Evidence for specific tetraspanin homodimers: inhibition of palmitoylation makes cysteine residues available for cross-linking

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It is a well-established fact that tetraspanin proteins, a large family of integral membrane proteins involved in cell motility, fusion and signalling, associate extensively with one another and with other transmembrane and membrane-proximal proteins. In this study, we present results strongly suggesting that tetraspanin homodimers are fundamental units within larger tetraspanin complexes. Evidence for constitutive CD9 homodimers was obtained using several cell lines, utilizing the following four methods: (1) spontaneous cross-linking via intermolecular disulphide bonds, (2) use of a cysteine-reactive covalent cross-linking agent, (3) use of an amino-reactive covalent cross-linking agent, and (4) covalent cross-linking via direct intermolecular disulphide bridging between unpalmitoylated membrane-proximal cysteine residues. In the last case, incubation of cells with the palmitoylation inhibitor 2-bromopalmitate exposed membrane-proximal cysteine residues, thus effectively promoting 'zero-length' cross-linking to stabilize homodimers. Similar to CD9, other tetraspanins (CD81

and CD151) also showed a tendency to homodimerize. Tetraspanin homodimers were assembled from newly synthesized proteins in the Golgi, as evidenced by cycloheximide and Brefeldin A inhibition studies. Importantly, tetraspanin homodimers appeared on the cell surface and participated in typical 'tetraspanin web' interactions with other proteins. Whereas homodimers were the predominant cross-linked species, we also observed some higher-order complexes (trimers, tetramers or higher) and a much lower level of cross-linking between different tetraspanins (CD81–CD9, CD9–CD151, CD81–CD151). In conclusion, our results strongly suggest that tetraspanin homodimers, formed in the Golgi and present at the cell surface, serve as building blocks for the assembly of larger, multicomponent tetraspanin protein complexes.

Key words: CD9, cross-linking, cysteine, oligomerization, palmitoylation, tetraspanin.

INTRODUCTION

Proteins of the tetraspanin family contain four transmembrane domains, connected by one small (EC1) and one large extracellular loop (EC2) and short intracellular N- and C-terminal tails [1]. The EC2 loop contains conserved 'signature' cysteine residues (usually six but sometimes four or eight) disulphide-bonded to stabilize the loop structure [2]. Each cell type expresses a distinct repertoire of tetraspanins; some tetraspanins (such as CD81 and CD151) are widely distributed, whereas others [CD37, CD53, UPIa/Ib (urolakin Ia/Ib) and ROM1] are limited to a few cell types. Whereas most tetraspanins are abundantly expressed on the cell surface, others (e.g. CD63) localize extensively to intracellular vesicles such as lysosomes, endosomes and various secretory granules [3–5]. There are 32 distinct mammalian tetraspanins, and some have been implicated in various biological activities, including cell migration, differentiation and fusion, tumour metastasis and cell–virus interaction (reviewed in [6–8]).

Tetraspanins contain at least five functionally important subregions [9]. Besides the functionally relevant C-terminal tail [10], there are transmembrane polar residues suspected to mediate intra- and inter-molecular interactions [9] and intracellular juxta-membrane cysteine residues (e.g. in CD9, CD63, CD81 and CD151) that undergo palmitoylation [11–14]. Within CD9, cysteine residues at 9, 78, 79, 87, 218 and 219 are palmitoylated [11]. Palmitoylation affects tetraspanin subcellular distribution, morphology and lateral associations with other proteins, including other tetraspanins [11,12,14]. Within the EC2 loop is a variable

region containing sites for specific interaction with other proteins [15–18]. Also the EC2 loop contains a 'stalk' region, conserved in many, if not all, tetraspanins [2] and suggested to be involved in homodimerization [19,20]. However, the extent to which dimerization in the CD81 crystal structure is relevant to full-length CD81 or other tetraspanin molecules is not yet clear. Tetraspanins peripherin/rds and ROM1 form homodimers that further assemble into tetramers, octamers and other higher-order structures [21,22]. However, it is not clear whether features of peripherin/rds and ROM1, required for specialized functions in the retina, can be extrapolated to other tetraspanins. Indeed, other specialized tetraspanins, UPIa and UPIb, form heterodimers rather than homodimers [23].

Tetraspanins are thought to be 'molecular facilitators' organizing a 'tetraspanin web' of multiprotein complexes on the cell surface [7,24,25]. In this respect, they associate laterally with many other transmembrane proteins (e.g. integrins, MHC I and II proteins, CD4, CD8, CD19 and CD21) as well as with each other, and with signalling molecules, e.g. conventional protein kinase C isoforms [26] and phosphoinositide 4-kinase [27,28]. From the vast literature describing tetraspanin associations, it appears as if most tetraspanins can interact with most other tetraspanins, as well as many other proteins, in a seemingly unlimited and random manner. However, since TEMs (tetraspanin-enriched microdomains) emerge as critical functional entities on nearly all cell types [1,6,29], it is essential to begin to define the core protein–protein interactions and distinguish them from the abundant weak and indirect interactions.

Abbreviations used: BMB, 1,4-bismaleimidobutane; BMH, 1,6-bis(maleimido)hexane; 2-BP, 2-bromopalmitate; DMEM, Dulbecco's modified Eagle's medium; DSP, dithio-bis-succinimidylpropionate; DTME, dithio-bismaleimidoethane; FBS, foetal bovine serum; GFP, green fluorescent protein; NEM, N-ethylmaleimide; RD, rhabdomyosarcoma; TEM, tetraspanin-enriched microdomain; UPIa, urolakin Ia.

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In the present study, we have utilized four different biochemical approaches to study homodimers of multiple tetraspanins in multiple cell types. Our results suggest that homodimers are a common core unit within many tetraspanin complexes. A useful and novel technical approach was to treat cells with 2-BP (2-bromopalmitate), a palmitate analogue that inhibits protein palmitoylation [30], thereby exposing tetraspanin membrane-proximal cysteine residues. Intermolecular linkage of these cysteine residues, either via direct disulphide bridging or thiol-specific cross-linkers, allowed the capture and detection of a 'pre-existing' state of homodimerization. Importantly, the presence or absence of palmitate itself does not alter the detection of pre-existing homodimers.

