INVESTIGATING POST-TRANSLATIONAL MODIFICATIONS OF TETRASPANINS: PALMITOYLATION OF CD81 AND GLYCOSYLATION OF TSPAN-2

by

CAROLINE DELANDRE

B.S., Université de Rennes I, France, 2002

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Division of Biology
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Abstract

Members of the protein superfamily of tetraspanins are best defined by a simple structure comprising four transmembrane domains, two extracellular loops of unequal size, and short cytoplasmic regions. Despite their small size, tetraspanins are able to participate in multiple functions, as diverse as B cell activation, cancer metastasis, and viral infection. To compensate for a lack of intrinsic enzymatic activity, tetraspanins have gained the fascinating ability of associating with numerous different proteins. In addition, tetraspanins interact with each other forming a network on the plasma membrane: the tetraspanin web. In this way, functionally related proteins binding to different tetraspanins can be brought into close vicinity, thereby enhancing signaling pathways. The tetraspanin web is a dynamic environment and its regulation has grasped the attention of several research groups in the past few years. Particularly, several tetraspanins have been found to be palmitoylated, a post-translational modification attaching a palmitic acid to cysteine residues in a reversible manner. Palmitoylation is thought to be important for the integrity of the tetraspanin web.

We examined the effect of disrupting putative palmitoylation sites on the tetraspanin CD81 by mutating its juxtamembrane cysteines. By flow cytometry, we observed a decrease in the detection of mutant CD81 at the cell surface. This was not due to defects in protein trafficking or antibody affinity, and might reflect an abnormal CD81 distribution in a membrane environment that prevents the exposure of the epitope recognized by the CD81 antibody. Immunoblotting analysis revealed a novel CD81 processing event that was impaired in the mutant CD81 proteins compared to wild-type. Finally, co-immunoprecipitation assays showed a reduction in binding of tetraspanin CD9 and Ig superfamily member EWI-2 to mutant CD81. Taken together, these results suggest the importance of juxtamembrane cysteines (via palmitoylation or protein conformational changes) in protein interactions of CD81 within the tetraspanin web.

Although 33 tetraspanins are expressed in humans, less than half of them have been well studied. Among the “orphan” tetraspanins awaiting further examination is

Tspan-2. Here, we provide the first elements for the characterization of mammalian Tspan-2 by investigating expression patterns, N-glycosylation status, and association with other tetraspanins.

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Acknowledgements

While I am writing this dissertation, I look back to all the years I have spent in Graduate School, and I realize what an amazing and challenging journey it has been. I am now getting ready to close this chapter of my life and start a new one. A few years ago, however, reaching this point seemed almost impossible to me, as I have encountered many obstacles on my way. Without the unfading support of my advisor, Dr. Rollie Clem, I am not sure I would be writing these lines. The thoughts of quitting have crossed my mind multiple times, but Dr. Clem's encouragements were always there to erase them and keep going. I still remember the first time I met Dr. Clem. I was then a naïve undergraduate student who had just arrived in the States and was taking Advanced Cell Biology, which he was teaching. I was eager to learn and discover research in a lab. Many years have past since then, and I have finally gone beyond the label of "student" to become a scientist. Dr. Clem has had a major influence in this progressive transformation. I will never forget his excellent mentorship.

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“Par la persévérance dans la recherche, on finit par acquérir ce que j’appelle volontiers l’instinct de vérité.”

Through perseverance in research, we end up acquiring what I gladly call the instinct of truth.

- Louis Pasteur –

CHAPTER 1 - The Tetraspanin Web

Four transmembrane domains, two extracellular loops, one conserved motif (Cys-Cys-Gly), and several additional cysteine residues in the large extracellular loop: these are the essential structural features that define members of the tetraspanin superfamily of proteins. Tetraspanins are ubiquitously found in most, if not all, multicellular organisms. Despite a seemingly simple structure with relatively short extra- and intracellular domains, tetraspanins are able to undertake a surprisingly broad array of functions in processes as diverse as the immune system, virus infectivity, viability of the retina, and cancer metastasis. How are tetraspanins able to achieve such functional diversity? Since their discovery in the early 1990's, the advancements made in the tetraspanin field have revealed a fascinating picture where tetraspanins and their associated proteins form a dynamic network at the plasma membrane, known as the tetraspanin web. Depending on the proteins they interact with, tetraspanins can influence several processes. This review will explore the tetraspanin web and its multiple cellular roles.

The building blocks: the tetraspanins

What are tetraspanins?

A tetraspanin protein can be divided into four major structural domains: the large extracellular loop (LEL), the small extracellular loop (SEL), the transmembrane domains (TMs), and the cytoplasmic regions (Fig. 1.1). The structure of tetraspanins is tightly packed resulting in an overall cylindrical shape that sticks out of the lipid bilayer by 3 to 5 nm[1, 2].

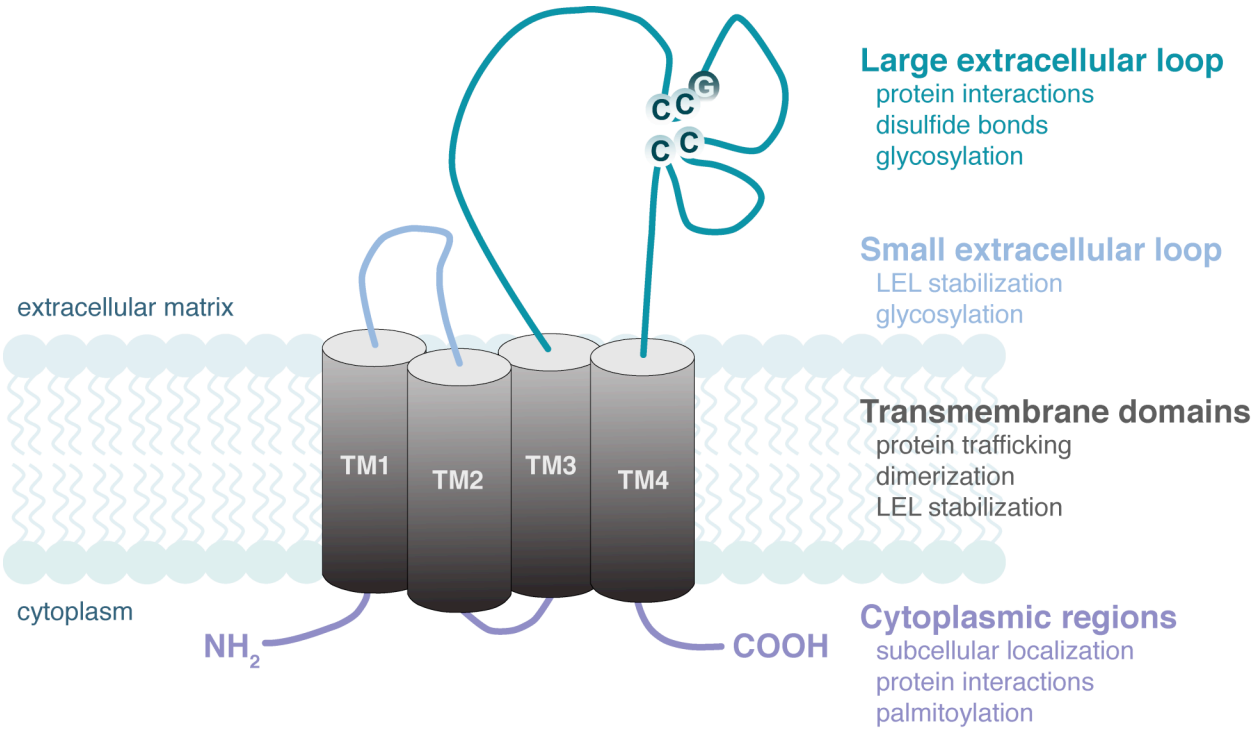

Figure 1.1 Basic tetraspanin structure and domains

Large extracellular loop

The LEL has received most of the attention since early findings reported it to be crucial for interactions with other proteins. This interest led to the X-ray analysis of tetraspanin CD81 LEL by Kitadokoro *et al.*[3]. This study revealed the LEL as a mushroom-shaped structure with two main regions: the so-called stalk region formed by helices A and E, and the head subdomain containing helices B, C, and D. The sequence corresponding to the head subdomain is highly variable among members of the tetraspanin superfamily except for the presence of a CCG motif in addition to two to six cysteines. These residues are distinctive of tetraspanins and have a role in stabilizing the LEL via disulfide bonds[3]. The significance of these covalent bonds is illustrated by the lack of binding of most tetraspanin-specific monoclonal antibodies under reducing conditions[4, 5]. This observation also underscores the relevance of the LEL since it is the target of most of these antibodies[6, 7].

The head subdomain region plays a fundamental role in protein-protein interactions. Because of the hypervariability of its sequence, it can confer great specificity in interactions with a particular tetraspanin. Entry of hepatitis C virus (HCV)

is mediated by this region of CD81[8]. Residue F186 is particularly important for this function as its mutation inhibits CD81 binding to HCV glycoprotein E2[4]. Tetraspanin CD151 also depends on its LEL to interact with the α_3 subunit of integrin $\alpha_3\beta_1$[6]. The LEL of another member of the superfamily, CD9, is required for binding to HB-EGF and for facilitating sperm-egg fusion[9, 10].

The stalk of the LEL has been linked to homodimerization of CD81[3]. However, further modeling of the full CD81 molecule by Seigneuret suggested that this region is mostly hidden by the SEL[1].

The LEL is also the region where N-glycosylation sites are mostly found. These sites vary among tetraspanins providing another level of specificity in this domain[7]. Some tetraspanins do not have predicted glycosylation sites (CD81), and in the case of CD9, the sites are located in the SEL[7].

Small extracellular loop

Less is known about the tetraspanin SEL. It is thought to have a role in supporting the proper structure of the LEL[2].

Transmembrane domains

The importance of the transmembrane domains was first highlighted by Cannon and Cresswell in a report where they found that a CD82 construct lacking the first TM domain was unable to exit the endoplasmic reticulum (ER)[5]. Surprisingly, co-expression with a separate construct containing TM1 restored proper transport to the plasma membrane, indicating a strong interaction between TM domains and a role in tetraspanin protein trafficking[5]. CD9 constructs lacking the TM2-TM3 region or containing only the TM3-LEL-TM4 region were unable to efficiently reach the cell surface[11]. The TM1-TM3 region was also found to be important for proper localization of CD151[6].

The transmembrane domains are required to ensure correct folding of the LEL. Indeed, deletion of TM domains in the previously mentioned CD9 constructs also prevents recognition of the LEL by conformational monoclonal antibodies[11].

Several polar residues embedded in the transmembrane domains are highly conserved among tetraspanins (N in TM1 and E/Q in TM3 and TM4)[7]. These residues have been proposed to be involved in inter-helical interactions between TM domains via

hydrogen bonding, thereby allowing more efficient packing of TM domains[1, 2]. Mutation of TM3 residue E102 in the tetraspanin uroplakin Ib prevents its export to the cell surface[12]. Substitutions of other TM polar amino acids in CD81 resulted in the inhibition of HCV entry[13].

Recent evidence supports an additional role for TM domains in tetraspanin dimerization and interaction with other proteins. CD81 TM1 is required for proper transport of its binding partner CD19 to the plasma membrane[14]. Homodimerization of CD9 was found to depend on its TM domains[15]. In addition, the primary interaction between tetraspanin uroplakins and their non-tetraspanin partners also requires the LEL and TM domains[2].

Cytoplasmic regions

Despite their short length, the cytoplasmic regions of tetraspanins can participate in various functions. Tetraspanin subcellular localization might be regulated by interaction with clathrin-dependent adaptor proteins. A specific tyrosine motif in the C-terminal domain of several tetraspanins is known to bind to adaptor protein complexes (YXXΦ where X is any amino acid and Φ is a bulky hydrophobic residue)[16]. Distribution of CD63 to intracellular vesicles is mediated by binding of AP-3 to this tyrosine motif[17]. CD151 internalization and antibody-stimulated CD82 endocytosis are impaired when this motif is mutated[16, 18]. However, some tetraspanins might not require this motif for proper localization, suggesting that other mechanisms are in place[16]. Moreover, syntenin-1 has been shown to bind the C-terminal domain of CD63 and influence its internalization in an adaptor protein-independent way[19].

The cytoplasmic regions are also involved in protein interactions. Several tetraspanins are able to interact specifically with important signaling molecules: phosphatidylinositol-4 kinase (CD9, CD63, CD81, CD151, and A15), protein kinase C (CD9, CD53, CD81, CD82, and CD151), 14-3-3 (CD81), and G protein subunits (CD9 and CD81)[20-23]. The C-terminal tail of CD81 also associates with ERM (ezrin-radixin-moesin) proteins, providing a bridge between the tetraspanin web and the cytoskeleton[24].

Finally, the cytoplasmic regions are the sites of palmitoylation. Several tetraspanins have already been shown to be labeled with palmitic acid, and more members of the superfamily are likely to join the list since most tetraspanins have

juxtamembrane cysteines, which are potential palmitoylation sites[25]. Mutation of these cysteines and use of a specific inhibitor have resulted in alterations in the organization of the tetraspanin web (described below, p22).

Where are tetraspanins found?

Expression in organisms throughout the evolutionary tree

From simple eukaryotic organisms such as sponges and fungi to arthropods and vertebrates, tetraspanins are ubiquitously found across the evolutionary tree. These proteins seem to be mostly specific to multicellular organisms as best illustrated by their presence in several multicellular pathogenic fungi whereas none were found in the genomes of 17 single-celled fungi species (including *Saccharomyces cerevisiae*)[26]. Perhaps due to historical reasons—tetraspanins were first discovered in mice and humans—most of the knowledge in this field has been largely concentrated on mammalian tetraspanins (33 members are expressed in humans), and this bias is reflected throughout this review. Although *Drosophila melanogaster* has 37 tetraspanins, only three of them have been studied: Late bloomer (role in neuromuscular junction formation), Sunglasses (light-induced retinal degeneration), and Tsp68C (hemocyte proliferation)[27-29]. Interestingly, the fruit fly genome has a cluster of 18 tetraspanins in the 42E region of the second chromosome[30]. When half of the genes in this cluster were deleted, flies developed normally, indicating a strong compensatory mechanism by other tetraspanins, a property that seems to be common to tetraspanins in general[31]. This particular cluster is specific to insects in the fly branch as other arthropods, such as the mosquito *Anopheles gambiae* and the honey bee *Apis mellifera*, do not encode it in their genomes[26]. Research on *Caenorhabditis elegans* has been limited to one of its 20 members, TSP-15, and its role in the maintenance of the hypodermis[32]. Several reports from Lebrun's group have shown the relevance of tetraspanins in the pathogenicity of several fungi including *Magnaporthe grisea*, an economically important rice pathogen[33-35]. Without the tetraspanin Pls1, *M. grisea* is unable to penetrate the host plant tissue[34]. The tetraspanin D76 in the moth *Manduca sexta* plays a role in innate immune responses through binding to an integrin on hemocytes[36]. Mosquito *Aedes albopictus* cells upregulate the tetraspanin C189 when infected by dengue virus[37]. Even in the primitive

sponges, a tetraspanin is thought to be a component of cell junctions in the epithelium[38]. Therefore, it is likely that tetraspanins emerged from duplications of a common ancestor acquired during the unicellular-to-multicellular transition of eukaryotes[26]. It is interesting to note that even several plants (including *Arabidopsis thaliana*) possess tetraspanin-like proteins (lacking the CCG motif in the LEL)[26].

Expression in multiple tissues

Tetraspanins are not only found in most organisms, they are also found in a wide variety of tissues. In mammals, almost all cell types, except erythrocytes, express multiple members of the tetraspanin superfamily, an important factor for the building of the tetraspanin web as described in the next section[39]. However, tissue distribution of individual tetraspanins can vary substantially. Tetraspanins CD9, CD63, CD81, CD82, and CD151 are characterized by a wide expression pattern[7, 39]. Other tetraspanins are limited to specific organs or tissues: uroplakins Ia and Ib are exclusively expressed in the bladder because of their unique function in the assembly of the urothelial plaques; ROM-1 and peripherin-2 expression is restricted to the outer segment discs of rod photoreceptor cells; and CD37 and CD53 are specific to immune cell types[12, 40, 41]. Additionally, this wide distribution is also present in *Drosophila melanogaster*; a study of 35 tetraspanins showed great variation in their expression patterns among tissue progenitors at the embryonic stage, indicating this might be a common aspect of tetraspanins across organisms[31].

