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Differential stability of tetraspanin/tetraspanin interactions: role of palmitoylation

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Abstract The tetraspanins associate with various surface molecules and with each other to build a network of molecular interactions, the tetraspanin web. The interaction of tetraspanins with each other seems to be central for the assembly of the tetraspanin web. All tetraspanins studied, CD9, CD37, CD53, CD63, CD81, CD82 and CD151, were found to incorporate [^{3}H]palmitate. By site-directed mutagenesis, CD9 was found to be palmitoylated at any of the four internal juxtamembrane regions. The palmitoylation of CD9 did not influence the partition in detergent-resistant membranes but contributed to the interaction with CD81 and CD53. In particular, the resistance of the CD9/CD81 interaction to EDTA, which disrupts other tetraspanin/tetraspanin interactions, was entirely dependent on palmitoylation.

Key words: Tetraspanin; Interaction; Palmitoylation; Detergent-resistant membrane

1. Introduction

The tetraspanins are homologous proteins characterized by four transmembrane domains delimiting two extracellular regions of unequal size, as well as a particular fold in the large extracellular loop [1–4]. They localize at the cell surface and for some of them within intracellular compartments [5,6]. If the tetraspanins have various patterns of expression, all cell types express several of them, except erythrocytes. Increasing genetic evidence has shown the functional importance of these molecules. For example, knock-out of CD9 has proved its critical role in sperm–egg fusion [7–9]. Targeted deletion of CD81 resulted in impaired lymphoid B cell function [10]. Moreover, a relation between mutations of Talla-1/TM4SF2 and certain cases of X-linked mental retardation was demonstrated [11]. Additionally, CD81 is a co-receptor for hepatitis C virus [12] and several tetraspanins, including CD9 and CD82, provide a metastasis inhibitory signal [13,14] (for review see [1,15]). In vitro studies have also implicated tetraspanins in many cellular functions such as adhesion, migration, co-stimulation and signal transduction [1,16].

The mechanisms that support these pleiotropic functional observations are not understood. However, a striking property of tetraspanins is their presence in molecular complexes organized in different levels. Primary complexes contain one tetraspanin linked to a specific molecular partner. They combine through tetraspanin/tetraspanin interactions to form higher-order complexes that are collectively called the tetraspanin web [1,2]. Several of the primary complexes have been identified, for example (tetraspanin/partner) CD81/CD19, CD81/integrin α4β1, CD151/α3β1, CD151/α6β1, CD9 or CD81/CD9P-1 (also called FPRP or EWI-F) and CD9 or CD81/PGRL (also called EWI-2) [17–24]. The use of chimeric molecules indicated that the second half of tetraspanins, which includes the large extracellular loop, contributes to these interactions [1]. Although a major step in the assembly of the tetraspanin web, the way tetraspanins interact with each other is poorly characterized, except that it does not involve the large extracellular loop [25].

Tetraspanins are present in part in a detergent-resistant membrane (DRM) environment, as shown by the recovery of a fraction of these molecules into low-density fractions of sucrose gradients after lysis with cold detergents [26–28]. These DRM were suggested to be different from previously described cholesterol- and sphingolipid-rich lipid microdomains (also called rafts [29]). These data raised the hypothesis that the apparent association of tetraspanins could in fact reflect their co-localization in DRM. Targeting to rafts of cytoplasmic molecules such as src kinases [30] and certain transmembrane proteins [31] is dependent on fatty acylation of the proteins (for review, see [32,33]). However, not all palmitoylated transmembrane proteins are present in rafts [34], suggesting that palmitoylation may have different roles for other transmembrane proteins. Two tetraspanins, CD9 and CD81, have previously been reported to be acylated [35,36]. In this paper we have demonstrated that all tetraspanins studied are palmitoylated. Palmitoylation of CD9 occurs at any of the four internal juxtamembrane regions and contributes to the formation and/or stabilization of interactions with other tetraspanins, but not to its partition into DRM.