EXPERIMENTAL

Cell culture, inhibitors and metabolic labelling

Human epidermoid carcinoma A431 cells and rhabdomyosarcoma (RD) cells were grown in DMEM (Dulbecco's modified Eagle's medium) with 10% (v/v) FBS (foetal bovine serum), 20 mM Hepes and antibiotics. 2-BP (Fluka, Rolconkoma, NY, U.S.A.) and palmitic acid (Sigma) were used at 25–100 µg/ml, Brefeldin A, tunicamycin and nocodazole (all from Sigma) at 10 µg/ml, cycloheximide (Sigma) at 20 µg/ml and monensin (Sigma) at 10 µM. For experiments using 2-BP, 0.25% defatted BSA (Sigma) was added to cells before the addition of 2-BP. As a negative control for 2-BP, cells were treated with either palmitic acid or 0.05% DMSO. 2-BP treatment was typically for 10–16 h and treatment with other inhibitors for up to 6 h (see the Figure legends).

For ³⁵S-labelling, A431 cells at 70–80% confluency were starved for 1 h in DMEM lacking cysteine and methionine residues and serum (with inhibitors added as desired) and cells were then labelled for 2 h in starvation medium supplemented with 0.4 mCi/ml [³⁵S]methionine/cysteine (NEN Bioscience, Boston, MA, U.S.A.), 5% dialysed FBS and inhibitors. Cells were then transferred on to ice and washed once with DMEM and twice with HBSM (25 mM Hepes/NaOH, pH 7.4/150 mM NaCl/2 mM MgCl₂), and processed for lysis and immunoprecipitation as described below. For [³H]palmitate-labelling, A431 cells at 80–90% confluency were serum-starved for 2 h and then pulsed for 2 h in a medium containing 0.2 mCi/ml [³H]palmitic acid (NEN Bioscience) and 5% dialysed FBS. 2-BP (50 µM) or 0.05% DMSO was present for 12 h before labelling and throughout starvation and labelling.

Antibody, immunoprecipitation and immunoblotting

The following antibodies were used: anti-CD9, Alb6 (Immuno-tech, Miami, FL, U.S.A.) and C9-BB [24] for immunoprecipitation and Western-blot analysis respectively; anti-CD81, M38 [31]; anti-CD151, 5C11 [28]; anti-integrin α3, A3-X8 [32]; anti-GFP (where GFP stands for green fluorescent protein), a monoclonal (JL-8) and a full-length polyclonal antibody (Clontech, Palo Alto, CA, U.S.A.). For immunoprecipitation, cells were washed twice with HBSM buffer and lysed in HBSM containing one of the following detergents: 1% Brij97 (Sigma) or 1% Triton X-100 (Roche, Indianapolis, IN, U.S.A.) or 1% Triton + 0.1% SDS or RIPA (1% Triton X-100/0.5% deoxycholate/0.1% SDS), supplemented with a complete protease inhibitor cocktail (Roche) for 1 h at 4 °C. To block free thiol groups, 10 mM NEM (*N*-ethylmaleimide) was added to the lysis buffer in some experiments. Lysates were clarified by centrifugation at 14 000 g for 15 min and then cleared with Protein G-Sepharose for 1 h.

After the addition of a specific antibody, immunocomplexes were collected for 2–12 h on Protein G beads. In re-precipitation experiments, protein complexes obtained from Brij97 lysis buffer in the first round of immunoprecipitation were dissociated by incubating the beads for 30 min with 1% Triton and 0.1% SDS in HBSM. The eluate was then utilized for a second round of immunoprecipitation with a specific second antibody and Protein G beads. For SDS/PAGE, proteins were eluted in sample buffer containing 1% SDS for 10 min at 37 °C in the absence of reducing agent. For experiments involving capture of cell-surface CD9 protein complexes, HBSM-washed cells were incubated with 2 µg/ml Alb6 antibody in HBSM + 1% FBS for 45 min at 4 °C and then washed three times with HBSM. Cells were lysed in Brij97 and immunocomplexes were precipitated with Protein G beads. Protein samples were typically analysed on 11% (w/v) polyacrylamide gels, followed by transfer on to a nitrocellulose membrane and immunoblotting with specific antibodies diluted in PBS + 5% (w/v) fat-free milk and 0.1% Tween-20, using a horseradish peroxidase-conjugated anti-mouse secondary antibody and an ECL[®] (enhanced chemiluminescence) detection kit (Amersham Biosciences). Radiolabelled samples were transferred on to a PVDF membrane (Bio-Rad Laboratories) and exposed to BioMax MS film (Kodak, Rochester, NY, U.S.A.) at –80 °C with an intensifying screen.

Chemical cross-linking and surface-labelling

The thiol-specific cross-linkers DTME (dithio-bismaleimidoethane), BMB (1,4-bismaleimidobutane) and BMH [1,6-bis(maleimido)hexane] and the amino-specific cross-linker DSP (dithio-bis-succinimidylpropionate) were obtained from Pierce Biotechnology (Rockford, IL, U.S.A.), and dissolved in DMSO at 10 mg/ml just before use. Thiol-specific cross-linkers were diluted to 0.2 mg/ml in HBSM for treatment of cells; DSP was added at 0.1–0.25 mg/ml to Brij97 detergent lysates prepared in HBSM. Cells were cross-linked for 45–60 min on ice, followed by two 5 min washes with HBSM containing 5 mM cysteine; in addition, lysis buffer contained 2 mM cysteine. Cross-linking with DSP in 1% Brij97 lysates (with or without 10 mM NEM) was performed for 1 h at 4 °C, followed by the addition of 20 mM Tris/HCl (pH 7.5) and 1% Triton X-100 and incubation for 15 min. Clearing with Protein G and immunoprecipitation was then performed at 4 °C. For cleavage of the reducible cross-linker DTME, samples were treated with 1% 2-mercaptoethanol for 10 min at 95 °C before electrophoresis. To label cell-surface proteins with biotin, A431 cells pretreated overnight with 50 µM 2-BP or 0.05% DMSO were washed twice with ice-cold HBSM and incubated with 0.2 mg/ml sulphy-NHS-LC-biotin (where NHS stands for *N*-hydroxysuccinimido; Pierce) in HBSM, with or without 0.2 mg/ml DTME, for 45 min on ice. Cells were washed three times with HBSM + 20 mM Tris/HCl (pH 7.5) and 5 mM cysteine/HCl and lysed in HBSM containing 1% Triton X-100.

CD9 constructs, mutagenesis and transfection

RD and HT1080 cells were transiently transfected with wild-type human CD9 cloned into vector pcDNA3 (Invitrogen, Carlsbad, CA, U.S.A.), using FuGene reagent (Roche) and harvested 48 h later. Mutation of CD9 cysteine residues to serine residues was achieved with mutagenic primers using standard PCR procedures. Mutant cDNAs were cloned in the expression vector pCMV-Tag3B (Stratagene, La Jolla, CA, U.S.A.), yielding CD9 protein with an N-terminal tag of approx. 2.5 kDa including the myc epitope. Fusion of the C-terminus of CD9 with GFP was performed

using vector pEGFP-N1 (Clontech); the fusion has a ten-amino-acid linker between CD9 and GFP. The GFP fusion of CD151 protein in pEGFP-N1 was performed as described in [14]. Tandem CD9 protein (CD9 $\times$ 2) was constructed by cloning two CD9-coding sequences one after another in a retroviral expression vector pLXIZ [33].