Subcellular expression

The diversity in tetraspanin expression distribution at the tissue level is also observed in their subcellular localization. Although tetraspanins are generally found at the plasma membrane, in several cases, they are enriched in various intracellular compartments. The best example is CD63; because of its tyrosine-sorting motif (described above, p4), it is commonly found in the lysosomes of multiple cell types. CD63 is also present in specialized vesicles in some cell types: dense and alpha-granules (platelets), azurophil granules (neutrophils), cytotoxic T lymphocyte granules, and Weibel-Palade bodies (endothelial cells)[42-46]. Together with CD37, CD81, and CD82, CD63 is also distributed to MHC class II-enriched multivesicular bodies (MIICs) in B

cells[47]. Multivesicular bodies are formed by the invagination of the limiting membrane, and in antigen-presenting cells, they can traffic to the cell surface and release internal vesicles called exosomes. Exosomes are thought to have a role in antigen presentation since they carry MHC class II molecules. Several groups have reported the localization of tetraspanins in exosomes: in B cells (CD37, CD63, CD81, and CD82), T cells (CD81), dendritic cells (CD9), and intestinal epithelial cells (CD9, CD81, and CD82)[47-50]. CD151 can also be concentrated in intracellular vesicles in endothelial and hemopoietic cell types[51, 52].

Distribution within the plasma membrane can also vary among tetraspanins. Highly specialized retinal tetraspanins ROM-1 and peripherin-2 are mostly found in the disc rims of rod photoreceptor cells[53, 54]. Uroplakin tetraspanins are restricted to the apical side of the bladder urothelium clustered in highly organized asymmetric unit membrane structures (described below, p8)[55]. Finally, most tetraspanins are thought to be found at specific times in specialized plasma membrane compartments called tetraspanin-enriched microdomains (TEMs), a key component of the tetraspanin web.

The tetraspanin web

The interaction with binding partners defines the essence of a tetraspanin. These associations are at the root of the tetraspanin web, a term coined to describe the protein network of members of the tetraspanin superfamily and their binding partners at the plasma membrane[56]. Assembled to provide proteins some sense of stability within the fluid environment of the lipid bilayer, the tetraspanin web can be a challenging concept to grasp at first hand. However, it can be simplified by dividing the network into two types of interactions. First, at the core of the web, there is the direct and strong binding between tetraspanins and their specific partners (primary interactions); and second, indirect binding between these primary groups brings the web together (secondary interactions). A great example to illustrate the building of such a network is the assembly of uroplakin tetraspanins and their binding partners into a bladder-specific epithelial structure called the asymmetric unit membrane (AUM).

The asymmetric unit membrane: a highly specialized tetraspanin web

The top-most layer of the bladder epithelium (urothelium) consists of highly differentiated cells that are characterized by the presence of plaques covering most of the bladder lumen[57]. These plaques can be seen in electron micrographs as composed of 16-nm particles organized in a crystal-like hexagonal lattice. Each particle can be divided in an inner and an outer ring, each containing six subdomains[57, 58]. These plaques are composed of four different types of proteins: tetraspanin uroplakins UP Ia and Ib, and single-transmembrane-domain uroplakins UP II and III[59, 60]. The protein content of these structures results in a thicker outer leaflet of the plasma membrane compared to the inner leaflet, a featured implied in the name "asymmetric unit membrane"[58]. The AUM is thought to be important in helping the bladder retain urine without damaging internal contents by acting as a permeability barrier[61]. The assembly of the AUM relies on the diverse interactions occurring among uroplakins (Fig. 1.2). The strongest associations are between the tetraspanin UP Ia/Ib and their binding partners UP II/III, respectively; they can be chemically cross-linked and only stringent detergents can break them apart[59]. These primary interactions are the base of the 16-nm particles by forming the subdomains (UP Ia/II in the inner ring and UP Ib/III in the outer ring)[2]. These subdomains are linked together into subunits via binding between the non-tetraspanin uroplakins UP II and III; and association between UP Ia and UP II from two neighboring subunits forms the particles[2]. These secondary interactions are weaker and less amenable to cross-linking. Finally, a third level of interactions brings together multiple particles into the lattice that makes the AUM[2].

By solving the structure of this bladder-specific tetraspanin web, Sun's group and their collaborators have provided a compelling model to the tetraspanin field in general, as several features of the AUM are likely to be extrapolated to other tetraspanin networks. Indeed, direct and indirect interactions between tetraspanin and their binding partners have already been the focus of a numerous studies.

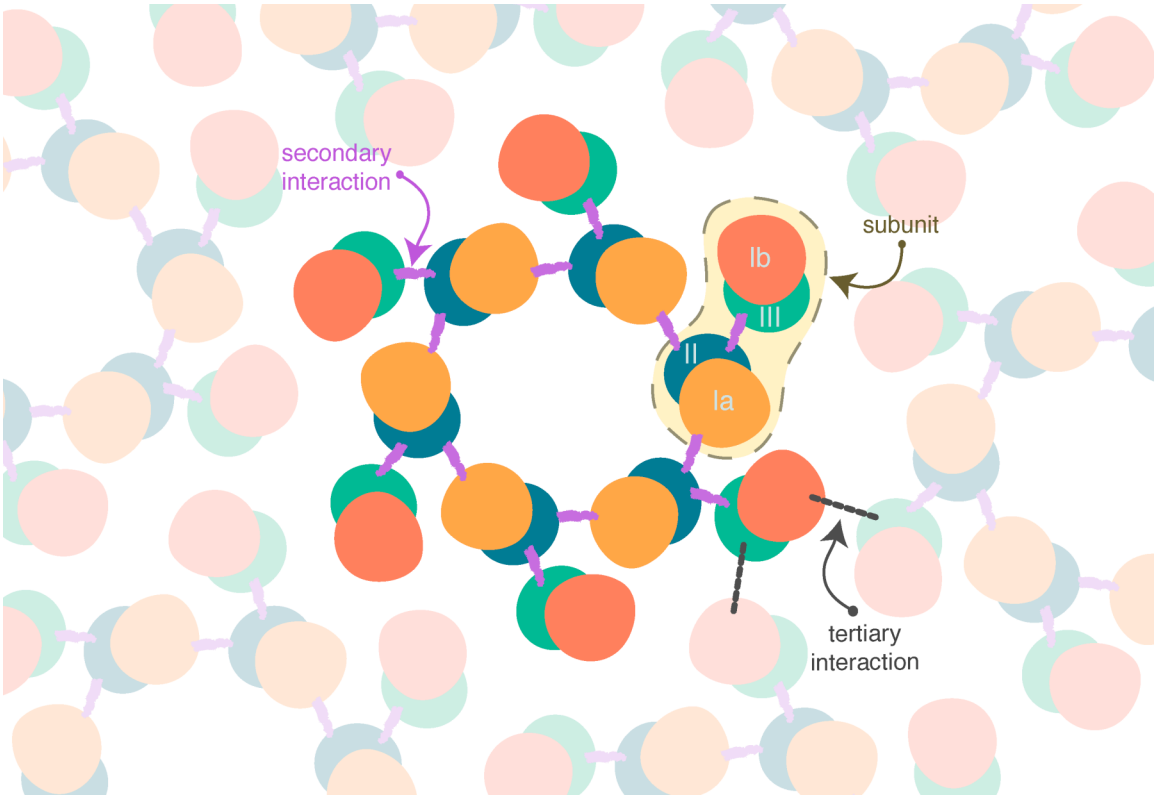

Figure 1.2 The asymmetric unit membrane

This figure depicts the assembly of the AUM by different interactions between tetraspanin uroplakins UP Ia/Ib and their respective non-tetraspanin binding partners UP II/III (adapted from ref. 2).

Direct interactions

One major difference, however, distinguishes the AUM from the classical tetraspanin web found in most tissues: whereas the tetraspanin/binding partner interaction is solely at the base of the AUM, tetraspanins homodimers are thought to be the central element of the tetraspanin web. A detailed study from Hemler's group analyzed tetraspanin homodimers by applying a novel cross-linking technique that takes advantage of spontaneous disulfide bonding that can occur between juxtamembrane cysteine residues of adjacent tetraspanins[62]. This cross-linking method, termed "zero-length" as compared to the use of chemical cross-linkers with variable arm lengths, allows the detection of molecules that are in very close proximity. Homodimers of CD9, CD81, and CD151 were found to be cross-linked, implying a direct interaction with themselves[62]. These results led the authors to propose that tetraspanin homodimers are the essential

components of the tetraspanin network from which all the other protein interactions are assembled[62]. Supporting this model, tetraspanins peripherin-2 and ROM-1 were also shown to form homodimers that could be cross-linked with glutaraldehyde[63].

The second type of primary interactions required to build a tetraspanin web is between tetraspanins and their specific binding partners[39]. Although multiple proteins are known to associate with tetraspanins, few of them show a direct interaction. Several criteria have been used to qualify proteins as direct binding partners: resistance to stringent detergents (Triton X-100, digitonin), ability to be chemically cross-linked, and/or high stoichiometry. The binding of CD151 to the integrin $\alpha_3\beta_1$ is a good example since it fulfills all these requirements[64, 65]. Two other tetraspanin/integrin associations (CD81/$\alpha_4\beta_1$ and CD151/$\alpha_6\beta_1$) are also highly efficient due to their presence under conditions preventing tetraspanin heterodimer formation (lysis with digitonin)[65]. Members of the Ig superfamily can also be found in primary complexes with tetraspanins: EWI-2 and EWI-F interact specifically with both CD9 and CD81, and CD19 depends on its direct association with CD81 for its maturation and functioning in the B cell coreceptor[14, 66-68].

Indirect interactions

The sensitivity to detergents of variable strengths has allowed the detection of the several levels characterizing the tetraspanin web. By lysing cells with weaker detergents (Brij-97, CHAPS), numerous additional proteins—including other tetraspanins—co-precipitate with tetraspanins[39]. For example, integrin $\alpha_3\beta_1$ can be co-precipitated with CD9, CD63, and CD81 only under mild conditions; with stringent detergents, only CD151 is co-precipitated with this integrin. CD9, CD63, and CD81 associate with $\alpha_3\beta_1$ indirectly, as part of the tetraspanin web[64, 69]. These secondary interactions are thought to occur via associations among different tetraspanins[39]. A striking feature of tetraspanins is their capacity to bind several other tetraspanins. These interactions, detected mostly under mild conditions, have been proposed to be the key elements linking the primary complexes together, thus forming the tetraspanin web[39]. This model illustrates why so many different proteins have been discovered in indirect association with tetraspanins, including integrins[69], Ig superfamily members[66, 68], growth factors[9], GPCRs[23],

metalloproteases[70], and various signaling molecules[20, 21]. By bringing these proteins close to each other, the tetraspanin web may facilitate signaling pathways, thereby influencing various cellular functions (described below, p13)[56]. The multiple interactions within this network are thought to be held in membrane compartments called tetraspanin-enriched microdomains.

Tetraspanin-enriched microdomains

Membrane microdomains are plasma membrane compartments characterized by a high protein:lipid ratio and a lipid content enriched in cholesterol and sphingolipids. The best-known examples are lipid rafts; many studies have shown their role in diverse signaling pathways such as B cell and T cell activation[71, 72]. Lipid rafts provide a delimited area within the plasma membrane where proteins required for specific functions can be recruited. Once considered the only type of membrane compartmentalization, lipid rafts are now becoming part of a more extensive list of membrane microdomains. The traditional criteria to define a lipid raft rely on its isolation method: when cells are lysed with 1% Triton X-100 (TX-100) and analyzed by a sucrose gradient, detergent-resistant membranes (DRMs) will float to the upper layers of the gradient[71]. These insoluble complexes contain the lipid rafts. Other methods have been used to confirm the existence of these rafts in living cells, but sucrose gradients are still commonly used to isolate lipid rafts. The hypothesis that other types of microdomains co-exist in the plasma membrane was brought to light upon discovering DRMs with different properties compared to classical lipid rafts. One example is the tetraspanin-enriched microdomain (TEM).

Because of the multiple associations found in the tetraspanin web under mild lysis conditions, several groups tested the idea that this network could be held together within membrane microdomains such as lipid rafts. However, several tetraspanins (CD9, CD63, CD81, and CD151) were not found in DRMs of cells lysed with TX-100. Instead, when a milder detergent (Brij-97) was used, tetraspanins were readily found in the top layers of sucrose gradients[73, 74]. These microdomains are sensitive to the amount of cholesterol, as treatment with methyl-β-cyclodextrin (MβCD), a chemical that depletes cholesterol from the plasma membrane, reduces the level of tetraspanins in DRMs[74, 75].

Whereas isolation of lipid rafts is temperature-sensitive (cell lysis needs to be performed at 4 ºC), the tetraspanin DRMs remain stable at 37 ºC[73]. Caveolin, a protein normally found in a subset of lipid rafts, is not present in tetraspanin DRMs, and no glycosylphosphatidylinositol (GPI)–anchored proteins, usually enriched in lipid rafts, have been found to associate with tetraspanins[71, 74]. Therefore, from the biochemical properties and protein content, these microdomains are novel membrane compartments different from lipid rafts; because of their high tetraspanin concentration, they were named tetraspanin-enriched microdomains.

In another report, CD9, CD81, and CD82 were found in the insoluble light fractions of gradients loaded with 0.5% TX-100 lysates from T cells in a cholesterol-dependent manner[76]. CD81 was also distributed in lipid raft-like microdomains upon coligation of the B cell receptor and its coreceptor on B cells[77]. The use of TX-100 to isolate TEMs contrasts with the previous reports, indicating the possibility of technical discrepancies or cell-dependent differences, and also illustrates the limitations of this assay. Indeed, there are several concerns over the possible artifacts that can be the result from lysing cells with a detergent[78]. How can one be sure that the results reflect the physiological environment?

Thus, other methods have been applied to confirm the relevance of TEMs *in vivo*. Silvie *et al.* reported the method of using a monoclonal antibody (MT81w) that binds mouse CD81 only when it is clustered with its associated proteins [79]. This antibody was proposed as a marker for the tetraspanin web. Binding of MT81w was decreased when cells were treated with MβCD, supporting the hypothesis that cholesterol is important in maintaining the tetraspanin web, probably through TEMs[79]. However, TEMs seemed mostly an abstract model until a recent report by Thali's group allowed, for the first time, the visualization of TEMs[80]. By using fluorescence microscopy, the localization of CD9, CD63, CD81, and CD82 (three at a time) at the surface of HeLa cells was determined. Several hundred TEMs were found; half of them contained all three tetraspanins tested. This was further supported by electron micrographs showing CD9 and CD63 together in restricted microdomains[80].

Tetraspanin functions

What do tetraspanins do? At first glance, this seems a very challenging question to answer when one is presented with the variety of biological functions tetraspanins have been linked to, from immunology to cancer and infectious diseases. However, an in-depth examination reveals a common factor among these roles: tetraspanin functions deeply rely on their association with binding partners and their belonging to the tetraspanin web. Thus, depending on the proteins they interact with, tetraspanins can influence multiple processes. This section will present the major functions that tetraspanins are known to influence. However, these probably represent just the tip of the iceberg: as more tetraspanins are studied and more binding partners are discovered, this list is likely to grow extensively in the near future.

Immunology

CD81 and the B cell coreceptor

The B cell coreceptor is composed of CD19, CD21, and the tetraspanin CD81. It plays a role in regulating the threshold of activation of the B cell receptor (BCR)[81]. CD21 is a receptor for complement molecule C3d. When the BCR and the B cell coreceptor are both engaged by C3d-bound antigen, the threshold of activation is considerably lowered. As described before, CD81 interacts directly with CD19. Other tetraspanins have been shown to co-immunoprecipitate CD19; however, these associations only occur under conditions maintaining the tetraspanin web, suggesting they probably bind CD19 via CD81[82]. The specificity of the CD19/CD81 complex was further confirmed by the phenotype of mice lacking CD81: B cells lacking CD81 express a reduced level of CD19 at the cell surface. Similar results were obtained by three independent groups[83-85]. Moreover, this phenotype is characteristic of CD81-null mice only; B cells of mice lacking CD9, CD37, or Tssc6 present normal surface levels of CD19[86].

This reduction of CD19 at the plasma membrane is thought to be due to a defect in its maturation, and the first transmembrane domain of CD81 is sufficient for proper CD19 trafficking to the surface[14, 87]. CD81 also has a role in stabilizing the B cell coreceptor and the BCR within microdomains. Upon costimulation of those complexes,

the BCR and B cell coreceptor distribute to lipid rafts. However, this localization is prevented in B cells lacking CD81[77].

T cell – antigen presenting cell interaction

Another phenotype of CD81-null mice is a reduction in Th2 responses. Th2 responses lead to the activation of the humoral branch of the immune system to fight extracellular pathogens, and are characterized by the production of specific cytokines such as IL-4. It is possible to artificially stimulate this type of T cell dependent signaling events by injecting mice with antigens combined with certain adjuvants. In this way, it was found that CD81-null mice were not able to produce normal amounts of antibody upon stimulation of Th2 responses[88]. These mice also lacked the ability to undergo airway hyperreactivity (AHR), a Th2-dependent event that is a characteristic of asthma[89]. Chimeric mice lacking CD81 only in B cells also have defects in their Th2 responses, suggesting that the role of CD81 in regulating Th2 responses depends on its expression in B cells[88]. However, another study pointed to the importance of CD81 in T cells in order to influence Th2-dependent signaling[90]. CD81 might play a role during the T cell – B cell interaction, a major event in Th2 responses. Supporting this hypothesis, CD81 has been found to localize within the immune synapse, a cluster of proteins facilitating the binding of peptide-loaded MHC class II molecules to the T cell receptor (TCR)[91]. CD81 is also known to associate with CD4 and MHC class II proteins[92, 93].

