagement [107–109]. Two groups observed a reduction of B1 cells in the peritoneum, and the Th2-dependent IgG1 production appeared to be reduced. Consistent with the Th2-deficient response, allergen-induced airway hyperreactivity was found to be diminished in CD81-deficient mice in relation to a dramatic reduction of IL-4, IL-5 and IL-13 secretion [110].

Mice deficient in CD37, a tetraspanin of mature B cells, exhibited a reduced humoral response to T-dependent antigens, suggesting a role of CD37 in mediating B and T cell interactions [111].

Interestingly, despite expression of CD9 on B and T lymphocytes, the CD9 knockout did not result in significant alterations of the immune system, since lymphoid organs and subpopulations of B and T cells appeared normal. Immunisation with influenza virus led to normal humoral and cellular responses [unpublished results].

Tetraspanins and cancer

There is a large literature comparing the expression of tetraspanins in normal tissues, primary tumours and metastasis. The use of immunohistochemical methods limits the interpretation of results, since these methods are more qualitative than quantitative. Some work was done by quantitative polymerase chain reaction (PCR) but results must be interpreted carefully since many tetraspanins are expressed not only on tumour cells but also on nontumour cells of normal neighbouring tissue or stromal component. Another level of difficulty was linked to the necessity of using frozen sections because the epitopes recognised by mAbs to tetraspanins usually do not resist fixation procedures.

For several cancers, however, the level of expression of certain tetraspanins can be related to the stage of tumour progression or the appearance of metastases and constitutes a prognostic factor. CD63 is strongly expressed in the early stages of the melanomas, whereas it is repressed in the advanced stages [112]. In tumours of the breast, lung, colon and pancreas, CD9 and CD82 are less expressed on primary tumours when metastases are present [113–121], and their decreased expression predicts a lower rate of survival [113, 114, 116, 118–125]. In general, these antigens are also less strongly expressed on metastases. In lung cancer and breast cancer, the simultaneous reduction in the expression of CD9 and CD82 was correlated with a greater metastatic potential and a rate of survival lower than when the expression of only one of these two antigens was reduced [122, 126].

Tetraspanins seem to play an active role in the formation of metastases. The transfection of CD9 or CD63 in melanoma cells reduces the metastatic potential of these cells [79, 127]. CD82 was even cloned again on its capacity to suppress the formation of metastases in prostate cancer [114].

An opposite effect is seen with the tetraspanin Co-029; induction of its expression was observed in subclones of colon or pancreas carcinoma rat cell lines with strong metastatic potential. The transfection of Co-029 in these cell lines increases that potential [128].

How tetraspanins affect tumour cells behaviour again calls attention to the tetraspanin complexes and especially the association with integrins. Cell migration and adhesion are part of the metastatic process, and how they are influenced by tetraspanins has been discussed. There are also many reports showing the critical role of integrins on tumour development, invasion and metastasis [129–132]. However, considering the multitude of molecules associated with tetraspanins, how tetraspanins control tumour progression may not be limited to their interaction with integrins.

The situation of the tetraspanin Talla-1 (T-acute lymphoblastic leukemia-associated antigen 1), expressed in acute neuroblastomas and T-lymphoid leukemias [10], is different. Its expression is correlated in leukemic cell lines with that of the Tal1 transcription factor, whose gene is rearranged and expressed in certain translocations observed in T cell acute leukemias. The Tal1 transcription factor acts in cooperation with the rhombotin gene products cofactors RBTN1 and RBTN2, and transfection of Tal1 and RBTN1 can induce the expression of tetraspanin Talla-1 [133]. Whether Talla-1 behaves as an oncogenic mediator of Tal1 in leukemia is presently unknown.

Tetraspanins and nervous system

Several tetraspanins have been found to be expressed in the nervous system, where association with integrins has been confirmed [134–136]. CD9, Tspan-2 and Tspan-3 were found to be expressed on myelin. CD81 was found to be strongly positive on post-injury microglia and astrocytes in the rat [137]. Tspan-5/NET-4 is highly expressed in brain cortical structures [138].

The physiological importance of tetraspanins is suggested by the effect of CD9 [139], CD81 and CD151 [136] mAbs in neurite outgrowth and the upregulation of CD81 after nervous system injury. Furthermore, neuromuscular synapse formation is delayed in mutant embryos of late bloomer, a *Drosophila* member of the tetraspanin surperfamily transiently expressed on motor axons, growth cones and terminal arbours. [140]. In addition, the evidence that a translocation t(X;2) disrupting the tetraspanin Talla-1/TM4SF2 gene was associated with a case of X-linked mental retardation prompted the study of other patients with this disease. In two other families a truncating mutation and a C > A mutation resulting in a nonconservative amino acid substitution (P172H) in the PxSCC motif (x = P) present in EC2 were observed. This points

out a critical functional site in the protein [141]. Also, numerous mutations of RDS/peripherin are associated with retinal dystrophies, which often result in a dominant phenotype [15]. The loss of expression of this molecule in mice resulted in the absence of photoreceptor outer segment formation [142], whereas mouse Rom-1 knockouts formed disorganized outer segments and rod photoreceptors underwent apoptosis [143].

Tetraspanins and infectious diseases

The role of tetraspanins in virus-induced syncytium formation and virus replication has been mentioned above. In addition, CD81 is an attachment receptor for the hepatitis C virus (HCV), which infects 170 million people in the world. Hepatitis C can evolve to hepatocellular cirrhosis and carcinoma, and immune disorders related to B-lymphoid cells (cryoglobulinemia, lymphoma, autoantibody production) [144]. It was recently shown that the envelope protein E2 interacts with the large extracellular loop of CD81 [145]. Sequence comparisons between human and monkey, and mutagenesis have allowed the identification of amino acids essential for recognition [146]. However, CD81 alone is not sufficient for entry of the virus into the cells, which suggests the presence of a coreceptor. In addition, the presence of CD81 in the CD19/CD21/CD81/Leu13 complex provides a possible explanation for the immunological disorders observed during infection by HCV. It was reported that like mAbs to components of this complex, a recombinant E2 protein induced homotypic aggregation and inhibited proliferation of the B-lymphoid cell line Daudi [147].

A link between CD9 and PrP was shown by differential display analysis of mouse brain infected with the agent of transmissible spongiform encephalopathy. Immunohistochemistry demonstrated upregulation of CD9, especially on astrocytes, both in humans and in the mouse model [148].

Conclusion

Different clinical observations, particularly the relation between increased metastatic properties and downregulation of tetraspanins, show that understanding the molecular functions of tetraspanins may have important medical consequences. Knockout mice have opened the way to a possible role of tetraspanins in female infertility and immunological diseases. Over the years, it has become clear that tetraspanins play an important role in membrane biology, possibly by being ‘organizers’ of multimolecular complexes. Among major questions remaining unresolved are the relation between the existence of these complexes and the functional effects observed in physiological or pathological conditions.

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