RESULTS

Human CD9 protein can oligomerize

Initial evidence for CD9 oligomeric structures was obtained from transiently transfected human HT1080 and RD cells (which have low endogenous CD9 levels). From 1% Brij97 lysate, CD9 immunoprecipitation and immunoblotting were performed under non-reducing conditions. In addition to CD9 bands of 25 and 27 kDa (presumably N-glycosylated CD9), distinct bands of approx. 38 kDa were observed together with additional weaker bands of approx. 52–54 and approx. 70–72 kDa (Figure 1A). None of these additional bands were visible in vector-transfected cells. As shown below (e.g. Figure 7A), the appearance of these additional bands is due to spontaneous, intermolecular disulphide bonding of membrane-proximal cysteine residues.

In contrast with transiently overexpressed CD9 (Figure 1A), endogenous CD9 in human carcinoma A431 cells appeared as 25 and 27 kDa bands, without the larger bands (Figure 1B). However, if cells were treated with 2-BP, to inhibit protein palmitoylation, an additional CD9 band of approx. 38 kDa was seen (Figure 1B). Other compounds (such as Brefeldin A, monensin, nocodazole and tunicamycin) did not yield a 38 kDa protein (Figure 1B and results not shown). Results in Figure 1(C) confirmed that 2-BP treatment completely eliminates the incorporation of [3 H]palmitate into CD9. Whereas the [3 H] signal disappeared (upper panel), a fraction of CD9 protein was shifted to a slightly smaller mass, consistent with the loss of as many as six palmitoyl moieties (lower panel).

Whereas the 38 kDa form of CD9 was observed in 1% Brij97 lysates (Figure 1B and lane 1 in Figure 1D), it was not seen when 2-BP-treated cells were lysed in the more stringent Triton X-100 or RIPA conditions (Figure 1D, lanes 3 and 4). However, if initial lysis was done in Brij97 and then Triton X-100 was added just before immunoprecipitation, the 38 kDa form still remained (lane 2). These results suggest that the 38 kDa protein arises from a protein–protein interaction that is initially Triton X-100-sensitive, but then undergoes time-dependent post-lysis stabilization, leading to Triton X-100 resistance. As shown below, there is no time-dependent change in the protein–protein interaction itself, but only in the spontaneous disulphide cross-linking that stabilizes the interaction so that it can be detected by SDS/PAGE. In addition, the absence of any 38 kDa protein from the Triton X-100 lysate (Figure 1D, lane 3) serves as a critical negative control for subsequent experiments in Figures 2(B), 2(C), 4(A), 6(A–D), 8(A), 9(A) and 9(B). In those experiments, involving cross-linking of intact cells followed by Triton X-100 cell lysis, we can be assured that the additional bands that appear are entirely due to the capturing of *in situ* cell-surface structures before cell lysis.

CD9 oligomerization involves cysteine residues

From the results in Figures 1(B) and 1(D), we hypothesized that 2-BP inhibition of palmitoylation made intracellular CD9 cysteine residues available for spontaneous intermolecular disulphide bonding after cell lysis. In support of this, inclusion in A431 cell

Figure 1 Spontaneous oligomerization of CD9 protein

(A) Human HT1080 and RD cells were transfected to express vector control or human CD9 protein. After 48 h, cells were lysed in a buffer containing 1% Brij97; then, CD9 immunoprecipitation was performed, followed by CD9 Western-blot analysis. CD9-immunoreactive bands of approx. 52 and 70 kDa from HT1080 cells are marked with asterisks. The weak band of approx. 31 kDa was non-specific, and a CD9 band of approx. 22–24 kDa seen in some transfection experiments (e.g. RD transfection in A) was presumably a degradation product. (B) Human A431 cells were treated with 50 μ M 2-BP for 12 h or with DMSO, nocodazole, monensin or Brefeldin A for 6 h. Cells were lysed and CD9 protein was analysed as in (A). (C) A431 cells were treated overnight with 50 μ M 2-BP or 0.05% DMSO and then labelled for 2 h with [3 H]palmitate in the continued presence of 2-BP or DMSO. Cells were lysed in a buffer containing 1% Triton X-100 and CD9 was immunoprecipitated. (D) A431 cells treated with 100 μ M 2-BP for 11 h were either lysed in 1% Brij97, 1% Triton X-100 and RIPA or lysed in 1% Brij97 followed by the addition of 1% Triton X-100 after 1 h. CD9 protein was analysed as in (A, B). IP, immunoprecipitation.

lysis buffer of a thiol-blocking agent, NEM, eliminated the appearance of 2-BP-inducible higher-molecular-mass CD9 bands, in both whole cell lysates and in CD9 immunoprecipitations (Figure 2A). To show that the 38 kDa CD9 protein could be stabilized on intact cells, A431 cells were treated with 2-BP and then with a thiol-specific, membrane-permeable cross-linker, DTME, before lysis in 1% Triton X-100. The use of Triton X-100 disrupts CD9 oligomers that have not been stabilized before lysis, as shown in Figure 1(D). Treatment of intact cells with DTME allowed the recovery of a substantial proportion (approx. 25%) of CD9 protein as a Triton-resistant 38 kDa form (Figure 2B). After treatment with 2-BP, a fraction of monomeric

Figure 2 CD9 cross-linking via cysteine residues

(A) A431 cells were treated overnight with 50 μ M 2-BP or DMSO used as control and lysed in 1% Brij97 in the presence or absence of 10 mM NEM. Cell lysates and CD9 immunoprecipitates were analysed by CD9 blotting. (B) A431 cells were treated with 2-BP as in (A) and then incubated with 0.2 mg/ml of the thiol-specific cross-linker DTME for 45 min at 4 °C. Cells were lysed in a buffer containing 1% Triton X-100 and 0.1% SDS and CD9 protein was analysed as in (A). (See text for details of bands marked 'u' and 'p'). (C) The experiment was performed as in (B), but using thiol-specific cross-linkers DTME, BMH and BMB.