Other tetraspanins share the ability to bind antigen-presenting proteins. In addition to CD81, tetraspanins CD9, CD53, CD63, and CD82 associate with MHC class II molecules[93-95]. These interactions are thought to occur within microdomains. Several tetraspanins are present in MHC class II-enriched compartments originating in multivesicular bodies (discussed above, p6). Tetraspanins are also enriched in microdomains containing a subset of MHC class II proteins loaded with specific peptides and characterized by the CDw78 epitope[96]. Although the functional consequence of these associations in antigen presentation remains unclear, it is possible that tetraspanins have a role in the stability of these proteins within membrane microdomains.

A common feature among mice lacking CD37, CD81, CD151, and Tssc6 is the hyperproliferation of T cells upon TCR stimulation, implying tetraspanins might have a negative regulatory effect on T cell signaling[40, 85, 97, 98]. However, antibodies recognizing

CD9, CD53, CD81, and CD82 have a costimulatory activity on T cells, also leading to an increase in proliferation[99-101]. For CD9 and CD81, this effect has been shown to be independent of CD28, a major player leading to costimulatory signals[102, 103]. These results seem to contradict data obtained from knockout mice. However, a model has been proposed to reconcile them where the tetraspanin-specific antibodies would act by bringing TEMs in close proximity with the immune synapse, and facilitating TCR signaling. On the other hand, the absence of specific tetraspanins in the knockout mice might cause rearrangements in the immune synapse, also leading to T cell activation[104]. This hypothesis, however, remains to be confirmed with experimental data.

Leukocyte adhesion

An important step in infiltration of leukocytes from the bloodstream to a site of infection is the attachment of circulating leukocytes to endothelial cells (leukocyte rolling). Cells from CD81-null mice have a defect in integrin-dependent attachment to VCAM-1 (an adhesion molecule required for rolling) under shear flow in an assay designed to mimic the force of circulating blood[105]. Moreover, knockdown of CD9 and CD151 also lowers the efficiency of attachment in a similar assay[106].

Cancer

Correlation between tetraspanins and metastasis

The relationship between tetraspanins and cancer goes back over 20 years when the first tetraspanins were isolated in cancer-related screens. Most of the attention has been focused on two tetraspanins: CD82 and CD9.

In the early 1990's, a screen was designed to identify genes able to inhibit metastatic potential of the rat prostate cancer cell line AT6.1. The major gene isolated in this screen was named KAI1, for "kang ai" meaning anticancer in Chinese[107]. KAI1 is still used today as an alternative name for CD82. AT6.1 cells transfected with CD82 were introduced into nude mice resulting in a decrease in the number of lung metastases compared with injection of control AT6.1 cells[107]. Numerous groups have further confirmed this function in the regulation of metastasis. The expression levels of CD82 in cancers of advanced metastatic stages was found to be reduced when compared to early

stages in many types of cancers: pancreatic, esophageal, prostatic, non-small cell lung, colorectal, hepatocellular, breast, bladder, cervical, ovarian, and endometrial cancers[108-118]. In several cases, the loss of CD82 expression was related to poor prognosis[111, 119]. Moreover, when comparing CD82 expression in cell lines corresponding to various types of cancers vs. normal cell lines, there was an inverse correlation between CD82 levels and cancer progression[120]. Interestingly, the downregulation of CD82 seems to be restricted to the late, metastatic, stages of cancer: in pancreatic cancer, CD82 was actually upregulated during the early stages of tumor progression before being downregulated in advanced stages[108]. Moreover, mice injected with AT6.1 cells expressing CD82 did not show any difference in primary tumor size compared to mice injected with control AT6.1 cells, suggesting that CD82 suppresses metastatic potential, but not early events of tumor progression[107]. The molecular mechanism causing downregulation of CD82 in cancer remains obscure. It probably acts at the transcriptional level since both mRNA and protein are affected[112]. No mutation has been found in the CD82 gene from cancerous tissues[110, 117]. Surprisingly, very little is known about the transcriptional regulation of tetraspanins, and future studies to determine the factors involved in tetraspanin gene expression will be useful to understand their regulation in cancer.

Similarly, CD9 expression is inversely correlated with cancer malignancy in multiple tissues: skin (melanoma), breast, lung, colon, pancreas, and esophagus[109, 119, 121-125]. Another tetraspanin, CD63, was discovered as the antigen recognized by an antimelanoma antibody, and its expression is also inhibited in advanced stages of melanomas[126]. However, less is known about the differential expression of CD63 in other cancers, even though it is ubiquitously expressed throughout the human body. The effect of CD63 may be limited to melanoma. *In vitro* studies have supported the histological evidence by showing that overexpression of CD9 and CD82 in metastatic cell lines is able to inhibit cell motility[127-131].

The involvement of other tetraspanins in cancer development is less clear. CD151, in contrast with CD9 and CD82, functions as a positive regulator of metastasis. In non-small cell lung cancer, high CD151 expression levels are correlated with a poor prognosis[132]. Overexpression of CD151 in HeLa cells stimulates motility, and cells from CD151-null mice have impaired migration [97, 133, 134]. The evidence concerning CD81 is

inconclusive: on one hand, CD81 is expressed at higher levels in squamous cell carcinoma relative to normal tissues, but on the other hand, metastatic potential is correlated with low CD81 expression in hepatocellular carcinoma[135-137].

Possible molecular mechanisms

Even though the correlation between tetraspanins and malignancy has been known for a while, the molecular mechanism behind it remains unclear. However, several models have been proposed. The main one involves the well-documented association between tetraspanins and integrins (discussed above, p10). Since integrins are major players in binding and responding to the extracellular matrix, it seems logical that tetraspanins would regulate cell motility in an integrin-dependent manner. With this hypothesis in mind, Zhang *et al.* examined the activation state of molecular components downstream of integrins in a prostate metastatic cell line stably expressing CD82[127]. These cells have reduced motility compared to cells lacking CD82. Protein $p130^{CAS}$ was found to be downregulated in the presence of CD82, and $p130^{CAS}$ overexpression in CD82-expressing cells stimulated their motility to a similar level as cells without CD82[127]. Thus, $p130^{CAS}$, a protein downstream of integrin signaling, appears to be important for CD82-dependent motility. In another report, the same group found that a CD151 mutant protein that is not internalized as efficiently as the wild-type protein is unable to stimulate integrin-dependent migration[18]. The authors proposed that the regulation of integrin endocytosis by CD151 leads to an increase in migration.

Another model to explain how tetraspanins regulate motility is through growth factor receptors. CD82 associates with EGFR and overexpression of CD82 reduces EGF-induced cell motility[130]. CD82 has a negative impact on the dimerization of EGFR and activation of downstream signaling upon EGF stimulation[130, 138].

The integrin and growth factor receptor hypotheses are not mutually exclusive as illustrated by recent work from Miranti's group, who reported that CD82 expression suppresses integrin-mediated cell migration and invasion of metastatic prostate cells[129]. Importantly, integrin-based activation of c-Met, the receptor for hepatocyte growth factor/scatter factor (HGF/SF), was also reduced in these cells upon CD82 expression[129]. Activation of Src and downstream molecules, including $p130^{CAS}$, was also inhibited by

CD82[129]. Therefore, the authors suggested that CD82 regulates integrin-mediated migration through the growth factor receptor c-Met.

CD82 and DARC

Recently, an exciting new mechanism has been proposed to explain the role of CD82 as a metastasis suppressor gene. In an attempt to identify novel CD82-binding proteins, the chemokine receptor DARC (Duffy antigen receptor for cytokines) was isolated by yeast two-hybrid[139]. CD82 on the surface of cancer cells that have entered the bloodstream is thought to associate with DARC proteins expressed at the cell surface of endothelial cells[139]. This interaction results in senescence and inhibition of proliferation of the cancer cells[139]. Strikingly, the number of lung metastases caused by the injection of CD82-expressing cells into mice was greatly increased in a DARC-null background, supporting the essential role of DARC in CD82-mediated metastasis[139]. This is a rare example of a *trans* interaction (*i.e.*, the proteins are found opposite to each other in two separate cells) between a tetraspanin and another protein, and these results open the field to new possibilities and indicate that more than one mechanism are likely to be involved in the regulation of metastasis progression by tetraspanins.

Infectious diseases

CD81 and hepatitis C virus

During the past decade, CD81 has captured the attention of virologists because of its role in hepatitis C virus (HCV) infectivity. HCV is an RNA virus from the family *Flaviviridae* that can cause liver cirrhosis and subsequent hepatocellular carcinoma. The viral glycoprotein E2 is essential for virus binding and entry into hepatocytes. CD81 was isolated in a screen designed to identify E2-binding proteins[140]. The LEL of CD81 was found to mediate this interaction[140]. This association is specific to human CD81 (hCD81) as other tetraspanins (CD9, CD63, and CD151) and the African green monkey CD81 (AgmCD81) homolog are not able to bind a recombinant version of E2[141]. AgmCD81 has only four different amino acids in the LEL relative to hCD81; therefore, the lack of E2 binding to AgmCD81 was particularly useful in mapping the LEL region important for

E2 association. Phenylalanine 186, a residue located within the head subdomain of CD81 LEL, was shown to be critical for binding[4].

The *in vivo* relevance of this protein interaction was not clear until CD81 was confirmed to be required for HCV cell entry. For a long time, a major obstacle in HCV research has been the lack of a cell culture system. However, recently, two novel tools were made available to overcome this problem: 1) HCV pseudoparticles (HCVpp) consisting of HIV pseudoparticles and E1 and E2 glycoproteins from HCV, and 2) a particular HCV strain that is able to replicate in cell culture (HCVcc)[8, 142]. CD81-specific antibodies or downregulation of CD81 by RNA interference inhibit HCVpp infection of permissive cells[8]. Similarly, HCVcc infectivity was dependent on CD81[142]. Moreover, in a more physiologically relevant assay, Molina *et al.* demonstrated the importance of CD81 in the infection of primary hepatocytes using serum isolated from patients infected with HCV[143].

However, although CD81 is necessary for HCV infection, several pieces of evidence indicate that CD81 is not sufficient to confer permissivity to HCV. First, CD81 has a wide distribution throughout the human body, whereas HCV infection is predominantly restricted to the liver. Second, the CD81 homolog in tamarins is able to bind E2 even though this species is refractory to HCV infection[144]. Third, Shaw *et al.* found that CD81 could not associate with E2 proteins from a specific HCV genotype[145]. Fourth, transgenic mice expressing the human form of CD81 are not infected by HCV. And fifth, other proteins have been identified to associate with E2, including scavenger receptor class B type 1 (SR-B1), DC-SIGN and L-SIGN, low-density lipoprotein receptor, and claudin 1 (CLDN1)[146-149]. Using an HCVpp cell entry assay, both CD81 and SR-B1 were found to be required for entry[150]. However, expression of these two proteins was not enough to allow HCV entry into non-hepatic non-permissive cells[150]. Thus, it was suggested that additional proteins present in liver cells might be involved in HCV cell entry. Recently, CLDN1, a protein distributed within tight junctions and enriched in liver cells, was found to play a role in HCV entry[149]. Among all the proteins found to mediate HCV entry, CLDN1 is the only one that is known to allow HCV entry into non-hepatic non-permissive cells[149]. A subsequent report showed the association between CLDN1 and CD81 by using FRET analysis[151]. However, even the presence of CLDN1,

CD81, and SR-B1 is not sufficient in some cell lines to mediate HCV entry[149]. Therefore, infection of hepatocytes by HCV is likely to be a highly complex mechanism involving multiple factors. Interestingly, EWI-2wint, a cleavage product of EWI-2 not present in hepatocytes, was reported to inhibit HCV entry by preventing the association of CD81 and E2[152].

Other tetraspanin-virus functional associations

A role for tetraspanins in viral infection is not unique to CD81. CD82 has a negative effect on the cell-cell fusion event occurring during infection by human T-cell lymphotropic virus type 1 (HTLV-1)[153]. A monoclonal antibody against CD63 (but not CD9, CD81, and CD82) can inhibit CCR5-dependent infection of macrophages by human immunodeficiency virus (HIV)[154]. Also, HIV-1 Env and Gag proteins are distributed in TEMs at the cell surface, together with cellular proteins required for virus budding[80]. A CD9-specific antibody blocks feline immunodeficiency virus (FIV) infectivity[155]. However, unlike the function of CD81 in HCV, this inhibition is not specific for cell entry[155]. CD9 has also been linked to the infectivity of canine distemper virus (CDV)[156]. Finally, a new tetraspanin was recently isolated from the mosquito *Aedes albopictus* because of its higher expression levels after Dengue 2 virus infection[37].

CD81 and Plasmodium sporozoites

The malaria-causing parasite (*Plasmodium* spp.) undergoes a complex life cycle involving multiple stages. One of these stages, the infection of liver cells by sporozoites, is thought to be dependent on CD81. *Plasmodium yoelii* (a rodent parasite) is not able to infect mice lacking CD81[157]. Mice lacking CD9 were unaffected, implying a specific role for CD81. Moreover, a monoclonal antibody recognizing CD81 is able to block infection of hepatocytes by the human parasite *Plasmodium falciparum*[157]. No *Plasmodium*-specific protein has been found to interact with CD81, suggesting that CD81 does not act as a receptor in this case (in contrast with its role in HCV infection)[157]. The antibody MT81w, which binds to CD81 only when associated with other proteins within the tetraspanin web, can also inhibit *Plasmodium* infectivity[79]. Moreover, infection of hepatocytes is cholesterol-dependent, supporting a role for membrane microdomains in

infectivity[79]. Thus, it has been proposed that TEMs are important for *Plasmodium* infection.

Additional potential tetraspanin roles in pathogenicity of microorganisms include the mediation of PLS1 in host plant invasion by fungi, and the characterization of uroplakin Ia as a receptor for *Escherichia coli* strains causing urinary tract infection[34, 158].

Others

CD9 is known for its role in fertility: only about half of female CD9-null mice are able to reproduce as compared to wild-type littermates[159]. This is caused by a defect in sperm-egg fusion[159]. However, the molecular mechanism remains unknown.

Several tetraspanins play roles in neurobiology. *Drosophila* Late bloomer is important for neuromuscular junction formation[27]. CD9 may play a role in the formation of paranodes (structures found at the axoglial junctions of myelinated axons)[160]. The integrity of rod photoreceptor disc rims is thought to rely on the expression of peripherin-2 and ROM-1[54]. *Drosophila* Sunglasses mediates internalization of rhodopsin upon light activation[28]. And CD81 has recently been found to play a role in phagocytosis of photoreceptor outer segments by retinal pigment epithelial cells[161].

Tetraspanin uroplakins are essential for the maintenance of bladder structure and function as a permeability barrier[61].

Regulation of the tetraspanin web

Glycosylation

Even though most tetraspanins are thought to be glycosylated, only a few reports have focused on the effect of glycosylation on tetraspanin function (surprisingly, at the time this review was written, only 13 hits result from a PubMed search using "tetraspanin glycosylation" as key words). Mutation of all three N-glycosylation sites of CD82 enhances its binding to integrin α_5, and CD82 glycosylation is thought to be important for its role in cell motility[162, 163]. Interestingly, the level of CD82 glycosylation seems to be variable when comparing various cancer cell lines[120]. During the maturation of dendritic cells, CD63 is modified by the addition of polylactosaminoglycans[164]. Dendritic cell

maturation is characterized by the distribution of MHC class II complexes from intracellular vesicles to the cell surface for antigen presentation. Because of the reported association of CD63 with MHC class II proteins in MHC class II-enriched intracellular compartments (discussed above, p6 and 14), the change in CD63 glycosylation was proposed to play a role in this maturation event[164]. Uroplakin tetraspanins UP Ia and UP Ib are both glycosylated. Nevertheless, despite a high sequence identity (about 40%), there is a major difference in the sugar molecules they carry[165]. Whereas UP Ia is modified with high mannose glycans, UP Ib carries a complex type of glycosylation. This difference has functional consequences since only UP Ia is a receptor for uropathogenic *E. coli* FimH, a protein that preferentially recognizes terminal mannose molecules[165]. The mechanism behind the differential glycosylation between uroplakin tetraspanins remains unknown. Additionally, mutation of glycosylation sites in UP Ib did not prevent its trafficking to the cell surface[12].