2. Materials and methods

2.1. Monoclonal antibodies

Anti-tetraspanin monoclonal antibodies (mAbs) used in this study were SYB-1, ALB-6 (CD9), Z81 (CD81), TS53 (CD53), TS81 (CD81), TS82, TS82b (CD82), TS151, 11B1G4 (CD151). As a negative control, we used the CD55 mAb 12A12 [21].

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Abbreviations: DRM, detergent-resistant membrane

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2.2. Cell lines, cell culture and transfection

The lymphoid B cell lines Raji and Daudi, the megakaryocytic cell line HEL and CHO (Chinese hamster ovary) cells were cultured as described [18,21]. CD9-transfected Daudi and Raji cells have previously been described [17], and a similar procedure was used to obtain Daudi cells expressing the mutant CD9 with no residual juxtamembrane residue ($CD9^{plm}$). CHO cells were transfected as previously reported [21].

2.3. Site-directed mutagenesis

Mutation of cysteines at juxtamembrane locations (positions 9, 78, 79, 87, 218 and 219) of CD9 cloned in pBluescript KS+ were made with the Quickchange Kit (Stratagene) according to the manufacturer's instructions. All mutants were entirely sequenced and subcloned into pcDNA3 (Invitrogen).

2.4. Metabolic labeling with [^{3}H]palmitate

Cells were radiolabeled as previously described by Seehafer et al. [36]: CHO cells stably or transiently transfected with wild-type or mutant CD9 cDNA were incubated in Petri dishes with 300 μCi/ml [^{3}H]palmitate (NEN, Boston, MA, USA) in RPMI 1640 containing 2% fetal calf serum (500 μl for a 10 cm^2 dish), for 3 h at 37°C. Raji and HEL cells were radiolabeled with [^{3}H]palmitate at a concentration of 10^7/ml under the same conditions. After three washes in Hanks' buffered saline solution, the cells were lysed in the presence of Triton X-100 and immunoprecipitated as previously described [21].

2.5. Analysis of protein interactions

Cells were lysed at a concentration of 2×10^7/ml in the lysis buffer (1% Brij 97 (Sigma, St. Louis, MO, USA), 10 mM Tris pH 7.4, 150 mM NaCl, 0.02% NaN_3, proteases inhibitors, and either 1 mM $CaCl_2$ plus 1 mM $MgCl_2$ or 1 mM EDTA) [21]. After 30 min at 4°C, the insoluble material was removed by centrifugation at $10\,000\times g$ and the cell lysate was precleared for 2 h by addition 1/200 volume heat-inactivated goat serum and 1/40 volume protein G-Sepharose beads (Amersham-Pharmacia, Rainham, UK). Proteins were then immunoprecipitated by adding 1 μl ascitic fluid and 10μl protein G-Sepharose beads to 250 μl of the lysate. After 2 h incubation at 4°C under constant agitation, the beads were washed five times in lysis buffer. The immunoprecipitates were separated by SDS–polyacrylamide gel electrophoresis under non-reducing conditions and transferred to a polyvinylidene difluoride membrane (Amersham Bioscience, Saclay, France). Western blotting on immunoprecipitates was performed using biotinylated mAbs (SYB-1, Z81, TS82b, 11B1G4, TS53) and a streptavidin-biotinylated horseradish peroxidase complex (Amersham), which was revealed by enhanced chemiluminescence (NEN, Boston, MA, USA).

2.6. Equilibrium density gradient centrifugation

The cells were lysed as above and equilibrium density gradient centrifugations were performed as previously described [37]: after lysis for 30 min on ice, the preparation was mixed with 60% w/w sucrose in lysis buffer without detergent to yield a final concentration of 40% sucrose. Then, 0.8 ml of the lysate–sucrose mixture was sequentially overlaid with 2 ml of 30% sucrose and 1 ml of 4% sucrose prepared in the same buffer, without detergent, and centrifuged at $200\,000\times g$ for 14–16 h in a SW55Ti rotor (Beckman). The gradient was fractionated in 0.5 ml fractions from the top of the tube and analyzed by Western blot. All steps were at 4°C.