unpalmitoylated CD9 appeared (bands marked 'u') that was slightly smaller than palmitoylated CD9 (bands marked 'p'). Since only unpalmitoylated CD9 can be cross-linked by thiol agents, this must be taken into account when estimating cross-linking efficiency. For example, in Figure 2(B), the intensity of the homodimer is at least as high as the unpalmitoylated fraction of CD9 (marked 'u'), indicating at least 50% cross-linking efficiency. In addition to DTME (13.3 Å spacer; 1 Å = 0.1 nm), two other thiol-specific reagents, BMH (16.1 Å) and BMB (10.9 Å) yielded similar quantities of cross-linked 38 kDa products, whereas a somewhat higher proportion of the 52 kDa band was seen with BMB (Figure 2C). The 38 and 52 kDa CD9 bands were virtually absent if 2-BP or thiol cross-linking agents were omitted (Figures 2B and 2C). Hence, free intracellular cysteine residues in CD9, exposed by blocking of palmitoylation, can be efficiently utilized as substrates for thiol-specific cross-linking. Notably, the pattern of cross-linked bands observed post-lysis and without added cross-linker (Figure 2A) is virtually identical with the pattern obtained by adding cross-linkers to intact cells (Figure 2B). Hence, spontaneous post-lysis disulphide

Figure 3 Stabilization of CD9 oligomers by amino-specific cross-linking

(A) A431 cells were treated with 50 μ M 2-BP or palmitate for 14 h and lysed in 1% Brij97 buffer containing 10 mM NEM; then, 0.25 mg/ml amino-specific cross-linker DSP (or 2.5% DMSO) was added. After maintaining the cells for 1 h at 4 °C, 20 mM Tris/HCl and 1% Triton X-100 were added. CD9 was then immunoprecipitated and blotted. (B) RD cells were transfected to express myc-tagged CD9, in which all six intracellular cysteine residues were mutated to serine residues. Lysis, cross-linking with DSP (0.1 mg/ml) and CD9 analysis were performed as in (A).

cross-linking is as specific and reliable as prelysis cross-linking by homobifunctional thiol-specific agents.

CD9 oligomerization is not artificially created owing to the absence of palmitoylation or exposure of cysteine residues

We considered that either removal of palmitate or exposure of membrane-proximal cysteine residues could create CD9 oligomers that would not otherwise exist. To address this issue, we utilized the amino-specific cross-linker DSP. This agent cross-linked endogenous CD9 with a similar efficiency in both 2-BP-treated and untreated A431 cells (Figure 3A), again yielding a major approx. 38 kDa band, as seen in Figures 1 and 2. The slightly smaller mass of CD9 cross-linked after 2-BP treatment (Figure 3A, lane 4) is consistent with the idea that CD9 is less palmitoylated. The low level of background 38 kDa band seen in lane 3 but not in lane 1 is consistent with 2-BP-induced cysteine exposure, leading to direct disulphide bridging (as also seen in Figures 1B, 1D and 2A).

Transiently expressed in RD cells, a palmitoylation-deficient CD9 mutant, with all six intracellular cysteine residues replaced by serine residues (CD9/Cys0), was nonetheless still readily cross-linked with DSP (Figure 3B). Consistent with the absence of cysteine residues, there was no spontaneous cross-linking of CD9/Cys0 in the absence of DSP. The somewhat increased mass of the cross-linked products in Figure 3(B) (approx. 43 and 67 kDa) is due to the myc tag (approx. 2.5 kDa; myc epitope plus a linker sequence) on the N-terminus of the protein. These results demonstrate that the capability of CD9 to oligomerize is unaffected by inhibition of palmitoylation and is not dependent on the presence of palmitoylation sites.

The predominant CD9 oligomer is a homodimer

To determine whether the major cross-linked CD9 adduct (38 kDa) is a dimer of 25 kDa CD9, we first analysed components

Figure 4 Evidence for homodimerization of CD9

(A) A431 cells were metabolically labelled with [^{35}S]methionine/cysteine for 2 h in the presence of 100 μM 2-BP or palmitate, and cross-linked with 0.2 mg/ml DTME before lysis. Cells were lysed in a buffer containing 1% Triton X-100 + 0.1% SDS and CD9 immunoprecipitations were analysed under reducing or non-reducing SDS/PAGE, followed by autoradiography. (B) RD cells were transfected to express a protein consisting of two tandem CD9 polypeptides ($\text{CD9} \times 2$). CD9 immunoprecipitates from mock- and $\text{CD9} \times 2$ -transfected cells were probed by CD9 Western-blot analysis. The band seen just above the endogenous 25 kDa CD9 band is probably a cleavage product of the $\text{CD9} \times 2$ molecule.

of ^{35}S -labelled, DTME-stabilized 38 kDa CD9. After reduction to cleave the cross-linker, no additional ^{35}S -labelled protein bands were visible (Figure 4A, lane 8), which were also absent from lanes 5–7. Hence, 38 kDa CD9 did not contain any obvious ‘non-CD9’ protein candidates for heterodimeric linkage to CD9. In addition, we prepared and analysed a ‘tandem’ CD9 molecule ($\text{CD9} \times 2$), with two CD9 sequences linked (via a short spacer) to produce one polypeptide. This CD9 molecule was stably and efficiently delivered to the cell surface (T. V. Kolesnikova, unpublished work). When introduced in RD cells, the covalent CD9 dimer migrated at approx. 38 kDa, rather than 50 kDa (Figure 4B), thus supporting our conclusion that the CD9 homodimer is of 38 kDa.

To analyse further the homo-oligomerization of CD9, we transiently introduced GFP-tagged CD9 in RD cells stably pretransfected with untagged CD9 or with vector control. CD9 oligomerization was probed by treating cells with 2-BP starting midway through transfection and cross-linking them with BMB before lysis in Triton X-100. A CD9 protein was precipitated from lysates and analysed by CD9 and GFP blotting (Figure 5). As shown in Figure 5 (lanes 3 and 5), there was a pronounced homodimerization of 40–42 kDa GFP–CD9, resulting in a band of approx. 80–85 kDa on both CD9 and GFP Western blots. Analysis of negative control CD9 (CD9^{Cys0} –GFP, in which all six cytoplasmic cysteine residues are replaced by serine residues) revealed no such band (Figure 5, lanes 4 and 6). Moreover, when CD9^{WT} –GFP was introduced into RD cells stably expressing untagged CD9 (where WT is wild-type), there appeared a band of approx. 54–56 kDa, which was immunoreactive with both CD9 and GFP antibodies (lane 5). This band was notably absent from

cells expressing CD9 alone (lane 2) or CD9–GFP alone (lane 3). Hence, the approx. 54–56 kDa band is entirely consistent with a CD9/CD9–GFP cross-linked dimer. Also there was a decrease in the amount of untagged CD9 dimer and trimer seen when CD9^{WT} –GFP was introduced (Figure 5, upper panel, cf. lanes 2 and 5), consistent with untagged CD9 cross-linking with CD9–GFP to form CD9/CD9–GFP (54–56 kDa) as well as higher-order complexes of > 100 kDa (see lane 5 in both CD9 and GFP Western blots).