Palmitoylation

Even though acylation of CD9 and CD81 was known for almost 20 years, the role of palmitoylation in the regulation of tetraspanins was practically unknown until a report by Rubinstein's group in 2002[166-168]. Palmitoylation is a reversible post-translational modifications resulting in attachment of palmitic acid to intracellular cysteine residues[169]. The palmitoylation of CD9, CD37, CD53, CD63, CD81, CD82, CD151, and A15 have been reported[168, 170].

Membrane microdomains

The palmitoylation state of several proteins has been associated with their localization within membrane compartments such as lipid rafts[169]. Does this property apply to tetraspanins? As discussed previously, CD81 is required for the stabilization of the B cell coreceptor and the BCR in lipid rafts upon coligation. Cherukuri and colleagues showed that palmitoylation of CD81 is important for this function[171]. First, CD81 palmitoylation levels increase when both the BCR and its coreceptor are stimulated. This inducible palmitoylation is dependent on the presence of lipid rafts. Second, when an inhibitor of palmitoylation (2-bromopalmitate or 2-BP) is added to the cells, the BCR and coreceptor do not partition into lipid rafts. Thus, the authors proposed

that the coligation of both receptor complexes leads to their distribution into microdomains, and subsequent activation of downstream signaling, via CD81 palmitoylation[171]. It is interesting to note that CD81 is constitutively palmitoylated in the absence of stimuli. Moreover, treatment with 2-BP was unable to inhibit this basal level, suggesting differences between the mechanisms controlling inducible vs. constitutive palmitoylation of CD81[171]. These results also imply that the partitioning of CD81 into lipid rafts is not mediated by palmitoylation in an all-or-none fashion. Indeed, basal levels of CD81 palmitoylation are not sufficient to trigger this change. This process seems to be more subtly regulated by the level of palmitoylation. At this point, it is not clear whether the increase in CD81 palmitoylation reflects an increase in number of palmitoylated proteins or in the number of palmitate molecules per CD81 protein.

Research on palmitoylation of other tetraspanins has focused on constitutive palmitoylation levels. By mutating juxtamembrane cysteines—potential palmitoylation sites—palmitoylation-deficient mutant versions of CD9 and CD151 were generated[168, 170, 172]. One of the major questions addressed concerned their distribution within TEMs as analyzed by flotation in sucrose gradients. For both tetraspanins, palmitoylation-deficient proteins were found in DRMs in amounts comparable to their wild-type counterparts[168, 170, 172]. A similar result was obtained when cells were treated with 2-BP[172].

Subcellular distribution

Another common property of palmitoylation in other membrane proteins is the ability to influence their trafficking to the plasma membrane[169]. In the case of CD82, a similar effect was found. When a palmitoylation-deficient CD82 construct was stably expressed in prostate cancer cells, its detection by flow cytometry was reduced to about half the level of wild-type CD82[173]. This was explained by an increased presence of the mutant proteins in intracellular vesicles (endosomes and lysosomes). The intracellular distribution of a palmitoylation-deficient CD151 was also found to be altered in a report from Hemler's group; in this case, the mutant proteins have lost the ability to traffic to endosomal and lysosomal vesicles[170]. However, the surface expression of CD151 remains unchanged in the absence of palmitoylation. These results contrast with another study by Berditchevski *et al.* where they found no difference in intracellular localization of their mutant CD151[172]. The authors indicated that this might be due to the differences between

the constructs used. The first report mutated four cysteines in order to prevent palmitoylation, whereas Berditchevski *et al.* showed that six cysteines needed to be replaced in order to efficiently inhibit palmitoylation[170, 172]. Another possible problem is the presence of a GFP tag in the former CD151 construct[170].

A palmitoylation-deficient CD9 construct showed no difference in surface expression compared to the wild-type protein[168]. On the other hand, surface detection of a CD81 construct lacking six cysteines is markedly reduced relative to wild-type[79]. Therefore, the effect of palmitoylation on subcellular localization might depend on the tetraspanin studied. However, it is too early to rule out discrepancies in the methodology used. Future work applying additional methods to inhibit palmitoylation of various tetraspanins will be helpful in resolving this question.

Protein associations

A major consensus among studies using palmitoylation-deficient proteins of CD9, CD82, and CD151 is the importance of palmitoylation in tetraspanin-tetraspanin interactions—a key component in assembling the tetraspanin web. Yang *et al.* showed a decrease in the association between mutant CD151 and tetraspanins CD9 and CD63[170]. The CD151 mutant construct reported by Berditchevski *et al.* is unable to efficiently immunoprecipitate CD63 and CD81[172]. Similarly, palmitoylation-deficient CD82 has a weakened interaction with CD9 and CD81 compared to wild-type CD82[173]. Work on a CD9 mutant protein by Rubinstein's group adds a level of complexity[168]. The authors make the distinction between two types of tetraspanin-tetraspanin interactions. Their detection depends on the presence of divalent cations (Ca^{2+} and Mg^{2+}) or EDTA in the lysis buffer. Only highly stable associations between tetraspanins are thought to remain in the presence of EDTA. Interestingly, the effect of CD9 palmitoylation appears to be restricted to the EDTA-insensitive CD9 associations with CD53 and CD81; associations detected in the presence of divalent cations are not dependent on the palmitoylation state of CD9[168]. It should be noted that the results described above on the associations of palmitoylation-deficient CD82 and CD151 with other tetraspanins were obtained in lysis buffers lacking EDTA. The functional relevance of a possible role of divalent cations in tetraspanin interactions remains unknown.

Whereas palmitoylation is important for tetraspanin-tetraspanin associations, it does not seem to facilitate the primary interactions between tetraspanins and their specific binding partners. Indeed, mutation of juxtamembrane cysteines in CD9 does not impair binding to CD9P-1 (EWI-F)[168]. Similarly, mutant CD151 associates with integrin $\alpha_3\beta_1$ as efficiently as wild-type CD151[170].

Homodimerization of tetraspanins is also thought to be palmitoylation independent. Formation of CD9 dimers, as shown by chemical cross-linking, remains unaltered when its putative palmitoylation sites are mutated[62]. However, a subsequent report found that the further clustering of CD9 homodimers (homoclustering) is dependent on palmitoylation[174]. This result was obtained by using a monoclonal antibody that recognizes CD9 only when it is clustered. Binding of this antibody to cells expressing a palmitoylation-deficient CD9 is reduced compared to wild-type. Palmitoylation was proposed to facilitate the switch between heteroclustering and homoclustering. When CD9 is palmitoylated, it tends to cluster with itself and exclude other proteins. In this way, a palmitoylation-deficient CD9 is able to bind EWI-2 more efficiently compared to wild-type[174].

As an alternative approach to study the effect of palmitoylation in protein interactions, 2-BP was used. However, in contrast with the previous results, 2-BP does not have a significant effect on the association between CD9 and CD151[172]. The authors proposed that palmitoylation plays a role only in the early stages when tetraspanins first form interactions with other tetraspanins while they traffic to the plasma membrane. Once those associations are established, palmitoylation is no longer required and other types of binding come into place to maintain the tetraspanin web. This would explain why the addition of 2-BP has no effect since these interactions would be already in place before the treatment. Future studies will be needed to confirm this result.

Functional relevance

Expression of the palmitoylation-deficient tetraspanins described above has resulted in the alteration of several cellular functions. CD82 has been shown to inhibit cell motility (discussed above, p15) and absence of palmitoylation prevents CD82 from inhibiting the motility and invasion of prostate cancer cells[173]. Mutant CD151 protein

expression leads to changes in cell morphology in one report, and to an increase in the number of focal adhesions and in PI-3 kinase signaling in another report[170, 172].

A palmitoylation-deficient CD81 lacking six cysteines, however, does not have a significant effect on the infection of *Plasmodium yoelii* in hepatocytes[79]. Also, distribution of CD9 into exosomes is not dependent on its palmitoylation state[175]. Therefore, it is possible that not all tetraspanin functions depend on palmitoylation-dependent associations with other tetraspanins within the tetraspanin web.

Enzymatic palmitoylation of tetraspanins

Recent work from Hemler's group identified a palmitoyl acyl transferase that is responsible for the palmitoylation of CD9 and CD151: DHHC2[176]. DHHC2 is part of a group of enzymes named after the conserved DHHC motif required for their function. When DHHC2, and not other DHHCs, is overexpressed, it leads to an increase in the palmitoylation levels of CD9 and CD151. This function seems to be specific to tetraspanins, as other proteins tested were not affected by the expression of DHHC2. Supporting the role of palmitoylation in building the tetraspanin web, DHHC2 expression results in an increase in CD9/CD151 association. As expected, the primary interaction between CD151 and $\alpha_3\beta_1$ remains unaffected[176].

Surprisingly, downregulation of DHHC2 protein levels by RNA interference results in a decrease of CD9 and CD151 protein expression[176]. This is thought to be mediated by an increase in lysosomal degradation. Thus, degradation of tetraspanins might be dependent on palmitoylation. However, protein levels of palmitoylation-deficient tetraspanins remain unchanged relative to wild-type proteins. The authors suggest that this discrepancy might be due to unknown side effects caused by the mutation of cysteines in these constructs aside from inhibiting palmitoylation[176].

	palmitoylation-deficient constructs (compared to wild-type)		
	CD9[a]	CD82	CD151[a]
localization in TEMs	=	nd	=
surface detection	=	↘	=
intracellular distribution	nd	altered	?[b]
association with tetraspanins	↘	↘	↘
homodimerization	=	nd	nd
association with binding partners	= (EWI-F) ↗ (EWI-2)	nd	= ($\alpha_3\beta_1$)
functional consequences	nd	motility	cell morphology/ focal adhesions

[a] known to be palmitoylated by DHHC2

[b] found to be altered in only one out of two reports

nd: not determined

Figure 1.3 Summary of results obtained from palmitoylation-deficient tetraspanins

The following chapters examine these post-translational modifications in two tetraspanins. Chapter two is centered on the characterization of palmitoylation-deficient CD81. A previous report used a CD81 construct lacking five cytoplasmic cysteines to prevent palmitoylation[22]. However, the predicted three-dimensional structure of CD81 identified an additional cysteine (position 80) as a possible palmitoylation site[1]. Is the mutation of five cysteines enough to efficiently inhibit palmitoylation of CD81? In addition, a thorough characterization of palmitoylation-deficient CD81 has not been reported. Does the palmitoylation-deficient CD81 have similar properties compared to palmitoylation-deficient CD9, CD82, and CD151 proteins? These questions were addressed by generating CD81 constructs with mutations in five, six, seven, or eight juxtamembrane cysteines and analyzing their palmitoylation level, cellular expression, and association with other proteins. Chapter three is focused on Tspan-2 and its N-glycosylation. Among the 33 human tetraspanins, the functions of more than half of them, including Tspan-2, remain obscure. To gain a full understanding of the tetraspanin web, more attention needs to be given to these tetraspanins. Similarly, little is known about the role of glycosylation in the tetraspanin web. We provide the first steps for a full characterization of Tspan-2 by investigating its expression, glycosylation, and association with CD81. In this way, we hope to encourage future studies on Tspan-2 and other lesser-known tetraspanins.

CHAPTER 2 - Mutation of Juxtamembrane Cysteines in the Tetraspanin CD81 Affects Palmitoylation and Alters Interaction with Other Proteins at the Cell Surface

Summary

Palmitoylation of tetraspanins affects protein-protein interactions, suggesting a key role in the assembly of the tetraspanin web. Since palmitoylation occurs on intracellular cysteine residues, we examined whether mutating these residues in the human tetraspanin CD81 would affect the association of CD81 with other surface membrane proteins. Mutation of at least six of the eight juxtamembrane cysteines was required to completely eliminate detectable CD81 palmitoylation, indicating that several sites can be palmitoylated. Interestingly, these cysteine mutations resulted in reduced cell surface detection compared to wild-type CD81 as indicated by flow cytometry analysis. This was not due to a defect in protein trafficking, differences in protein stability, or reduced anti-CD81 antibody binding affinity. In contrast, total CD81 levels, as measured by immunoblotting, appeared to be increased with the mutant proteins. However, we observed that both endogenously and exogenously expressed CD81 underwent a novel processing event either in Golgi or at the cell surface, which was decreased in the mutant CD81 proteins compared to wild-type. This reduction in processing of full length CD81 explained the apparent increase in total CD81 levels for the mutant proteins. Finally, CD81 associations with CD9 and EWI-2 were also impaired with the mutant proteins. Taken together, these findings indicate that mutation of juxtamembrane cysteines alters the interaction of CD81 with other proteins, thereby affecting the detection of CD81 on the cell surface by antibodies and accessibility by proteases.[*]

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[*] A modified form of this chapter has been accepted for publication in *Experimental Cell Research* (Delandre, C., Penabaz, T.R., Passarelli, A.L., Chapes, S.K., and R.J. Clem. 2009. Mutation of juxtamembrane cysteines in the tetraspanin CD81

affects palmitoylation and alters interaction with other proteins at the cell surface. Exp. Cell Res. In press.). The publisher, Elsevier, allows authors the right to include the journal article, in full or in part, in a thesis or dissertation.

Introduction

CD81 is a ubiquitously expressed member of the tetraspanin superfamily of proteins. Tetraspanins are defined by four transmembrane domains and by the presence of conserved cysteines in addition to a CCG motif in the large extracellular loop. Their most striking property is the ability to interact with multiple proteins (*e.g.*, receptors, adhesion proteins, Ig superfamily members, and signaling molecules, as well as other tetraspanins). These associations are thought to occur mainly in membrane compartments such as lipid rafts and tetraspanin-enriched microdomains (TEMs), thereby forming a membrane network known as the "tetraspanin web". Since they regulate the function of their binding partners, tetraspanins play roles in diverse cellular processes including adhesion, proliferation, fusion, and signaling[86, 177]. However, redundancy among the members of the superfamily and the multiplicity of associations make the understanding of their specific molecular mechanism a challenging task.

Research on CD81 has largely focused on its regulatory role in the immune system since its discovery in 1990 as a target of an antiproliferative antibody on lymphoma B cells[86, 178]. Through its direct association with CD19, CD81 participates in the B cell coreceptor, a complex of integral membrane proteins (CD19/CD21/CD81) that lowers the threshold of activation of the B cell receptor (BCR) in response to opsonized antigens[68, 81]. CD81 is thought to facilitate trafficking of CD19 and stabilize the B cell coreceptor[14, 77]. Accordingly, B cells from CD81-null mice express less CD19 on the cell surface and present a weaker antibody response to Th2-inducing antigens[83-85, 88]. Lack of CD81 also results in a defect in allergen-induced airway hyperreactivity, a Th2-dependent reaction commonly found in asthma patients[89]. T-cell function is also influenced by CD81 since T cells isolated from CD81-deficient mice proliferate more rapidly after T-cell receptor cross-linking in comparison with wild-type littermates[85]. Moreover, CD81 interacts with CD4 and CD8, and is able to induce a T-cell costimulatory signal[92, 102, 179]. CD81 has also been found to dynamically concentrate in the immune synapse, a cluster of proteins formed during the close contact between T cells and antigen-presenting cells essential for T-cell activation[91]. Thus, all these

immunological CD81 functions depend on interactions and clustering with other proteins, but how CD81 is able to regulate these associations remains unclear.

It is possible that protein modifications have a role in these functions. Palmitoylation is a post-translational process that attaches palmitic acid to proteins via thioester linkages to cysteine residues. Since it is a reversible process, it can be considered an "on-off" switch similar to protein phosphorylation. Addition of fatty acids is thought to provide proteins more hydrophobic stability in cellular membranes, thus influencing their fate during trafficking and partitioning into membrane microdomains[169, 180]. Most tetraspanins possess cysteines in their intracellular domains that may be available for palmitoylation, and all tetraspanins tested so far have been shown to be acylated[22, 168, 170, 172, 173]. Palmitoylation-deficient constructs have been generated for several tetraspanins (CD9, CD81, CD82, and CD151), revealing a role for palmitoylation in protein association, cell motility, morphology, adhesion-dependent signaling, and oxidative stress[22, 170, 172, 173]. In B cells, coligation of the BCR with the CD19/CD21/CD81 complex induces CD81 palmitoylation, a necessary event resulting in the localization of this cluster of proteins into lipid rafts and signaling[171]. In contrast, palmitoylation of CD9 and CD151 does not seem to be required for their association with TEMs[168, 170, 172].

In this study, we examined whether mutation of intracellular cysteine residues that are sites of palmitoylation would affect CD81 trafficking and/or interactions with other proteins. To address this issue, we characterized CD81 mutant proteins with substitutions in five, six, seven, or eight cysteines in the cytoplasmic and transmembrane domains. In particular, we assessed CD81 expression at the plasma membrane, palmitoylation levels, association with direct binding partners, and protein stability. Our results highlight the importance of intracellular and transmembrane cysteines for the proper expression and post-translational modification of CD81 and its interaction with other proteins.