3. Results and discussion

3.1. Higher stability of CD9-containing complexes

The use of different detergents has suggested that the tetraspanin web is assembled by the combination of various primary complexes through tetraspanin/tetraspanin interactions [1,2]. Whether all tetraspanin/tetraspanin interactions are equivalent in terms of stability is not known, as they are similarly conserved or disrupted in the detergents tested so far (conserved in Brij 97, Brij 98 or CHAPS, disrupted in Triton X-100, NP-40 or digitonin). While testing different lysis conditions we found that supplementing a Brij 97-based lysis buffer with either $CaCl_2$ and $MgCl_2$ (our standard conditions [18]) or EDTA gave strikingly different results, depending on the cell type. In the presence of divalent cations, a strong level of tetraspanin/tetraspanin interactions was observed. For example, in some experiments using Raji cells, up to 50% of

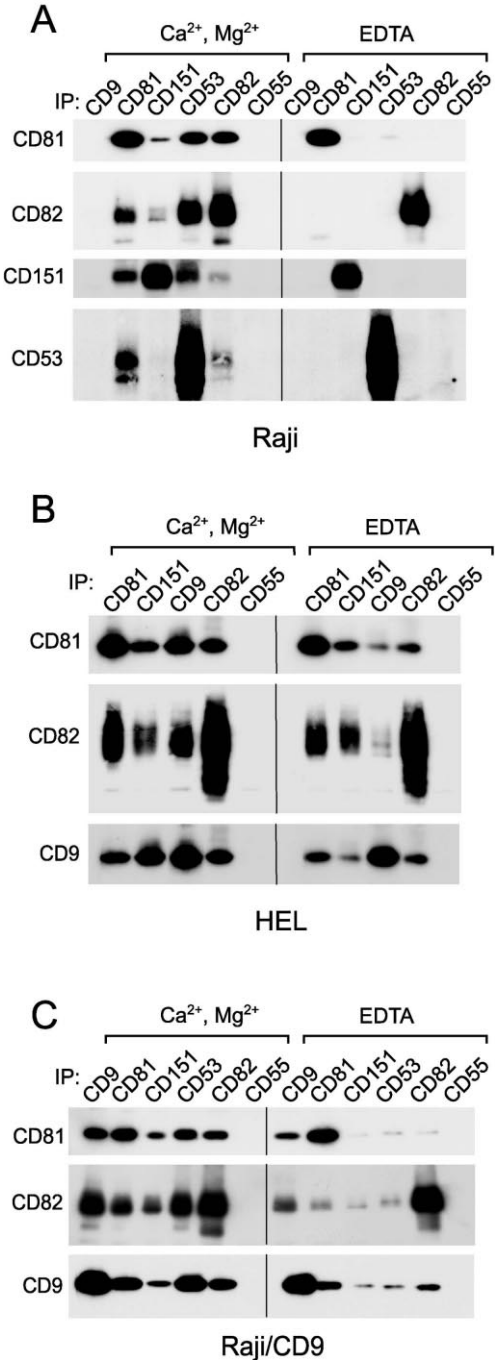

Fig. 1. Higher stability of CD9-containing complexes. Raji, Raji/CD9 and HEL cells were lysed in Brij 97 lysis buffer supplemented with either 1 mM $CaCl_2$ and 1 mM $MgCl_2$, or 1 mM EDTA as indicated. Immunoprecipitations were performed with anti-tetraspanin mAb or a CD55 mAb as a control, as indicated at the top of each lane, and the composition of the immunoprecipitates was analyzed by Western blot as indicated on the left. The ability of the CD55 mAb to immunoprecipitate the target antigen in the presence or absence of divalent cations has been checked in independent experiments using biotin-labeled cells.