Homodimers versus heterodimers

We next compared the extent of tetraspanin homodimerization and heterodimerization. As in previous experiments, intact A431 cells were treated with 2-BP (to expose cysteine residues) and DTME (to cross-link cysteine residues) and then lysed with 1% Triton X-100 + 0.1% SDS (to break up non-covalent tetraspanin associations). As expected, CD9 immunoprecipitation and CD9 blotting yielded major 38 and 52 kDa bands (CD9 homodimer and homotrimer; Figure 6A, lane 4). In the same experiment, CD81 immunoprecipitation and CD81 blotting yielded CD81-reactive bands of approx. 36 and 50 kDa (CD81 homodimer and homotrimer; Figure 6B, lane 8). Compared with the extent of homodimerization of CD9 and CD81, there was relatively little heterodimer detected. For example, CD81 blotting of immunoprecipitated CD9 yielded no detectable multimers (Figure 6B, lane 4), and CD9 blotting of immunoprecipitated CD81 yielded only traces of apparent CD9–CD81 heterodimer and heterotrimer (approx. 37 and 51 kDa respectively; Figure 6A, lane 8). CD151 blotting of the immunoprecipitated CD9 complex yielded CD151-reactive bands of approx. 42 and 58 kDa (Figure 6C, lane 4), corresponding to CD9–CD151 heterodimers and heterotrimers. Also, CD151 blotting yielded CD151–CD81 heterodimers and heterotrimers (Figure 6C, lane 8). However, highly efficient blotting of CD151 in Figure 6(C) may over-emphasize the levels of CD9–CD151 and CD81–CD151 heterodimers and heterotrimers (Figure 6C, lanes 4 and 8), since comparable bands were not detected in CD9 or CD81 Western blots (Figures 6A, lane 4 and 6B, lane 8). In control experiments (Figures 6A–6C), omission of 2-BP (lanes 1, 2, 5 and 6) or DTME (lanes 1, 3, 5 and 7) yielded little or no cross-linked proteins. In conjunction with DTME, we used 1% Triton X-100 (instead of 1% Brij97) to remove non-covalently associated tetraspanins. The effectiveness of this approach is evidenced by the relative absence of indirectly immunoprecipitated tetraspanin monomers in Figure 6 (A, lanes 6 and 8; B, lanes 2 and 4; C, lanes 2, 4, 6 and 8). The key lanes from Figures 6(A–C) are carefully aligned and shown again in Figure 6(D). Notably, the bands corresponding to CD9–CD9 and CD81–CD81 homodimers and homotrimers are predominant, with little evidence for heterodimers and heterotrimers (Figure 6D, lanes 1 and 3). Hence, levels of the CD9–CD151, CD81–CD9 and CD81–CD151 heterodimers and heterotrimers (lanes 2, 4 and 5) are relatively small compared with the homodimers and homotrimers.

Unequal cysteine contributions to homodimerization

To evaluate the contributions of different CD9 cysteine residues to the homodimer formation, we tested a series of CD9 mutants in which one or more intracellular cysteine residues were replaced by serine residues. These mutants were transiently transfected into RD cells and spontaneous disulphide formation (without 2-BP treatment) was analysed after lysis in Brij97. CD9 with all six membrane-proximal cysteine residues mutated readily homodimerized (Figure 3B); however, as expected, we could

Figure 5 Homodimerization of tagged and untagged CD9 proteins

RD cells, stably transfected with untagged CD9 (RD/CD9) or vector (RD/vect.) alone, were subsequently transfected with CD9^{WT}-GFP or CD9^{Cys0}-GFP constructs for 48 h. Cells were treated with 100 μ M 2-BP during the last 16 h of transfection, followed by cross-linking with 0.2 mg/ml BMB and lysis in a buffer containing 1% Triton X-100. CD9 was immunoprecipitated and then blotted for CD9 under non-reducing conditions (upper panel) or GFP under reducing conditions, since the BMB cross-linker is non-reducible (lower panel). As a control, non-transfected RD cells or stable CD9/RD cells were similarly treated with 2-BP and cross-linked and CD9 was immunoprecipitated and blotted (lanes 1 and 2).

not detect it by SDS/PAGE in the absence of spontaneous disulphide cross-linking (Figure 7A, lane 6). However, a single residue in the middle of the molecule (Cys-87) or two C-terminal cysteine residues (Cys-218 and Cys-219) did enable homodimer stabilization and detection (lanes 4 and 5), and all three of these residues together yielded an even more SDS/PAGE-resistant homodimer (lane 3). On the other hand, the presence of six cysteine residues in wild-type CD9 led to diminished homodimer detection by this spontaneous method (Figure 7A, lane 2), despite an abundance of homodimer being present (see e.g. Figure 3A, lane 2). We suspect that six cysteine residues may enhance the formation of intramolecular disulphide bonds, thereby decreasing the efficiency of intermolecular disulphides. Also, the detection of homodimerization of the single-cysteine CD9 excludes the possibility that intramolecular disulphide formation produces the 38 kDa form of CD9.

The tetraspanin CD151, stably introduced as a 52 kDa fusion with GFP into MDA-MB-231 cells, yielded only a trace of spontaneously stabilized approx. 105 kDa homodimer (Figure 7B, lane 2). However, the homodimer band was much more abundant

for CD151, with two cysteine residues (Cys-11 and Cys-15) replaced by serine residues, thus leaving only four remaining membrane-proximal cysteine residues (lane 3). Analysis of samples under reducing conditions revealed only the monomeric CD151-GFP form (lanes 5 and 6), consistent with the loss of disulphide-mediated cross-linking. CD151 with four mutated cysteine residues (Cys-11, Cys-15, Cys-241 and Cys-242), which retained only Cys-79 and Cys-80, also showed a substantial amount of spontaneously stabilized homodimer in SDS/PAGE (results not shown).