Experimental Procedures

Cell lines, antibodies, and plasmids

Baby Hamster Kidney (BHK-21) cells were generously provided by Carol Blair from Colorado State University. African green monkey COS-7, human embryonic kidney 293, and human fibrosarcoma HT1080 cells were obtained from Scott C. Todd (Kansas State University). Cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). Anti-CD81 monoclonal antibody (5A6) was obtained from Scott C. Todd and its specificity for CD81 has been described previously[178]. Anti-hEWI-2 monoclonal antibody (8A12) was a generous gift from Eric Rubinstein (INSERM U268, France). Anti-V5-HRP antibody was obtained from Invitrogen (Carlsbad, CA), and anti-hCD9 monoclonal antibody (M-L13) was purchased from BD Biosciences (San Jose, CA). Human V5-tagged CD9 and EWI-2 constructs were generated by cloning the corresponding cDNAs into pcDNA^{TM}3.1/V5-His TOPO® vector (Invitrogen).

Site-directed mutagenesis and transfection

The human CD81 (CD81-5C) mutant with mutations in the cysteines at positions 6, 9, 89, 227, and 228 in the intracellular domains has been described previously[22]. This earlier report, however, mistakenly labeled the residue in position 89 as being an alanine instead of a serine. The construct CD81-6C was obtained by mutating cysteine 80 to a serine residue using CD81-5C as a template and the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Similarly, cysteine 97 was replaced by a serine using CD81-6C as a template to obtain the construct CD81-7C. Finally, cysteine 104 was changed to a serine using CD81-7C as template to generate CD81-8C. Constructs CD81-5CA, -6CA, -7CA, and -8CA were generated using the same sequential scheme but replacing cysteines to alanines instead of serines. The constructs were sequenced to verify the substitutions and to assure other nonspecific mutations did not occur.

BHK, COS-7, 293, and HT1080 cells were transfected with different plasmids as indicated using Lipofectamine™ 2000 (Invitrogen) according to the directions of the manufacturer.

Flow cytometry

Transfected cells were harvested with 0.02% EDTA and stained with αCD81 antibody 5A6, αCD9, or αEWI-2 antibodies (1 μg/10^6 cells) for 20-30 min on ice. Cells were washed with phosphate-buffered saline (PBS) before incubation with Alexa Fluor® 488 goat anti-mouse IgG (BD Biosciences) (1 μg/10^6 cells) for 20 min on ice. Samples were analyzed with a BD FACSCalibur™ flow cytometer (BD Biosciences) and WinMDI 2.9 software (The Scripps Research Institute).

Metabolic labeling

To assess palmitic acid incorporation, COS-7 cells were plated on 100-mm dishes and transfected with a control vector expressing GFP or with vectors expressing wild-type or mutant CD81. At two days post-transfection, cells were serum starved for 3 hours and labeled overnight with 300 μCi/ml palmitic acid [9,10-^{3}H(N)] (PerkinElmer Life and Analytical Sciences, Wellesley, MA) in serum-free medium supplemented with 5% delipidated Bovine Serum Albumin (Sigma-Aldrich, St. Louis, MO). The cells were harvested and washed three times with PBS followed by immunoprecipitation with αCD81 antibody 5A6 as described below. The samples were separated by non-reducing SDS-PAGE, transferred to PVDF membranes, and membranes were exposed to Kodak BioMax MS Film (Eastman Kodak Company, Rochester, NY) for one week at -80 ºC.

To assess protein stability, cells were biosynthetically labeled with [^{35}S]-methionine/cysteine. BHK cells were transfected with vectors expressing wtCD81, CD81-6C, or CD81-8C. The next day, transfected cells were starved in DMEM Met/Cys-free medium (Mediatech, Inc., Herndon, VA) containing 5% dialyzed FBS (Pulse-labeling medium) for one hour. TRAN35S Label (MP Biomedicals, Solon, OH) was then added to the cells at 0.25 mCi/ml in pulse-labeling medium for one hour. After removing the medium containing [^{35}S]-methionine/cysteine, cells were incubated with pulse-labeling medium supplemented with 2 mM L-methionine and 2 mM L-cystine to start the

chase. Cells were harvested at the indicated time points, lysed in lysis buffer (150 mM NaCl, 20 mM Tris pH 7.5, 5 mM $MgCl_2$, 10 units/ml aprotinin, 1mM PMSF, and 1 μg/ml pepstatin A) containing 1% Triton-X 100 (Sigma-Aldrich), and CD81 was immunoprecipitated with αCD81 antibody as described below. After separation by SDS-PAGE, gels were incubated in destain solution (10% acetic acid, 30% methanol) overnight, soaked twice in DMSO, and treated with 22.5% fluor 2,5-diphenyloxazole (Acros Organics, Morris Plains, NJ) to enhance the signal. Following several washes in water, gels were dried and exposed to X-ray film for 2-24 hours at -80 ºC.

Immunoprecipitation

Cells were detached with 0.02% EDTA and lysed for one hour at 4 ºC in lysis buffer containing 1% Brij-99 (Sigma-Aldrich). The insoluble material was removed by centrifugation at 21,000g for 15 min at 4 ºC. The antibody (5A6) was captured on Protein G-Sepharose® beads (Sigma-Aldrich) for one hour. After washing off the excess antibody, the lysates were added to the antibody-bead mixture and left rotating overnight at 4 ºC. Suspensions were centrifuged at 2,300g for 5 min at 4 ºC, the supernatants were collected, and the pellets were washed twice with lysis buffer containing 1% Brij-99, separated by SDS-PAGE under non-reducing conditions, and transferred to an Immobilon-P membrane (Millipore, Billerica, MA).

Immunoblotting

Proteins immobilized on PVDF membranes were blocked for one hour at 4 ºC in Tris-buffered saline with 0.1% Tween-20 (TBS-T) containing 5% non-fat milk. To detect CD81, a solution of αCD81 antibody 5A6 was diluted 1:10,000 in TBS-T containing 1% non-fat milk and was incubated with the membrane for one hour at room temperature. After washing, ImmunoPure® antibody goat anti-mouse HRP (diluted 1:20,000) (Pierce Biotechnology, Rockford, IL) was used as the secondary antibody for 30 min at room temperature. For V5 detection, anti-V5-HRP antibody was used at 1:5,000 for 90 min at room temperature. All blots were visualized by applying SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology) and exposing to X-ray film.

Surface biotinylation labeling

Cells were detached with 0.02% EDTA, washed three times with PBS (pH 8), and incubated with EZ-Link Sulfo-NHS-LC-Biotin (Pierce Biotechnology) or PBS alone for 30 min at 4 ºC. Biotin was dissolved in PBS (pH 8) just before use. Following incubation, cells were washed three times with PBS (pH 7.2) containing 100 mM glycine to remove unbound biotin. Cells were lysed for one hour at 4 ºC in lysis buffer containing 1% Triton X-100, insoluble material was removed by centrifugation at 21,000g for 15 min at 4 ºC, and biotinylated proteins were pulled down with Streptavidin-Agarose beads (Sigma-Aldrich) overnight at 4 ºC. Samples were analyzed by immunoblotting as described above.

Saturation binding assay

BHK cells transfected with a control vector, or with vectors expressing wtCD81 or CD81-8C, were harvested with 0.02% EDTA, and incubated with increasing concentrations of αCD81 antibody 5A6-FITC (34, 67, 201, 402, 670, and 1072 nM) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for one hour on ice. Samples were analyzed with a BD FACSCalibur^{TM} flow cytometer (BD Biosciences). Saturation isotherm curves and K_D values were obtained using GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA) and the following equation: $Y=(B_{max}.X)/(K_D+X)$, where X is the antibody concentration and Y is the mean fluorescence intensity value. Mean Fluorescence Intensity (MFI) values from control vector samples (nonspecific binding) were subtracted from MFI values from wtCD81 and CD81-8C samples to obtain specific binding values. To confirm that the antibody concentrations were saturating under these conditions, supernatants were collected after antibody incubation to measure fluorescence from unbound 5A6-FITC molecules and compared to fluorescence from total number of 5A6-FITC molecules added to the cells using a VICTOR^3 plate reader (PerkinElmer Life and Analytical Sciences). No difference in binding among samples was observed when an isotype control (normal mouse IgG_1-FITC, Santa Cruz Biotechnology, Inc.) was used (data not shown).

Brefeldin A treatment

The medium was removed from cells at one day post-transfection and replaced with fresh medium containing absolute ethanol (vehicle control wells) or brefeldin A (Sigma-Aldrich) at 10 μg/ml for 10 hours in the 37 ºC incubator. Cells were then detached and assayed for CD81 expression by flow cytometry or immunoblotting as described above.

Results

Expression of CD81 palmitoylation mutants is altered compared to wild-type CD81

To study the effect of palmitoylation on CD81 function, we generated a mutant of human CD81 where eight intracellular and transmembrane cysteines were replaced by alanines or serines (CD81-8C), potentially inhibiting palmitoylation of CD81. Intermediate constructs lacking six or seven cysteines were also obtained, and named CD81-6C and CD81-7C, respectively (Fig. 2.1). These mutants were compared to a previously described construct with mutations in five cysteines, CD81-5C (referred to as triple mutant CD81 in ref. 22). A second set of constructs was generated where all of the substituted cysteines were replaced by alanines, and named CD81-5CA, -6CA, -7CA, and -8CA.

To determine whether the introduced mutations affected CD81 expression, total CD81 protein levels were determined by immunoblotting using monoclonal 5A6, a conformation-dependent antibody recognizing a specific epitope in the large extracellular loop of human CD81 (Fig. 2.2*A*)[178]. Baby Hamster Kidney (BHK) cells transfected with the plasmids were lysed in 1% Brij-99 prior to electrophoresis and immunoblot analysis. Consistent with previous findings[168], we observed a difference in migration between wild-type CD81 (wtCD81) and the mutants, which migrated faster than wild-type, possibly due to the absence of bound palmitate. In addition, CD81 protein levels were higher in cells transfected with the mutant constructs. This difference, especially between wtCD81 and CD81-8C, was consistently observed in multiple (more than 10) independent experiments using different plasmid preparations (*e.g.*, Fig. 2.3*B*). Interestingly, mutation to serines in CD81-6C and -7C resulted in even higher CD81 protein levels than the alanine substitutions in CD81-6CA and -7CA. There was also a change in the ratio between monomers and dimers (white arrow) of CD81. It has been reported that these dimers form after lysis when spontaneous disulfide bonds form between intracellular and transmembrane cysteines[62]. The lack of such cysteines in CD81-8C and -8CA would explain why no dimers were observed with these constructs

(Fig. 2.2*A*). However, the variability among the other constructs (with CD81-6C having the highest level of dimers) may depend on whether the specific available cysteines are involved in intra- or inter-molecular interactions. We observed that mutation to serine or alanine residues could also become a relevant factor for dimer formation (note the increase in dimer intensity for CD81-5CA and -7CA compared to -5C and -7C, respectively). The observation that the migration of the CD81 homodimers was faster than expected is consistent with previous results obtained with CD9 homodimers[62].

Since CD81 is mainly targeted to the plasma membrane, we examined whether cysteine mutagenesis affected its localization at the cell surface. BHK cells were transfected with the mutant constructs and the cells were stained for flow cytometry using αCD81 antibody 5A6 (Fig. 2.2*B*). In contrast to the immunoblotting results, we observed an overall decrease in the detection of CD81 mutant proteins compared to wtCD81. In the case of the serine mutants (CD81-5C, -6C, -7C, and -8C), there was a correlation between an increased number of mutated cysteines and reduced CD81 detection at the cell surface. The alanine mutants (CD81-5CA, -6CA, -7CA, and -8CA), however, had an intermediate phenotype, between the levels of their corresponding serine mutants and wtCD81. A similar pattern was observed when another mAb also specific for the large extracellular loop of CD81 (JS81) was used (data not shown). Furthermore, only surface protein detection was affected by these point mutations since all serine mutant CD81 transcript levels were comparable to wtCD81 as measured by real time RT-PCR (data not shown). Surface detection by 5A6 was also tested in COS-7 cells transiently expressing wtCD81 and CD81-8C, and similar results were obtained (data not shown).

To test whether the difference in protein levels seen by immunoblotting was independent of the antibody used, we tagged wtCD81, CD81-5C, and -8C with a V5 epitope at their carboxyl termini. As observed with the untagged constructs, CD81-5C-V5 and CD81-8C-V5 were detected at a higher level than wtCD81-V5 by immunoblot using αV5 antibody (Fig. 2.2*C*), but flow cytometry measurements using 5A6 showed reduced surface levels of CD81-8C-V5 compared to wtCD81-V5 (Fig. 2.8*A*).

Mutation of six cysteines is necessary for complete inhibition of CD81 palmitoylation

To analyze if palmitoylation was defective in our constructs, we biosynthetically labeled COS-7 cells expressing the CD81 mutants with [^{3}H]-palmitic acid. After Brij-99 lysis, samples were immunoprecipitated with 5A6, resolved by SDS-PAGE, and transferred to PVDF membranes. Duplicate membranes were then exposed to X-ray film or probed with 5A6. Figure 2.3*C* shows the level of palmitic acid incorporation in CD81 relative to the expression of each construct (Fig. 2.3*B*). In contrast to an earlier report[22], substituting only five cytoplasmic cysteines was not sufficient to completely inhibit CD81 palmitoylation. We observed that palmitoylation was reduced by 75% in CD81-5C, while additional mutation of the cysteine residue at position 80 reduced palmitoylation to 11% of wtCD81. Further mutation of cysteines at positions 97 and 104 did not appreciably decrease palmitoylation further. Therefore, mutation of at least six juxtamembrane cysteines is necessary to fully inhibit CD81 palmitoylation.

Association of EWI-2 or CD9 with palmitoylation-deficient CD81 is impaired

Since interactions with other molecules are critical for tetraspanin-mediated functions[177], we performed co-immunoprecipitations to examine the effect of cysteine mutagenesis on CD81 associations with other tetraspanin web members. We tested the ability of the CD81 mutants to bind to the tetraspanin CD9 and the Ig superfamily member EWI-2, two proteins normally found associated with CD81[177]. Our results indicated that the amount of CD9 and EWI-2 co-immunoprecipitated by CD81 depended on the number of cysteines in the cytoplasmic and transmembrane domains (Fig. 2.4*A* and *B*). We observed a slight but consistent reduction in CD9 association to the palmitoylation-deficient proteins that was most pronounced when all eight cysteines were replaced (Fig. 2.4*A*). However, CD9 interaction with CD81-5C did not appreciably differ from wtCD81. Longer exposure of the blot revealed upper bands that were stronger when CD81-6C or CD81-7C were pulled down (white arrow). Similar to the bands seen in figure 2.2*A*, they may be the result of post-lysis formation of CD9 homodimers. When we assessed CD81 association with EWI-2, all mutated proteins co-immunoprecipitated EWI-2 at lower levels, with CD81-8C being the lowest (Fig. 2.4*B*). We also examined the

surface levels of CD9 and EWI-2 when co-expressed with CD81-8C but did not detect any significant difference relative to co-expression with wtCD81 (Fig. 2.4*C*). In conclusion, the data suggest that mutating these cysteine residues affects the association of CD81 with CD9 and EWI-2 but does not affect the expression of CD9 or EWI-2 on the plasma membrane.

CD81 protein stability remains unchanged after mutation of six or eight cysteines

One possible explanation for the apparent increase in total expression of the mutant CD81 proteins is that the mutations affected the stability of CD81. To address this, we performed pulse-chase labeling on CD81-transfected BHK cells using [^{35}S]-methionine/cysteine. Proteins were labeled for one hour and chased for various time intervals. Protein stability during the next 2 to 3 days was measured for wtCD81, CD81-6C, and -8C (Fig. 2.5). Since CD81-6C and 8C had lower radiolabeling than wtCD81 (as expected, due to the absence of multiple cysteines), we normalized the densitometry values for each protein by taking time zero as 100%. No significant change in stability was observed among these proteins, with each having a half-life between 12 and 22 hours (values estimated from four independent experiments). Additionally, we observed a small upward shift in wtCD81 migration in the early time points that is not apparent in CD81-6C or -8C (Fig. 2.5, compare 0 and 1 hour to 6 hours and later). We hypothesize that this decrease in migration is due to the addition of palmitic acid during CD81 maturation. These results indicate that palmitoylation does not affect the stability of CD81, and alterations in protein stability cannot explain the differences observed in total protein expression between mutant and wtCD81.