CD81 and CD82 associated with CD53 (Fig. 1A). In Raji (Fig. 1A) or Daudi (not shown) cells, tetraspanin/tetraspanin interactions (CD81, CD53, CD82, CD151) were totally disrupted in the presence of EDTA. A low level of CD81 could, however, be observed in some experiments in the CD53 immunoprecipitate. This effect of EDTA could be neutralized by supplementing the lysis buffer with higher levels of $CaCl_2$ and $MgCl_2$ (data not shown). In contrast, in HEL (Fig. 1B) and HeLa (not shown) cells, only a partial dissociation of the tetraspanin/tetraspanin complexes was observed. In particular, the CD9/CD81 association was barely modified by EDTA.

Contrary to Raji or Daudi cells, HEL and HeLa express CD9. Thus we examined whether the presence of CD9 could modify the effect of Brij 97/EDTA. In CD9-transfected Raji cells (Raji/CD9) the CD9/CD81 association was only slightly affected by the presence of EDTA and the association of CD9 with other tetraspanins was partially resistant to Brij 97/EDTA disruption (Fig. 1C). Moreover, the expression of this molecule rendered other tetraspanin/tetraspanin complexes slightly more resistant. The simplest explanation is that some of these associations occur indirectly through CD9. In contrast, overexpression of CD82 in Raji cells did not modify the sensitivity of tetraspanin/tetraspanin complexes to EDTA (not shown).

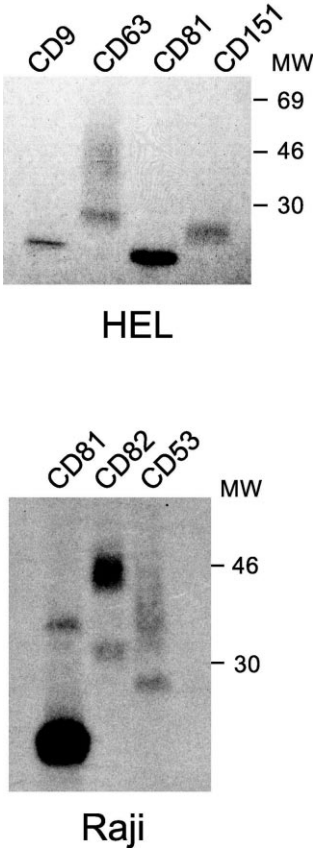

Fig. 2. Metabolic labeling of tetraspanins with [^{3}H]palmitate. Raji and HEL cells were radiolabeled with [^{3}H]palmitate and immunoprecipitations were performed with different anti-tetraspanin mAbs as indicated. The lower bands in the CD63, CD82 and CD53 immunoprecipitates most likely correspond to immature forms of the proteins. The identity of the higher band in the CD81 immunoprecipitate collected from Raji cells is unknown.

Further work will be necessary to determine why divalent cations influence the association of tetraspanins with each other. They may decrease the ability of the detergent to disrupt these interactions. Alternatively, divalent cations may be required for optimal tetraspanin/tetraspanin interaction. In any case, these data point out a higher stability of CD9/tetraspanin interactions, and in particular of CD9/CD81.

3.2. Tetraspanins are palmitoylated

We then considered the hypothesis that palmitoylation could contribute to tetraspanin/tetraspanin interactions. Among tetraspanins, only CD9 and CD81 were previously shown to be acylated [35,36]. We have confirmed these data and have found that all tetraspanins tested (CD53, CD63, CD82, CD151, and CD37) (Fig. 2 and data not shown) incorporated [^{3}H]palmitate. In several experiments, CD81 was the most efficiently labeled. Interestingly, the immature, poorly glycosylated forms of CD53, CD63 and CD82 were also labeled with [^{3}H]palmitate.