Homodimers of CD9 can appear on the cell surface and associate with integrins and other tetraspanins

To determine whether CD9 homodimers appear on the cell surface, A431 cells were treated with 2-BP to expose cysteine residues and then surface-labelled with biotin in the presence or absence of a DTME cross-linker. As indicated in Figure 8(A), an abundance of 38 kDa biotin-labelled CD9 homodimer was

Figure 6 CD9 homodimers versus CD9 heterodimers

A431 cells were treated with 25 μ M 2-BP or palmitate for 16 h and then either lysed in a buffer containing 1% Brij97 and 10 mM NEM ('- DTME, Triton') or first treated with 0.2 mg/ml DTME and then lysed in 1% Triton X-100 + 0.1% SDS ('+ DTME, Triton'). CD9 (lanes 1–4) and CD81 (lanes 5–8) were immunoprecipitated and then blotted for CD9 (A), CD81 (B) or CD151 (C). Each experiment was performed multiple times under identical conditions and using carefully aligned molecular-mass standards. Thus, in (D), it was feasible to excise and compare lanes A4, C4, B8, A8 and C8 side by side to appreciate better the mass differences.

captured on intact cells when DTME was added, but not when omitted. In an alternative approach, an anti-CD9 antibody was added to intact 2-BP-treated A431 cells, excess antibody washed away, cells were lysed in Brij97 (to retain homodimers while spontaneous disulphide cross-linking occurred) and then the cell-surface pool of CD9 selectively immunoprecipitated. Again, a spontaneous disulphide-stabilized CD9 homodimer was readily detected (Figure 8B, lane 2). Importantly, the proportion of 38 kDa CD9 homodimers detected relative to monomeric CD9 was similar for both cell-surface CD9 (lane 2) and total CD9 (lane 4). Hence, CD9 homodimers are well represented on the cell surface. Again, almost identical results were obtained either by treatment of intact cells with covalent cross-linker (Figure 8A) or by spontaneous, post-lysis disulphide stabilization of CD9 homodimer (Figure 8B), further emphasizing the specificity and reliability of the latter method.

Next, we asked whether CD9 homodimers engage in typical 'tetraspanin web' interactions with other tetraspanins and with

Figure 7 Variable contributions of cysteine residues to CD9 and CD151 dimerizations

(A) RD cells were transfected to express myc-tagged CD9 protein in which one or several cysteine residues were replaced by serine residues. Cys6, all six cysteine residues remaining; Cys3, Cys-87, Cys-218 and Cys-219 remaining; Cys2, Cys-218 and Cys-219 remaining; Cys1, Cys-87 remaining; Cys0, all cysteine residues removed. Cells were collected 48 h post-transfection, lysed in a buffer containing 1% Brij97 and analysed by CD9 immunoprecipitation and blotting. (B) MDA-MB-231 cells were stably transfected to express GFP-tagged wild-type CD151 protein or a Cys^{11,15} → Ser double-point mutant (C11,15S). Cells were lysed in a buffer containing 1% Brij97 and immunoprecipitated using an anti-GFP antibody, followed by GFP blotting.

integrins. To this end, A431 cells were treated with 2-BP to expose cysteine residues, homodimers captured using DTME cross-linker and the cells lysed under detergent conditions (i.e. Brij97) that preserve secondary 'tetraspanin web' associations. Then, directly immunoprecipitated CD9 (Figure 8C, lanes 1, 2, 5 and 6) was compared with CD9 that was indirectly immunoprecipitated in complexes with CD81, CD151 or α 3 integrin and subsequently recovered by CD9 re-immunoprecipitation (Figure 8C, lanes 3, 4 and 8). Although a little less CD9 was recovered from the indirect immunoprecipitations (Figure 8C, cf. lanes 3, 4 and 8 with lanes 2 and 6), homodimers were readily detectable (lanes 3, 4 and 8). Hence, CD9 homodimers engage in typical tetraspanin web

Figure 8 Localization of CD9 homodimer to the cell surface and its association with integrin and other tetraspanins

(A) A431 cells were incubated overnight with 50 μ M 2-BP and then surface-labelled with sulphy-NHS-LC-biotin in the absence or presence of 0.2 mg/ml DTME. Cells were lysed in a buffer containing 1% Triton X-100 and CD9 protein was immunoprecipitated. (B) A431 cells were treated overnight with 50 μ M 2-BP or palmitate and then either lysed immediately in a buffer containing 1% Brij97 or first incubated for 45 min at 4 °C with 2 μ g/ml of the anti-CD9 monoclonal antibody Alb6. CD9 was precipitated from Brij97 lysates with the monoclonal antibody Alb6 and Protein G beads. Antibody-treated cells were subsequently lysed in 1% Brij97, and Protein G beads were added to precipitate cell-surface immunocomplexes of CD9. (C) A431 cells were treated overnight with 50 μ M 2-BP or palmitate, cross-linked with DTME and then lysed in a buffer containing 1% Brij97. CD9 was immunoprecipitated either directly from the lysates or re-precipitated from CD81, CD151 and integrin α 3 immunoprecipitates after treating them with 1% Triton X-100 and 0.1% SDS to dissociate interacting proteins.

interactions. Furthermore, consistent with the results in Figure 6, a single band of CD9 homodimers was almost exclusively observed, even though conditions were designed to permit associations with other tetraspanins (such as CD81 and CD151).

Formation of a CD9 homodimer requires an intact Golgi apparatus

To determine where CD9 homodimers might form in the secretory pathway, A431 cells were treated with a variety of inhibitors. Treatment of cells with 2-BP alone (Figure 9A, DMSO vehicle control), as expected, led to the appearance of an unpalmitoylated pool of newly synthesized CD9 (marked 'u') with cysteine residues available for DTME cross-linking, leading to an SDS/PAGE-resistant 38 kDa homodimer. Simultaneous treatment with 2-BP and cycloheximide eliminated the approx. 24 kDa band of unpalmitoylated CD9 (marked 'u') and, hence, also eliminated the pool of proteins with exposed cysteine residues available to be cross-linked for the detection of a 38 kDa homodimer (Figure 9A). Importantly, disruption of the Golgi apparatus with Brefeldin A did not affect synthesis of 24 kDa unpalmitoylated

Figure 9 Requirement of an intact Golgi apparatus for the formation of CD9 homodimers

(A) A431 cells were treated for 6 h with 100 μ g/ml 2-BP, supplemented with either DMSO, cycloheximide, Brefeldin A, monensin or nocodazole. Cells were cross-linked with 0.2 mg/ml DTME before lysis in a buffer containing 1% Triton X-100. The lysates were analysed by CD9 immunoprecipitation and blotting. (See text for details of bands labelled 'p' and 'u'.) (B) The experiment was performed as in (A), with CD9 immunoprecipitates analysed also by CD151 blotting to reveal cross-linked CD9-CD151 complexes.