CD81-8C proteins are localized normally to the plasma membrane and bind to αCD81 antibody 5A6 normally, but their detection by 5A6 on the cell surface is hindered

The decrease in CD81 mutant surface detection relative to wtCD81 as measured by flow cytometry (Fig. 2.2*B*) could reflect a defect in trafficking of the protein to the plasma membrane. To address this question, we surface biotinylated BHK cells

transfected with V5-tagged wtCD81 or CD81-8C. Biotinylated CD81 proteins were detected by Streptavidin pull-down followed by αV5 immunoblotting. We observed an increase in biotinylated CD81-8C proteins levels compared to wtCD81 (Fig. 2.6*A*), similar to the increase seen in total protein levels of CD81 mutants (Fig. 2.2*A* and *C*), suggesting that CD81 transport to the plasma membrane is not affected by the cysteine mutations. Similar results were obtained using untagged wtCD81 and CD81-8C by 5A6 immunoblot (data not shown).

Another possible explanation for the apparent decrease in surface expression of the mutant proteins by flow cytometry is a defect in 5A6 binding to the CD81 mutant proteins. To test this hypothesis, we performed a saturation binding assay where transfected BHK cells were incubated with increasing concentrations of FITC-labeled 5A6 and analyzed by flow cytometry. From the saturation isotherm curves, we obtained similar K_D values for both wtCD81 and CD81-8C (Fig. 2.6*B*), suggesting that the affinity of 5A6 for CD81 remains unchanged when all eight cysteines are mutated. However, the CD81-8C curve reached a plateau at a level (B_{max}= 22.66) considerably lower than wtCD81 (B_{max}= 52.66), indicating a reduction in the number of 5A6 binding sites on the surface of cells expressing CD81-8C compared to wtCD81.

Cleavage of CD81 is altered when all eight cysteines are mutated

When we examined the expression of V5-tagged wtCD81 and CD81-8C proteins by immunoblot using αV5 antibody, we observed the appearance of additional faster migrating immunoreactive bands when blots were exposed for a longer time: one band at 26-28 kDa (grey arrows) and additional bands at 10-12 kDa (black arrows, Fig. 2.7*A*). Interestingly, there was a consistent difference in the amounts of these faster migrating bands for CD81-8C compared to wtCD81 across various cell types (Fig. 2.7*A*). In addition to the increase in full-length CD81-8C levels described above (Figs. 2.2 and 2.3), CD81-8C-V5 proteins were characterized by an increase in the 26-28 kDa band and a decrease of the 10-12 kDa bands compared to wtCD81-V5. We did not observe the faster migrating 10-12 kDa bands on immunoblots using αCD81 antibody 5A6. This is consistent with these 10-12 kDa bands arising due to proteolysis of CD81; since the 10-12 kDa fragments contain the C-terminal V5 tag, they would not be expected to include

the 5A6 epitope, based on their size. On the other hand, the 26-28 kDa form probably does not arise from this proteolysis event, since its levels correlated with those of full-length CD81 (Fig. 2.7*A*). Thus, a portion of CD81 appears to be proteolytically cleaved, and reduced cleavage of mutant CD81 explains the apparent increase in detection of full-length mutant CD81 by immunoblotting with either 5A6 or αV5 (Figs. 2.2, 2.3, and 2.7).

The presence of mutant CD81 also affects CD9 processing

Cells co-transfected with CD9-V5 (C-terminal tag) and wtCD81 or CD81-8C showed the presence of a faster migrating immunoreactive CD9 band around 18 kDa that was strikingly reduced in intensity when CD81-8C was co-expressed (Fig. 2.7*B*, black arrow), even though the levels of full-length CD9 remained unchanged (upper blot). We observed the same effect on CD9 when it was co-transfected with CD81-7C, -7CA, or -8CA (data not shown). On the other hand, CD9 homodimer formation was only diminished when wtCD81 was co-expressed (Fig. 2.7*B*, white arrow), consistent with the decreased interaction between CD9 and CD81-8C compared to wtCD81 (Fig. 2.4).

Brefeldin A treatment prevents CD81 cleavage

We used brefeldin A, an inhibitor of ER-Golgi transport, to examine whether CD81 targeting to the Golgi or plasma membrane was required for the processing event observed in figure 2.7. Transfected BHK cells were treated with brefeldin A for 10 hours and analyzed for surface CD81 by flow cytometry using 5A6. Surface levels of both wtCD81-V5 and CD81-8C-V5 were reduced by similar ratios after brefeldin A incubation (Fig. 2.8*A*), indicating similar kinetics of transport to the cell surface. Similar results were obtained with untagged versions of these constructs (data not shown). However, when similarly transfected cells were assayed by immunoblotting with αV5, the levels of full-length wtCD81 were increased by brefeldin A treatment, while the levels of the 10-12 kDa bands decreased (Fig. 2.8*B*). Brefeldin A also had a similar effect on CD81-8C-V5 but it was less pronounced. The appearance of the 26-28 kDa CD81 band was also inhibited by brefeldin A (grey arrow). Endogenous human full-length CD81 levels were also affected by brefeldin A treatment in HT1080 cells as determined by 5A6 immunoblotting (Fig. 2.8*C*), indicating that the observed processing of CD81 is not a result of protein overexpression but a *bona fide* cellular event. These results indicate

that the faster migrating forms of CD81 are due to posttranslational processing events that occur after CD81 enters the Golgi complex.

Discussion

This study presents novel data on the effects of juxtamembrane cysteine mutagenesis on CD81 behavior in cells. Previous reports have used similar constructs to examine whether acylation had an effect on specific CD81 functions, but a thorough characterization of a palmitoylation-deficient CD81 has not been previously published[13, 22, 79]. We found that complete inhibition of CD81 palmitoylation required mutagenesis of at least six juxtamembrane cysteine residues (Fig. 2.3). We detected what initially appeared to be reduced CD81 levels on the plasma membrane when cytoplasmic and transmembrane cysteines were replaced (Fig. 2.2*B*). This reduction was not due to a defect in CD81 transport to the cell surface (Fig. 2.6*A*), or in the affinity of αCD81 antibody 5A6 for its epitope, which lies in the large extracellular loop of CD81 (Fig. 2.6*B*). In contrast, total protein levels of the mutants, particularly CD81-8C, appeared to be higher than wtCD81 by immunoblotting (Fig. 2.2*A* and *C*), and surface biotinylation results indicated that higher amounts of CD81-8C were transported to the plasma membrane than wtCD81 (Fig. 2.6*A*). These results could not be explained by a difference in protein stability since wtCD81, CD81-6C, and -8C had similar half-lives (Fig. 2.5). However, when CD81 was C-terminally tagged with a V5 epitope, the appearance of faster migrating bands in immunoblots using αV5 antibody was reduced with CD81 mutant proteins compared to wtCD81 (Fig. 2.7*A*). These faster migrating CD81 forms are presumably due to proteolytic processing occurring in the Golgi or at the plasma membrane since their appearance is inhibited by brefeldin A (Fig. 2.8*B*). Finally, the associations of CD81 mutant proteins with CD9 and EWI-2 were impaired compared to wtCD81 (Fig. 2.4). The results obtained for wild-type CD81 and CD81-8C are summarized in figure 2.9. Taken together, a likely explanation for these data is that the mutation of juxtamembrane cysteines in CD81 results in altered interactions between CD81 and other membrane proteins, which results in masking or covering up of CD81 and reduced access by large molecules such as antibodies or proteases by steric hindrance.

Cysteine mutagenesis remains the most common method used to study the role of palmitoylation on a specific protein. Since no specific palmitoylation motif is known, substitution of all available cysteines is generally used to completely inhibit palmitic acid attachment. To generate palmitoylation-deficient CD81 proteins, other groups have replaced five cysteines located in the intracellular domains at positions 6, 9, 89, 227, and 228[13, 22]. However, the predicted three-dimensional conformation of the second transmembrane domain places another cysteine (position 80) at the plasma membrane-intracellular interface where it would be available for acylation[1]. Consistent with this model, we showed that CD81 proteins lacking only five cysteines still incorporate 25% of the wild-type levels of palmitic acid (Fig. 2.3). However, the contribution of cysteines 97 and 104 to palmitoylation is unclear, since the sensitivity of the labeling assay used in this study cannot differentiate among CD81-6C, -7C, and -8C. Since cysteines 97 and 104 are predicted to be embedded in the third transmembrane region[1], it is possible that their accessibility to palmitic acid is constrained by neighboring residues and phospholipids. Further studies using more sensitive methods such as mass spectrometry should provide more precise information on the number of cysteines that are available for palmitoylation.

Several groups have used this mutagenesis approach to inhibit palmitoylation in tetraspanins, but none have addressed the differences that could occur depending on the residue chosen to replace cysteines, either serines or alanines. Here, we report differences in apparent CD81 surface expression depending on the use of serines or alanines at positions 80, 89, 97, and 104, which are residues located inside or in close proximity to transmembrane domains (Fig. 2.2*A* and *B*). Because serine is more hydrophilic relative to cysteine, it could have an impact when introduced into a hydrophobic membrane environment by disrupting protein structure and interactions with other proteins. This difference might be relevant when mutagenesis of all transmembrane cysteines is needed to map interaction domains by cysteine-scanning mutagenesis. For example, we found an increase in dimer formation between CD81-7CA and CD81-7C, mutants that lack all but one cysteine at position 104 (Fig. 2.2*A*). The only difference between these two constructs is the amino acid used to replace all the other cysteines: only alanines (CD81-7CA) or a mixture of alanines and serines (CD81-7C). The observation that the surface

detection of CD81-7CA and CD81-8CA mutants is not as severely affected as CD81-7C or CD81-8C (Fig. 2.2B) indicates that alterations in protein structure due to amino acid substitution play at least some role in our results. However, the possibility remains that lack of palmitoylation may also have an effect, since detection of surface CD81-7CA and CD81-8CA is still decreased compared to wtCD81.

Palmitoylation is known to regulate trafficking to the plasma membrane of a number of integral membrane proteins (*e.g.*, CCR5, δ opioid receptor, thyrotropin receptor, and histamine H2 receptor)[169, 181-184]. Thus, the decrease in surface detection of the CD81 mutant proteins by flow cytometry (Fig. 2.2*B*) initially led us to think there was a defect in protein transport to the cell surface. However, this hypothesis was refuted when immunoblots displayed an increase in surface biotinylated CD81-8C compared to wtCD81 (Fig. 2.6*A*), corresponding to the levels of total CD81 protein (Fig. 2.2*A*). We then considered that the affinity of αCD81 monoclonal antibody 5A6 for the CD81 mutant proteins might be reduced, which would explain the decreased detection by flow cytometry. However, 5A6 binding to both wtCD81 and CD81-8C showed similar K_D values (Fig. 2.6*B*). One difference between these assays is the use of biotin, a relatively small molecule (0.5 kDa), versus an antibody (150 kDa) to detect surface expression. Therefore, to explain these apparently contradictory results, we propose that these CD81 mutant proteins are expressed and transported to the cell surface normally, but once at the surface they become inaccessible to bulky proteins such as antibodies. Either the lack of palmitoylation or structural alterations due to amino acid substitutions, or a combination of both factors, may cause CD81 to be displaced to a membrane compartment where it is surrounded by large neighboring proteins, thus preventing 5A6 binding by steric hindrance but still allowing access to biotin. It has been shown that palmitoylation induces translocation of CD81 to lipid rafts[171]. Additionally, association of the CD81 mutant proteins with CD9 and EWI-2 is decreased compared to wtCD81 (Fig. 2.4*A* and *B*), supporting the hypothesis that when these cysteines are mutated, the protein network surrounding CD81 is affected. However, palmitoylation-deficient CD9 and CD151 proteins have been shown to remain in TEMs similar to their wild-type counterparts. It is possible that the methodology used to detect membrane microdomains localization is not

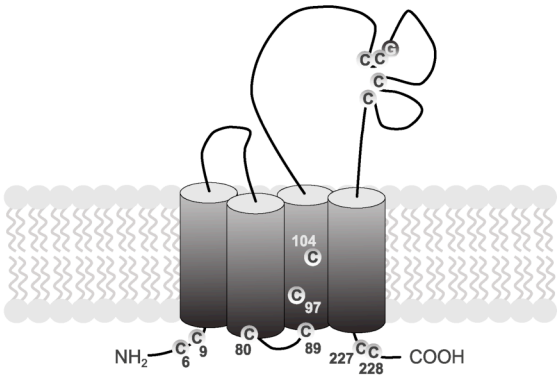
precise enough to detect subtle changes in protein displacement around the plasma membrane.

Another initially puzzling set of results was the apparent increase, using immunoblotting, in total CD81 protein levels when cysteines were mutated (Fig. 2.2*A* and *C*). We showed that protein stability was not affected in our mutants (Fig. 2.5), and levels of mRNA expressed from the serine mutant and wtCD81 constructs were shown to be similar (data not shown). Then, we observed additional faster migrating bands on immunoblots using an antibody specific for an epitope tag located at the C-terminus of CD81 (Fig. 2.7*A*), adding a new level of complexity to CD81 expression. An increase in full-length CD81 was observed when cells were treated with brefeldin A, which correlated with a decrease in the faster migrating CD81 bands. Thus, changes in full-length CD81 levels do not necessarily correspond to changes in total protein expressed; they may instead reflect disparities in CD81 processing. The functional significance of these faster migrating CD81 forms remains to be understood. Other groups have used brefeldin A (at similar concentrations) and reported no difference in levels of endogenous tetraspanins including CD81[170, 175]. However, when we tested the effect of brefeldin A on endogenous CD81 in a human cell line (HT1080), we observed an increase in full-length CD81 levels (Fig. 2.8*C*), indicating that a similar type of processing occurs under physiological conditions. To our knowledge, the presence of similar faster migrating bands on tetraspanin immunoblots has not been previously reported. It should be noted that most tetraspanin-specific antibodies would not detect these bands, since they usually bind at the large extracellular loop.

The decreased processing of CD81-8C (Fig. 2.7*A*) also supports our hypothesis that palmitoylation-deficient CD81 proteins are surrounded by different proteins compared to wtCD81. Indeed, this new environment may bring less or different proteases in close proximity to CD81, or inhibit access to CD81 by steric hindrance, thus reducing the proportion of processed forms. The reduction in the intensity of a similar faster migrating CD9 band and increase in CD9 homodimerization when CD9 was co-expressed with CD81-8C compared to wtCD81 might also be the result of a change in protein interactions surrounding CD9 and CD81 (Fig. 2.7*B*). At this time we cannot completely distinguish between the effects of inhibiting palmitoylation and possible

conformational effects due to the mutation of juxtamembrane cysteines in CD81. Thus, it remains possible that palmitoylation may contribute to the assembly of the tetraspanin network, but our results illustrate that caution must be used when interpreting the results of such mutational approaches on protein function.

Figures



	Amino acid position							
	6	9	80	89	97	104	227	228
wtCD81	C	C	C	C	C	C	C	C
CD81-5C	**A**	**A**	C	**S**	C	C	**A**	**A**
CD81-6C	A	A	**S**	S	C	C	A	A
CD81-7C	A	A	S	S	**S**	C	A	A
CD81-8C	A	A	S	S	S	**S**	A	A
CD81-5CA	A	A	C	**A**	C	C	A	A
CD81-6CA	A	A	**A**	A	C	C	A	A
CD81-7CA	A	A	A	A	**A**	C	A	A
CD81-8CA	A	A	A	A	A	**A**	A	A

Figure 2.1 Location of mutated cysteines in CD81 mutants

The CD81 mutant constructs used in this study were obtained by replacing cysteines present in the cytoplasmic or transmembrane (illustrated by cylinders) domains with alanines or serines as indicated via site-directed mutagenesis (wt, wild-type). Residues in bold indicate ones that were altered in comparison to the construct immediately above. The top line indicates the positions of the mutations.[†]

[†] CD81-6C, -7C, and -8C constructs were generated by Taryn R. Penabaz.