3.3. CD9 is palmitoylated at any of the four internal juxtamembrane regions

There is no clear consensus site for palmitoylation of transmembrane molecules, which typically takes place at one or several cysteine residues located adjacent to or just within the transmembrane domain [32,33]. CD9 contains six such cysteines located at each of the four internal juxtamembrane regions and was previously shown to be palmitoylated exclusively through *S*-acylation [36]. These residues were progressively mutated from the C-terminal extremity to the N-terminal end (Fig. 3A) and intermediate single mutants were also generated. The different constructs were expressed in CHO cells. All mutants were expressed at similar levels on the cell surface as determined by flow cytometric analysis (not shown), showing that palmitoylation is not involved in the transport of CD9 to the plasma membrane. The recognition of the mutant molecule with no residual juxtamembrane cysteine ($CD9^{plm}$) by different mAbs, as well as its ability to associate with CD9P-1, the major CD9 partner, indicates that these mutations do not affect the overall conformation of CD9 (not shown).

Palmitoylation occurs at any of the internal juxtamembrane regions of CD9 as judged by the progressive diminution of both relative molecular mass (Fig. 3B,C) and labeling with [^{3}H]palmitate (Fig. 3D). $CD9^{plm}$ failed to incorporate any [^{3}H]palmitate and its MW was reduced by 1.5 kDa (Fig. 3C), which is consistent with a previous study showing that removal of fatty acids from CD9 by hydroxylamine removes 1 kDa [36]. The lower intensity of CD81 labeling as compared to CD9 in Fig. 3B most likely reflects a lower expression after transfection (data not shown).

3.4. Partition of CD9 and $CD9^{plm}$ in DRM

We then investigated whether the palmitoylation of tetraspanins played a role in their partition to DRM. Brij 97 lysates of CD9- or $CD9^{plm}$-transfected Daudi cells were subjected to centrifugation in a sucrose gradient, and the different fractions were recovered and analyzed by Western blot (Fig. 4). When cells were lysed in the presence of divalent cations, both CD9 and $CD9^{plm}$ were partially recovered in the low-density fractions, indicating their presence in DRM. The localization of these molecules in DRM was strongly reduced