CD9, but nonetheless abrogated the appearance of a homodimer. Since a pool of proteins with exposed cysteine residues is available, Brefeldin A must be abolishing the actual 'formation' of the homodimer, and not just its 'detection'. Treatment of cells with monensin or nocodazole (Figure 9A) or tunicamycin (results not shown) did not diminish the efficiency of detection/formation of a CD9 homodimer. To test whether a low-level tetraspanin heterodimer (CD9-CD151) formation was also Golgi-dependent, the experiment in Figure 9(A) was repeated and CD9 immunoprecipitates were blotted with a highly sensitive anti-CD151 antibody. As shown in Figure 9(B), Brefeldin A again virtually eliminated DTME cross-linkable CD9 products and, at the same time, significantly decreased the amount of CD151 that is cross-linked to CD9. Monensin and nocodazole had only minor effects on CD9-CD9 or CD9-CD151 cross-linking. Thus, cellular assembly of CD9 into homodimers or CD9-CD151 heterodimers requires the intact Golgi, but not lysosomal, endosomal or microtubule-dependent trafficking.

DISCUSSION

Evidence for homodimers from cysteine cross-linking experiments

Numerous studies have shown that tetraspanin proteins can associate with each other and with many non-tetraspanin partners in a seemingly vast network of largely indirect interactions.

However, there has been little evidence presented for tetraspanin homodimerization *in situ*. In the present study, four different methods and several different cell types are used to demonstrate CD9 homodimerization, and this is supplemented with evidence for CD81 and CD151 tetraspanin homodimerization. In our first method, 38 kDa homodimer-like adducts were recovered from transiently transfected CD9 in RD or HT1080 cells. We surmise that protein overexpression resulted in an atypically high proportion of CD9 being synthesized without being palmitoylated, thus having cysteine residues available for spontaneous intermolecular disulphide bonding. Consistent with this interpretation, a 38 kDa homodimer, although present, was not detected in RD cells when all six membrane-proximal cysteine residues were mutated. In our second method, the detection of endogenous CD9 homodimers was enhanced by treating cells with 2-BP, thereby inhibiting palmitoylation and making cysteine residues available for intermolecular disulphide binding. This 'zero-length' cross-linking approach worked best if cells were lysed in a mild detergent (e.g. Brij97) to maintain hydrophobic interaction between CD9 monomers, thereby enabling time-dependent intermolecular disulphide formation after exposure of cell lysate to oxygen. Confirming the role of cysteine residues, homodimer detection was eliminated by the thiol-reactive agent NEM or by mutation of membrane-proximal cysteine residues in CD9. On the other hand, if copper phenanthroline [Cu(II)-(1,10-phenanthroline)₃] was added to catalyse disulphide bonding [34], homodimer detection was enhanced (results not shown).

In some experiments, cross-linking of exposed cysteine residues occurred 'post-lysis', thus suggesting the possibility that results could be spurious and, therefore, not representative of the situation *in situ*. However, we are convinced that 'post-lysis' results are valid for several reasons. First, essentially identical results were obtained whether cross-linking occurred before lysis or after lysis (e.g. cf. Figures 2A and 2B and Figures 8A and 8B). Secondly, if post-lysis results were artifactual, one would expect to see a diversity of results. Instead, we observed almost exclusively homodimers in 'post-lysis' experiments with three different cell lines and two different tetraspanins, each contributing several distinct cysteine residues. Thirdly, in previously published 'mixing experiments' involving tetraspanins CD81 and CD151, we failed to obtain any evidence for post-lysis formation of new complexes [28,35].

Our third and most efficient method for demonstrating homodimerization was to treat cells with 2-BP to expose membrane-proximal cysteine residues and then to cross-link tetraspanins on intact cells using the homobifunctional cysteine-reactive reagent DTME. Besides DTME (length, 13.3 Å), other cysteine-reactive cross-linking agents (BMH and BMB; length, 16.1 and 10.9 Å respectively) were also quite efficient. Cross-linking by cysteine-reactive agents such as these is diagnostic for direct protein-protein interaction [36–38]. The efficiency of cysteine cross-linking (up to 25% of the total CD9) is consistent with cysteine cross-linking efficiencies observed for other direct protein-protein interactions [36–38]. Indeed, the extent of CD9 homodimerization is underestimated, owing to an abundant pool of 'uncross-linkable' palmitoylated proteins present before cell treatment with 2-BP and remaining even after extended (10–16 h) incubation with 2-BP. Considering only the unpalmitoylated pool of CD9, cross-linking efficiency increases to approx. 50% or greater in some experiments. Cysteine residues are often introduced by mutagenesis and then cross-linked to evaluate protein associations and to map interaction interfaces [36,39]. However, to our knowledge, this is the first example of the use of inhibition of protein acylation to expose existing cysteine residues for subsequent chemical cross-linking. This novel approach

should have widespread utility as the list of palmitoylated proteins continues to grow [40].

Evidence for homodimers from amino cross-linking experiments

To complement cysteine cross-linking, the amino cross-linker DSP, a reagent with a long history of specific cross-linking both on intact cells and in cell lysates, was utilized [41–43]. Again, using a variety of cell lines and multiple conditions, tetraspanin homodimers were predominantly captured. Furthermore, since DSP acts through amino groups rather than cysteine residues, it was demonstrated clearly that tetraspanin homodimerization is not dependent on palmitoylation. Indeed, CD9 lacking all six membrane-proximal cysteine residues was readily cross-linked using DSP, and inhibition of palmitoylation by 2-BP did not diminish cross-linking by DSP. Hence, CD9 differs from the synaptic vesicle protein synaptotagmin, a molecule that undergoes palmitoylation-dependent homo-oligomerization [44].

Failure of 2-BP to promote cross-linking of CD9 by an amino cross-linker argues strongly against an inhibitory role for palmitoylation. However, in one RD cell experiment, wild-type CD9, with all of its palmitoylation sites, was readily cross-linked through amino groups, but less efficiently than mutant CD9 with no palmitoylation sites (results not shown). Such a result might suggest that palmitoylation inhibits homodimer formation. However, the more probable explanation is that amino groups in the fully palmitoylated CD9 are sometimes simply less accessible to the cross-linker. Indeed, palmitoylated CD9 shows increased association with other proteins [11,12,14] and we have often found that amino groups in tetraspanins associated with other proteins are less accessible to biotinylation (results not shown).