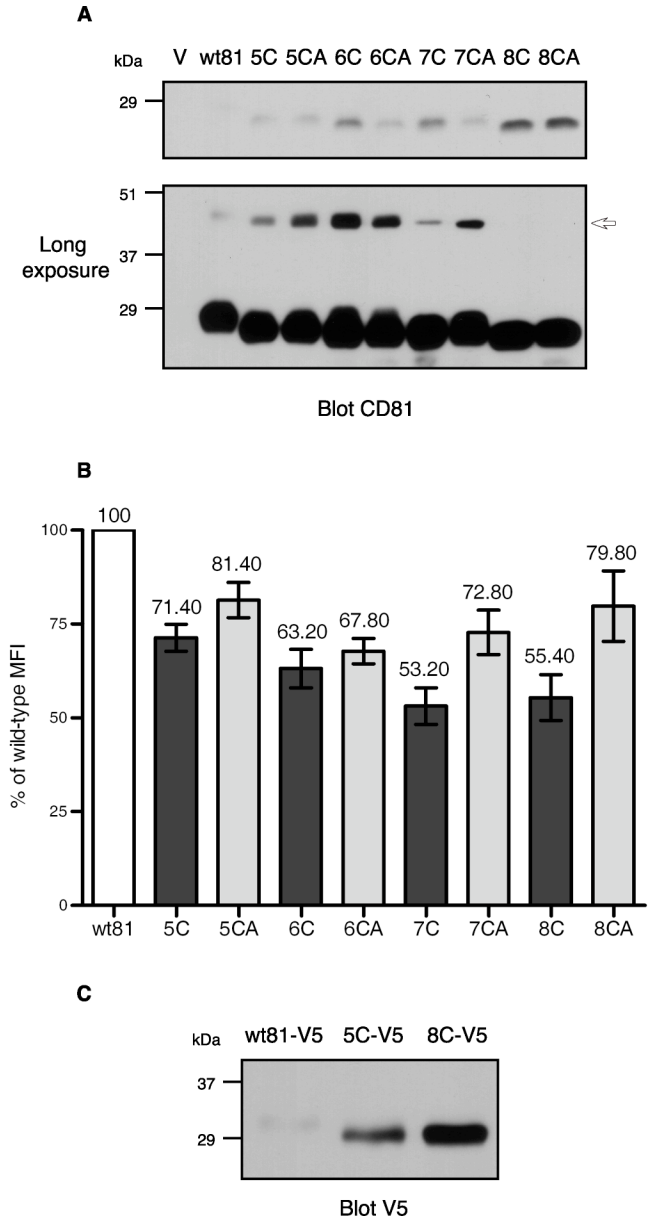


Figure 2.2 Expression of CD81 mutant proteins in BHK cells

BHK cells were transfected with the indicated CD81 constructs and analyzed for total CD81 levels by immunoblotting with the αCD81 mAb 5A6 after lysis with 1% Brij-99 (*A*), or for surface CD81 expression by flow cytometry using 5A6 (*B*). V5-tagged wtCD81, CD81-5C, and CD81-8C were similarly tested for expression by immunoblotting (*C*). Note that wtCD81 is barely visible at this exposure. In panel A, a longer exposure of the same blot is presented below to show dimer formation (white

arrow). In panel B, the values shown are means from 5 independent experiments normalized relative to wtCD81 levels (100%). Error bars indicate standard error of the mean (SEM). V, vector; MFI, mean fluorescence intensity.

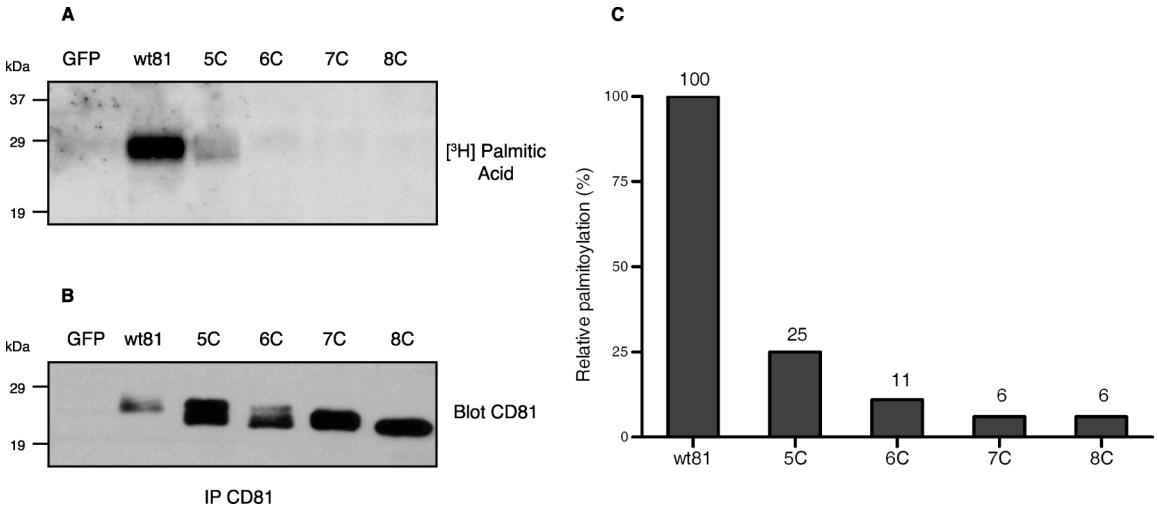


Figure 2.3 Palmitoylation levels of CD81 mutants

COS-7 cells transfected with the CD81 constructs were starved and labeled overnight with [^{3}H]-palmitic acid. Samples were then lysed with 1% Brij-99 and immunoprecipitated with αCD81 mAb 5A6. Following SDS-PAGE and transfer to PVDF membranes, samples were exposed to autoradiography for one week (*A*). As a control, the level of CD81 was assayed by immunoblotting duplicate membranes using 5A6 (*B*). Relative palmitoylation values were obtained by normalizing the amount of [^{3}H]-palmitic acid in panel A to the total amount of CD81 expressed in panel B, as assessed by densitometry (*C*). Palmitoylation of wtCD81 was set at 100%.‡

‡ This experiment was performed by Taryn R. Penabaz.

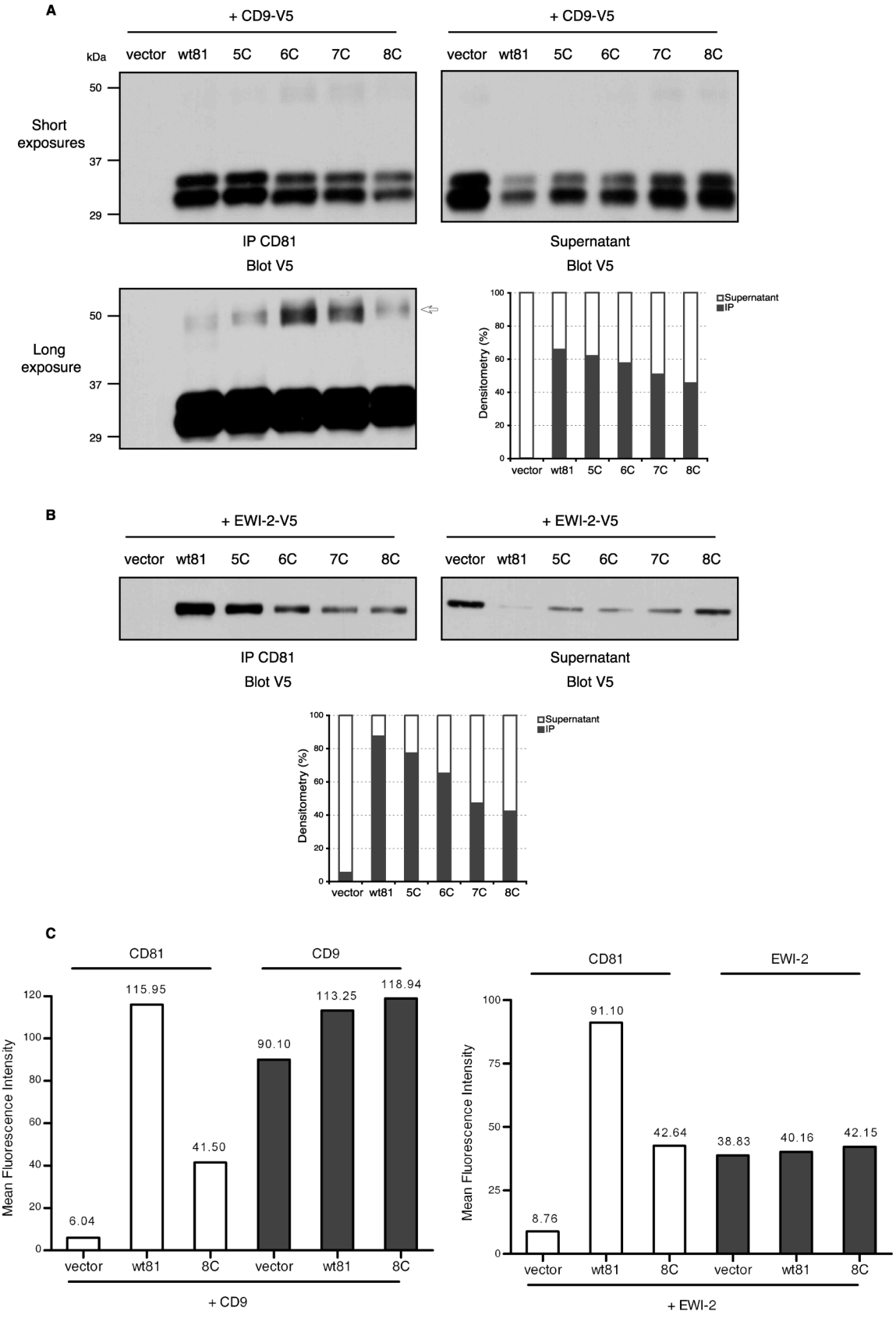

A
+ CD9-V5
+ CD9-V5
kDa
vector wt81 5C 6C 7C 8C
vector wt81 5C 6C 7C 8C
50
37
29
Short exposures
IP CD81
Blot V5
Supernatant
Blot V5
Long exposure
Densitometry (%)
Supernatant
IP
vector wt81 5C 6C 7C 8C
B
+ EWI-2-V5
+ EWI-2-V5
vector wt81 5C 6C 7C 8C
vector wt81 5C 6C 7C 8C
IP CD81
Blot V5
Supernatant
Blot V5
Densitometry (%)
Supernatant
IP
vector wt81 5C 6C 7C 8C
C
CD81
CD9
Mean Fluorescence Intensity
6.04
115.95
41.50
90.10
113.25
118.94
vector wt81 8C vector wt81 8C
+ CD9
CD81
EWI-2
Mean Fluorescence Intensity
8.76
91.10
42.64
38.83
40.16
42.15
vector wt81 8C vector wt81 8C
+ EWI-2

Figure 2.4 Association of CD81 mutants with CD9 and EWI-2

BHK cells transiently co-expressing the CD81 constructs and CD9-V5 or EWI-2-V5 were lysed with 1% Brij-99, and CD81 proteins were immunoprecipitated with αCD81 mAb 5A6. Immunoblots were probed with αV5 antibody to assess the co-immunoprecipitation of CD9-V5 (*A*) or EWI-2-V5 (*B*). Relative amounts of CD9 and EWI-2 in the immunoprecipitated material or the supernatant were obtained by densitometry. A longer exposure of the blot in (*A*) is presented below to show dimer formation (white arrow). Surface detection levels of CD9 and EWI-2 when co-expressed with wtCD81 or CD81-8C were assessed by flow cytometry using 5A6 (open bars) and αEWI-2 or αCD9 (closed bars) (*C*).

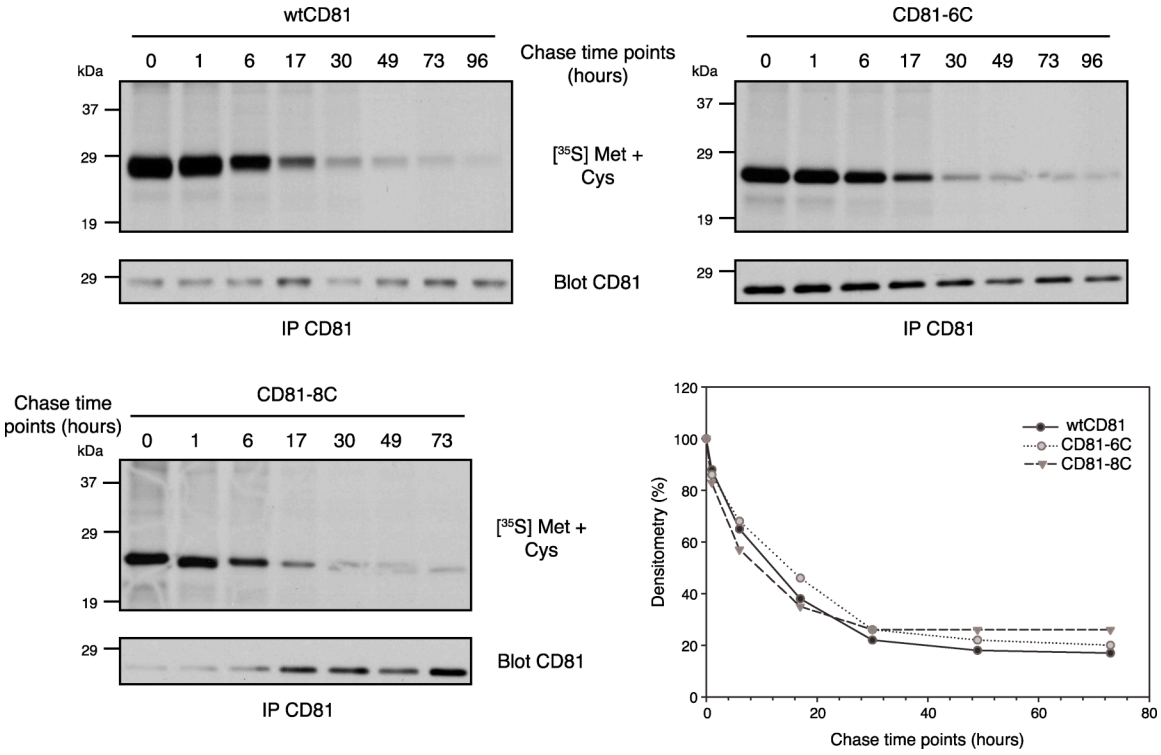


Figure 2.5 Stability of wtCD81, CD81-6C, and CD81-8C proteins

BHK cells expressing wtCD81, CD81-6C, or CD81-8C were methionine/cysteine-starved, pulsed with [^{35}S]-methionine/cysteine, and chased for the indicated time periods (hours). Following lysis and CD81 immunoprecipitation, samples were resolved by SDS-PAGE and gels were exposed to X-ray film. CD81 immunoblots were carried out using the same samples to show CD81 expression levels from each construct. Densitometry values were normalized by setting the value obtained for 0 hours at 100% for each protein.

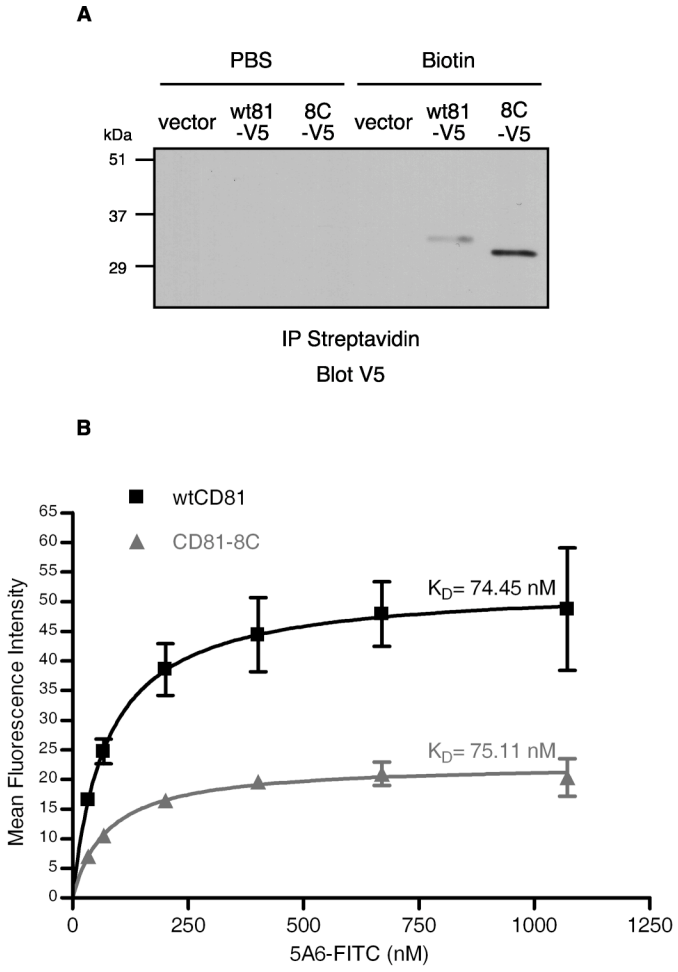


Figure 2.6 Surface detection of wtCD81 and CD81-8C proteins by biotinylation and αCD81 mAb 5A6 binding

BHK cells expressing V5-tagged wtCD81 or CD81-8C were surface biotinylated, lysed, and biotinylated proteins were pulled down with Streptavidin beads. Immunoblots were probed with αV5 antibody (*A*). 5A6 binding to CD81 was assayed by incubating BHK cells expressing wtCD81 or CD81-8C proteins with increasing concentrations of 5A6 followed by flow cytometry analysis (*B*). In panel B, data points represent the mean of two independent experiments (± SEM).