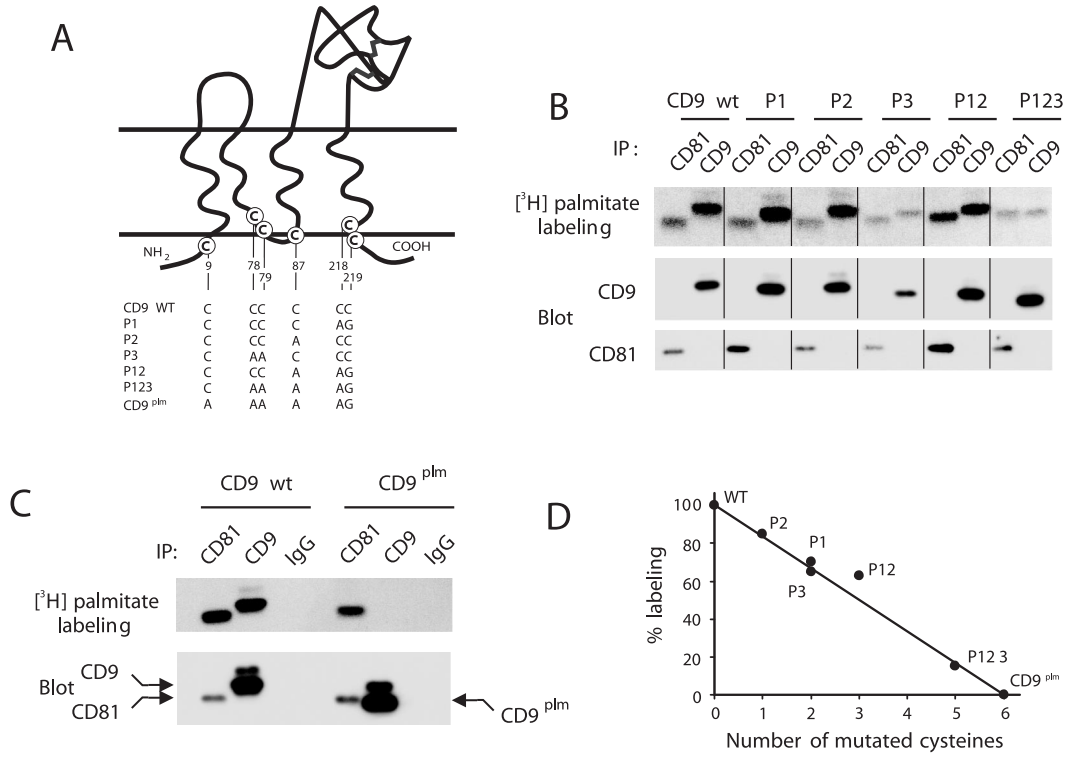

Fig. 3. Analysis of CD9 molecules mutated on one or several juxtamembrane cysteine residues. A: Schematic representation of CD9 with indication of the internal juxtamembrane cysteine residues, and their change in the mutant molecules. The numbers indicated correspond to the position of cysteines in the CD9 sequence. B: CHO cells were transiently transfected with CD81 together with different CD9 constructs and metabolically labeled with [^{3}H]palmitate. Cells were lysed and immunoprecipitations with CD9 and CD81 mAbs were performed. The extent of [^{3}H]palmitate labeling was determined by fluorography. A fraction of the immunoprecipitates was analyzed by immunoblotting using biotin-labeled CD9 or CD81 mAbs as indicated. C: Same as B, except that CHO cells stably expressing CD9 or CD9^{plm} and CD81 were analyzed. D: The intensity of [^{3}H]palmitate labeling of each mutant was estimated by densitometry and normalized according to the amount of protein determined by Western blot. The graph shows the labeling of each mutant relative to CD9 as a function of the number of mutated cysteines. The line corresponds to the expected values for an attachment of a palmitate on each CD9 juxtamembrane cysteine.

after lysis in the presence of EDTA, correlating with the disruption of most tetraspanin/tetraspanin interactions (Fig. 1). There was no difference between CD9 and CD9^{plm}. Thus, the palmitoylation of CD9 does not significantly contribute to its presence in DRM.

3.5. *Effect of cysteine mutations on the association of CD9 with other tetraspanins*

The contribution of CD9 palmitoylation to the interaction with other tetraspanins was examined in transfected Daudi cells. As observed in Raji cells, ectopically expressed CD9 associated with CD81 in a Brij 97/EDTA-resistant manner and rendered other combinations of tetraspanin/tetraspanin complexes more resistant (Fig. 5). Interactions of CD9^{plm} with other tetraspanins were detected after lysis in the presence of Brij 97 and divalent cations but were for the most part disrupted in the presence of EDTA (including with CD81). Similar results were observed in CHO cells transiently or stably expressing CD81 and CD9 or CD9^{plm}. In CHO cells stably expressing these molecules, and transiently transfected with CD82, CD82 behaved like CD53 in Daudi cells (data not shown).

3.6. *Concluding remarks*

Altogether our data show that the palmitoylation of CD9 contributes to the interaction with other tetraspanins, and in particular to the higher stability of the CD9/CD81 interaction.

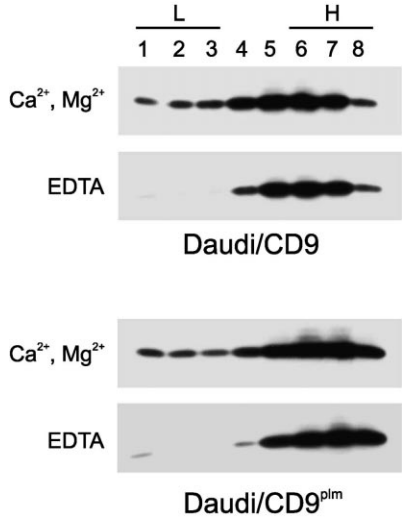

Fig. 4. Buoyant properties of CD9 and CD9^{plm}. Daudi cells expressing CD9 or CD9^{plm} were lysed with Brij 97 buffer supplemented with either 1 mM $CaCl_2$ plus 1 mM $MgCl_2$, or 1 mM EDTA as indicated. The extracts were directly subjected to equilibrium density gradient centrifugation. Gradient fractions were collected and analyzed by Western blot using the CD9 mAb SYB-1. The fractions are indicated at the top of each lane. Fractions 1–3 correspond to low-density fractions (L) and 5–8 to high-density fractions (H).