Homodimers and heterodimers

Our present results indicate that the predominant tetraspanin oligomeric form detected by SDS/PAGE after cross-linking is a homodimer. Cross-linking of CD9-GFP (40–42 kDa) yielded a molecule with twice the mass (80–85 kDa), and cross-linked CD151-GFP (approx. 52 kDa) appeared as a homodimer of approx. 105 kDa. Furthermore, cross-linking of a mixed CD9 population yielded an apparent CD9/CD9-GFP dimer, which blotted for both CD9 and GFP and appeared only when both tagged and untagged CD9 were present. Cross-linking of untagged CD9 yielded an apparent homodimer with a mass (38 kDa) less than that expected from two 25 kDa CD9 monomers. However, the 38 kDa band is indeed also a homodimer, since it co-migrates with an artificially engineered CD9 protein dimer, and reduction of metabolically labelled 38 kDa homodimer yielded only protein bands corresponding to a CD9 monomer. The 38 kDa band did not arise due to intramolecular disulphide reorganization, since it was obtained using either thiol- or amino-reactive cross-linkers and with membrane-proximal cysteine residues either present or absent. We suspect that untagged tetraspanins, such as CD9, CD81 and CD151, adopt a highly compact dimeric/oligomeric structure, resulting in a diminished apparent mass in SDS/PAGE. However, placement of a large GFP tag on the C-terminus of interacting tetraspanins yielded dimers of the expected mass, suggesting that aberrant conformations were prevented. In a previous study [45], peripherin/rds homodimers migrated in SDS/PAGE at approximately twice the mass of a monomer.

Whereas the predominant cross-linked proteins detected by SDS/PAGE corresponded to tetraspanin homodimers (and homotrimers), we did detect small amounts of cross-linked SDS/PAGE-resistant tetraspanin heterodimers. Since these heterodimers were

essentially undetectable relative to the levels of homodimers (in the experiments designed to observe homodimers), they represent a relatively minor population. For these experiments (shown in Figure 6), A431 cells that express comparably high levels of all tetraspanins tested were used. Thus, the low abundance of heterodimers does not result from a large difference in the levels of constituent monomers. It is well established that different types of tetraspanin proteins associate with each other [1]. We suspect that after 2-BP treatment and exposure of free cysteine residues, a small subset of cross-links occurs between monomeric units from two distinct but adjacent homodimers, thus yielding an apparent heterodimer on SDS/PAGE that may not actually exist as a simple heterodimer in intact cells.

The tetraspanin dimerization interface

Cysteine cross-linking has been used to map dimerization interfaces for proteins containing multiple transmembrane domains [36,39]. Our present results now point to the membrane-proximal cysteine region of tetraspanins as being part of, or in close proximity to, a homodimerization interface. Previous studies of the crystallized CD81 large-loop protein suggested that extracellular membrane-proximal helices A, B and E form a dimerization interface [19,20]. Hence, the dimerization interface should now be extended to include membrane-proximal extracellular and intercellular regions, as well as intervening transmembrane domains. The sensitivity of homodimers to a hydrophobic detergent such as Triton X-100 is consistent with such a hydrophobic interface. Cysteine residues from CD9 (Cys-87, Cys-218 and Cys-219) and CD151 (Cys-79, Cys-80 and perhaps also Cys-241 and Cys-242) contribute to cross-linking and homodimer detection while being adjacent to transmembrane domains 2, 3 and 4. Hence, transmembrane domains 2, 3 and 4 could contribute to the dimerization interface. Unexpectedly, the presence of six cysteine residues in wild-type CD9 and CD151 yielded lower levels of spontaneously cross-linked homodimers when compared with only three cysteine residues in CD9 and only two or four cysteine residues in CD151. In particular, the N-terminal cysteine residues (Cys-9, Cys-78 and Cys-79 in CD9; Cys-11 and Cys-15 in CD151) had a suppressive effect on homodimer detection, as more homodimer was seen when these residues were mutated. We doubt that these mutations are disproportionately decreasing palmitoylation, since each of the six membrane-proximal cysteine residues in CD9 makes an approximately equal contribution to CD9 palmitoylation [11]. Instead, we suspect that the N-terminal cysteine residues are forming competing, intramolecular disulphide bonds with the other cysteine residues, making them less available for intermolecular bonding.

Homodimers as fundamental units of tetraspanin physiology

Disruption of Golgi integrity by Brefeldin A prevented the formation of CD9 homodimers, detected either by DTME cross-linking of exposed palmitoylation sites (Figure 9) or by an amino-specific cross-linking of newly synthesized, metabolically labelled CD9 (results not shown). This result is consistent with homodimerization of newly synthesized CD9 occurring in the late Golgi compartment or in the *trans*-Golgi network. Whereas most multisubunit plasma membrane proteins assemble in the endoplasmic reticulum, there are examples of proteins, such as connexin43, that assemble in the Golgi [46]. Brefeldin A also inhibited formation of the cross-linked CD9–CD151 complex (Figure 9). This result is consistent with previous results showing association between different tetraspanins appearing in

the final stage of biosynthesis [12,14]. It remains to be seen why homodimerization is delayed until after the endoplasmic reticulum and whether homodimerization is a prerequisite for CD9 association with other tetraspanins.

Just as palmitoylation-deficient tetraspanin mutants are delivered to the cell surface [11,12,14], so also was CD9 that lacked palmitoylation owing to 2-BP inhibition. Cross-linked CD9 homodimers were detected on the cell surface by two different methods. One involved cell-surface biotinylation and the other utilized treatment of intact cells with antibodies. Not only did CD9 homodimers appear at the cell surface, but also they were readily detected in complexes with other tetraspanins (CD81 and CD151) and with integrin $\alpha 3 \beta 1$. On the basis of the high efficiency of homodimer cross-linking (discussed above), the appearance of tetraspanin homodimers at the cell surface and the detection of these homodimers in typical tetraspanin protein complexes (with other tetraspanins and with $\alpha 3 \beta 1$ integrin), we propose that the tetraspanin homodimer is a fundamental primary unit within TEMs. Our results obtained with tetraspanins CD9, CD81 and CD151 are consistent with the homodimer results obtained previously for the specialized retinal tetraspanins peripherin/rds and ROM1 [45]. We suggest that homodimer formation may apply to many, if not most, tetraspanins. The bladder tetraspanins UPIa and UPIb are an exception, since heterodimers rather than homodimers appear to be the basic structural units, although UPIa homodimers were observed [23].

In conclusion, we propose that tetraspanins (CD9, CD81, CD151 and possibly others) engage in a new type of primary (i.e. direct) interaction with themselves to form homodimers. These homodimers then serve as building blocks, around which weaker, secondary and tertiary interactions are assembled to form TEMs. In contrast with these secondary and/or tertiary tetraspanin interactions that can be promoted by palmitoylation [11,12,14], the homodimerization step is not enhanced by tetraspanin palmitoylation. Hence, without perturbing homodimer formation, unpalmitoylated cysteine residues could be utilized for homodimer detection. This approach has two important advantages over traditional amino cross-linking. First, it is more efficient, presumably since membrane-proximal cysteine residues from multiple tetraspanins are closely packed together. Secondly, it is more instructive, since the contributions of individual cysteine residues can be easily compared.

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