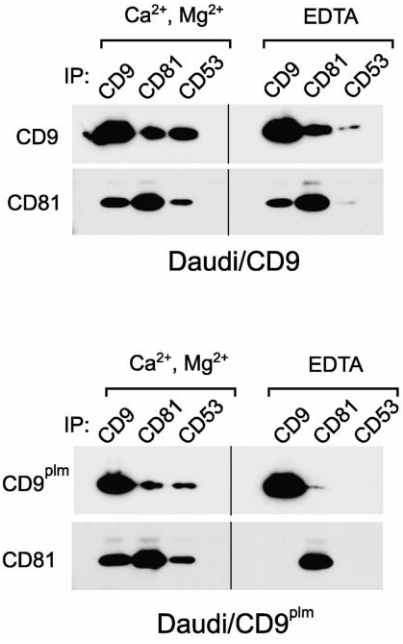

Fig. 5. Association of CD9 and CD9^{plm} with other tetraspanins. Daudi cells stably expressing CD9 or CD9^{plm} were lysed in a Brij 97 lysis buffer supplemented with either 1 mM $CaCl_2$ plus 1 mM $MgCl_2$, or 1 mM EDTA as indicated. Immunoprecipitations with CD9, CD81 and CD53 mAbs were then performed and the immunoprecipitates were analyzed by immunoblotting using biotin-labeled CD9 or CD81 mAbs.

The observation that CD9^{plm} interacts with other tetraspanins in the milder condition (in the presence of divalent cations) indicates the participation of additional mechanisms. It is unclear why the other tetraspanin/tetraspanin interactions are disrupted in the presence of EDTA in spite of the palmitoylation of the molecules. It is possible that the palmitoylation is involved but is not sufficient to stabilize the complexes under these conditions. In this regard, CD9 and CD81 may be the most palmitoylated tetraspanins, since both have six juxtamembrane cysteine residues as compared to four for CD82 and three for CD53. Moreover, CD81 was found to incorporate more [^{3}H]palmitate than the other tetraspanins although this may also reflect a different turn-over or a different subcellular localization (at least for a certain fraction of the molecule). Alternatively, among the tetraspanins studied here, CD81 is the only one that lacks *N*-glycans, and only a minor fraction of CD9 has such modifications [36]. It is possible that the presence of *N*-glycans on other tetraspanins contributes to a better dispersion of the tetraspanin/tetraspanin complexes in the more stringent conditions.

The observation that CD9 can be induced to redistribute into the high-density fractions of sucrose gradients by EDTA while maintaining the association with CD81 shows that these molecules interact outside DRM. This association is itself dependent on CD9 palmitoylation since it is lost upon mutation of palmitoylation sites. This clearly indicates that palmitoylation of CD9 plays a role in the interaction with CD81 (and probably other tetraspanins) independently of the interaction with DRM. To our knowledge, this is the first demonstration that acylation contributes to the formation and/or stability of multimolecular complexes involving transmembrane molecules. It is possible that tetraspanins interact through lipids, and that their palmitoylation facilitates this interaction. Indeed, a photoactivatable GM3 was recently found to crosslink to CD9 [38], and we speculate that GM3 and possibly other lipids may contribute to the formation of tetraspanin/tetraspanin complexes. We propose that when a sufficient level of tetraspanin interactions is achieved, the tetraspanins partition into DRM.

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