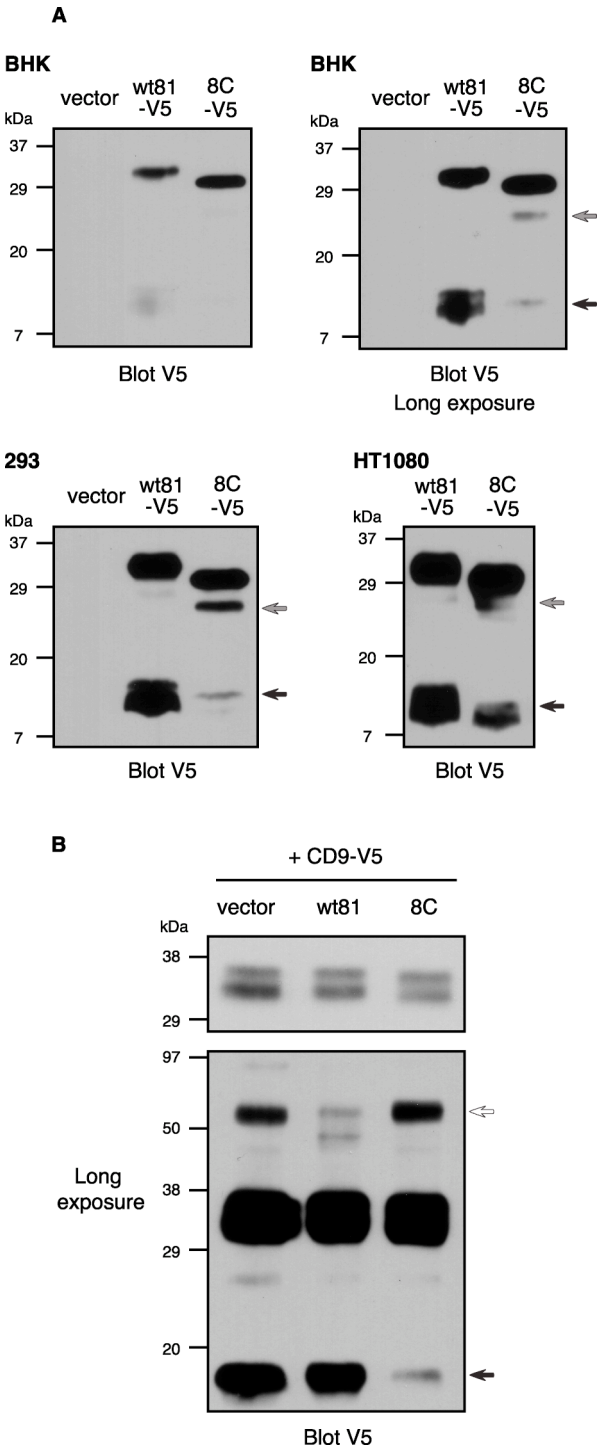

A
BHK
vector
wt81 -V5
8C -V5
kDa
37
29
20
7
Blot V5
BHK
vector
wt81 -V5
8C -V5
kDa
37
29
20
7
Blot V5
Long exposure
293
vector
wt81 -V5
8C -V5
kDa
37
29
20
7
Blot V5
HT1080
wt81 -V5
8C -V5
kDa
37
29
20
7
Blot V5
B
+ CD9-V5
vector
wt81
8C
kDa
38
29
97
50
38
29
20
Long exposure
Blot V5

Figure 2.7 Processing of wtCD81 and CD81-8C proteins, and of CD9 when co-expressed with wtCD81 or CD81-8C

BHK, 293, or HT1080 cells transfected with V5-tagged wtCD81 or CD81-8C were lysed, and CD81 protein expression patterns were assessed by immunoblotting using αV5 antibody (*A*). Faster migrating forms of CD81 are indicated by grey and black arrows. Two exposures of the same BHK immunoblot are presented to show the differences between wtCD81 and CD81-8C in the levels of full-length CD81 (*upper left panel*) and the faster migrating forms of CD81 (*upper right panel*). In panel B, Brij-99 lysates from BHK cells, co-transfected with CD9-V5 and wtCD81 or CD81-8C, were analyzed for CD9 expression by αV5-antibody immunoblot. Faster migrating forms of CD9 and CD9 homodimers are indicated by black and white arrows, respectively. A shorter exposure of the same blot (*upper panel*) shows the levels of full-length CD9 monomers.

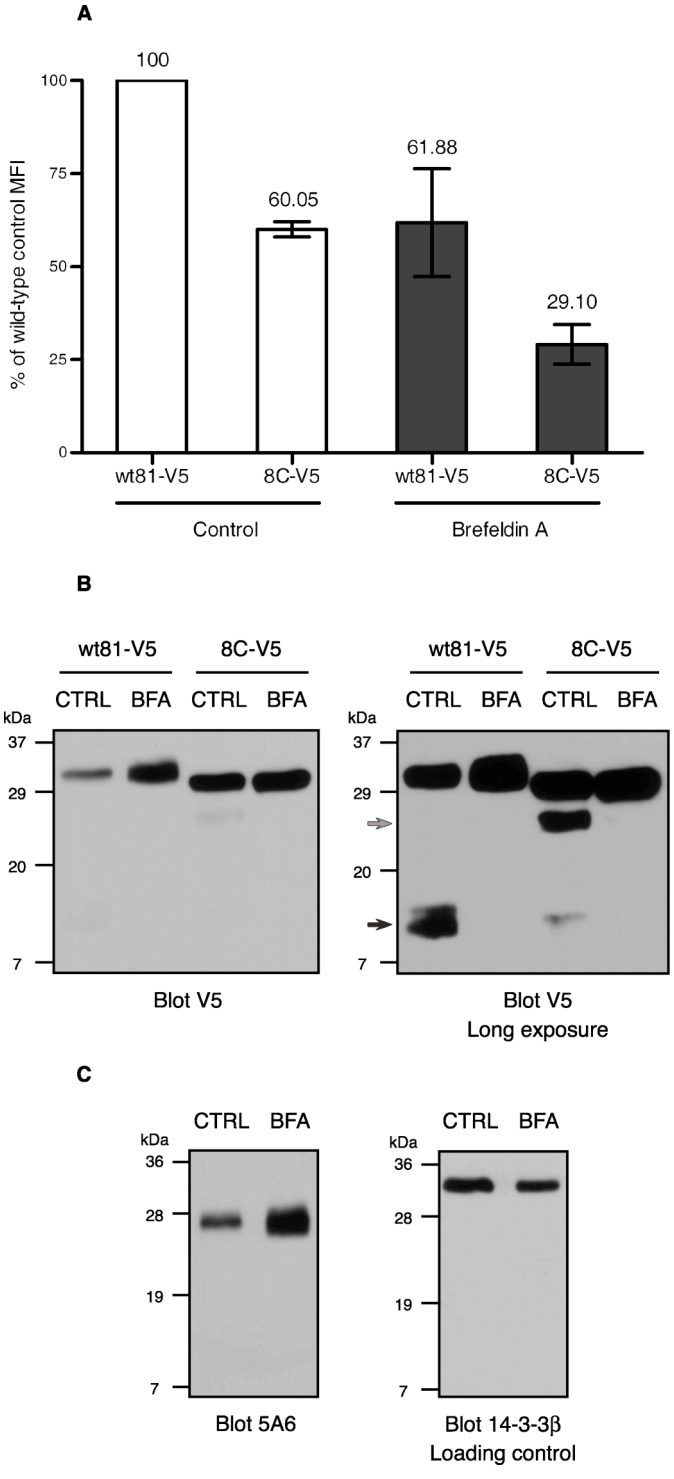

A
100
61.88
60.05
29.10
% of wild-type control MFI
100
75
50
25
0
wt81-V5
8C-V5
wt81-V5
8C-V5
Control
Brefeldin A
B
wt81-V5
8C-V5
CTRL
BFA
CTRL
BFA
kDa
37
29
20
7
Blot V5
wt81-V5
8C-V5
CTRL
BFA
CTRL
BFA
kDa
37
29
20
7
Blot V5
Long exposure
C
CTRL
BFA
kDa
36
28
19
7
Blot 5A6
CTRL
BFA
kDa
36
28
19
7
Blot 14-3-3β
Loading control

Figure 2.8 Effect of brefeldin A on wtCD81, CD81-8C, and endogenous CD81 protein processing

BHK cells transiently expressing V5-tagged wtCD81 or CD81-8C were treated with ethanol carrier (control cells) or brefeldin A (10 μg/ml) for 10 hours. Cells were analyzed by flow cytometry using αCD81 mAb 5A6 (*A*), or by αV5-antibody immunoblotting following Brij-99 lysis (*B*). In panel B, a longer exposure is presented (*right panel*) to show the CD81 processed forms (grey and black arrows). Human HT1080 cells were similarly treated with or without brefeldin A and endogenous human CD81 levels were analyzed by 5A6 immunoblotting (C). Levels of 14-3-3β were analyzed as a loading control. In panel A, flow cytometry percentages are means from 2 independent experiments normalized relative to wtCD81 control levels (100%) (± SEM). MFI, mean fluorescence intensity; CTRL, control; BFA, brefeldin A treatment.

	wtCD81	CD81-8C
palmitoylation levels	++	-
surface detection (flow cytometry)	++	+
surface localization (biotinylation) *	+	++
affinity to αCD81 5A6	=	=
total protein levels (immunoblot):		
➔ full-length	+	++
➔ 10-12 kDa	++	+
protein stability (half-life)	=	=
association with CD9	++	+
association with EWI-2	++	+

* only the full-length CD81 was determined

Figure 2.9 Summary of the results obtained for wtCD81 and CD81-8C

CHAPTER 3 - Characterization of Human and Mouse Tspan-2

Introduction

Tetraspanins are membrane proteins best defined by their structural features: four transmembrane domains, two extracellular loops of unequal size, and short cytoplasmic regions. Their function relies on the association with multiple proteins in a plasma membrane network called the tetraspanin web. The protein content of this network varies depending on the cell type, thus allowing tetraspanins to influence diverse cellular processes including B cell activation, tumor progression, viral infection, and bladder permeability. The tetraspanin web is thought to orchestrate associations among proteins to bring together proteins required for specific functions, thereby facilitating signaling pathways[86, 177]. Most, if not all, multicellular organisms express several tetraspanins. For example, 33 different tetraspanins are found in humans. However, less than half of human tetraspanins have been studied in detail. Included among these uncharacterized tetraspanins is Tspan-2.

There is only one previous major report focusing primarily on mammalian Tspan-2. Birling *et al.* discovered Tspan-2 in a subtractive cDNA library designed to find genes highly expressed in rat oligodendrocytes[185]. Northern blots showed that rat Tspan-2 expression was limited to the nervous system[185]. By using immunofluorescence, the authors confirmed that Tspan-2 is enriched in oligodendrocytes[185]. By extrapolating from known tetraspanin functions, it was speculated that Tspan-2 might participate in the communication between oligodendrocytes and the axons they surround[185]. Not surprisingly, CD9 was shown to precipitate Tspan-2 from rat brain tissue, suggesting that Tspan-2 might also belong to the tetraspanin web[186]. However, due to the lack of functional data, it remains too early to assign a particular role to Tspan-2. A different group did not find any Tspan-2 sequences in human EST libraries from nervous tissue[187]. However, because this study only included four ESTs, it is not possible to make any

meaningful conclusions. Indeed, at the time this dissertation was written, UniGene lists 10 human Tspan-2 ESTs in brain tissue, whereas none are found in the nerve pool. Finally, Tspan-2 was found in three different screens: two screens linked to tumor progression (breast and squamous cell cancers) and one searching for genes downstream of angiotensin II (a hormone linked to heart failure through its stimulation of cardiac remodeling)[188-190]. These screens indicate that human Tspan-2 is expressed outside of the nervous system, unlike the rat homolog, and hint to a possible role in tumor progression. Interestingly, several tetraspanins are known as metastasis suppressor genes, and they can influence cellular processes such as motility, adhesion, and proliferation—key events in the development of cancer[191]. Thus, Tspan-2 might have a similar role.

Here, we analyzed the expression of human and mouse Tspan-2, the N-glycosylation of human Tspan-2, and association with the tetraspanin CD81 in order to further characterize this relatively unknown tetraspanin.

Experimental Procedures

Cell lines, antibodies, and plasmids

Baby Hamster Kidney (BHK-21) cells were generously provided by Carol Blair from Colorado State University. Human embryonic kidney 293 cells were obtained from Scott C. Todd (Kansas State University). Cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). Anti-CD81 monoclonal antibody (5A6) was obtained from Scott C. Todd and its specificity for human CD81 has been described previously[178]. Anti-V5-HRP antibody was obtained from Invitrogen (Carlsbad, CA). Human V5-tagged human and mouse Tspan-2 constructs were generated by cloning the corresponding cDNAs into pcDNA^{TM}3.1/V5-His TOPO$^{®}$ vector (Invitrogen).

Site-directed mutagenesis and transfection

The construct N139Q was obtained by mutating asparagine 139 to a glutamine residue using human Tspan-2 as a template and the QuikChange$^{®}$ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The construct was sequenced to verify the substitution and to assure other nonspecific mutations did not occur.

BHK and 293 cells were transfected with different plasmids as indicated using Lipofectamine^{TM} 2000 (Invitrogen) according to the directions of the manufacturer.

Peptide: N-Glycosidase F treatment

Transfected cells were detached with 0.02% EDTA and treated with Glycoprotein Denaturing Buffer (0.5% SDS, 0.4 M DTT) diluted 1/10 in water at 100 ºC for 10 min. G7 Reaction Buffer (50 mM sodium phosphate), NP-40, and PNGase F (New England Biolabs, Ipswich, MA) were added, and samples were incubated overnight at 37 ºC (PNGase F was replaced with water in control samples). Tspan-2 expression was analyzed by immunoblotting as described below.

Immunoprecipitation

Cells were detached with 0.02% EDTA and lysed for one hour at 4 ºC in lysis buffer (150 mM NaCl, 20 mM Tris pH 7.5, 5 mM $MgCl_2$, 10 units/ml aprotinin, 1mM PMSF, and 1 μg/ml pepstatin A) containing 1% Brij-99 (Sigma-Aldrich). The insoluble material was removed by centrifugation at 21,000g for 15 min at 4 ºC. The antibody (5A6) was captured on Protein G-Sepharose® beads (Sigma-Aldrich) for one hour. After washing off the excess antibody, the lysates were added to the antibody-bead mixture and left rotating overnight at 4 ºC. Suspensions were centrifuged at 2,300g for 5 min at 4 ºC, the supernatants were collected, and the pellets were washed twice with lysis buffer containing 1% Brij-99, separated by SDS-PAGE under non-reducing conditions, and transferred to an Immobilon-P membrane (Millipore, Billerica, MA).

Immunoblotting

Proteins immobilized on PVDF membranes were blocked for one hour at 4 ºC in Tris-buffered saline with 0.1% Tween-20 (TBS-T) containing 5% non-fat milk. For V5 detection, anti-V5-HRP antibody was diluted at 1:5,000 in TBS-T containing 1% non-fat milk for 90 min at room temperature. All blots were visualized by applying SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology) and exposing to X-ray film.

Results and Discussion

Human and mouse Tspan-2 have different electrophoretic migration patterns as detected by immunoblotting

Sequence analysis of Tspan-2 shows that it is closely related to tetraspanins CD9 and CD81 (Fig. 3.1*A*). Amino acid identity percentages are of 39% and 46% for human Tspan-2/CD81 and Tspan-2/CD9, respectively. However, although CD9 and CD81 have been well studied, little is known about Tspan-2. To investigate the expression of human and mouse Tspan-2 in mammalian cell lines, we generated constructs for both of them having a V5 epitope tag at the C-terminus. Even though the sequences for human and mouse are very similar (Fig. 3.1*B*), we observed differences in the migration of the two proteins as seen by αV5 immunoblot (Fig. 3.2*A*). In particular, an additional lower band running around 27 kDa was observed for mTspan-2. The 38-kDa band observed for both proteins is likely to correspond to full-length Tspan-2. However, the expected size of Tspan-2 with the V5 tag is 28 kDa. Thus, we hypothesized that the 38-kDa proteins may be N-glycosylated forms of Tspan-2 since both human and mouse homologs have a conserved putative N-glycosylation site in the large extracellular loop (LEL; Fig. 3.1*B*). Interestingly, Birling *et al.* reported a single band of ~25 kDa for rat Tspan-2 by immunoblotting using a Tspan-2-specific antibody[185]. This antibody was generated against a peptide corresponding to a portion of the rat Tspan-2 LEL, located C-terminal to the CCG motif. The expected size of endogenous rat Tspan-2 is 24 kDa. Therefore, the difference in size between expected and experimental Tspan-2 was much smaller using the rat Tspan-2-specific antibody compared with our results. One possibility would be that this antibody only targets unglycosylated Tspan-2, and its epitope might be masked by the glycosylation of Tspan-2. Supporting this idea, we showed that removal of N-linked glycans form hTspan-2 increases its migration by at least 5 kDa (Fig. 3.3). However, this discrepancy might be due to a species-dependent difference in glycosylation patterns. Indeed, the 27-kDa band present only in the mTspan-2 lane migrates faster than unglycosylated full-length Tspan-2 (Fig. 3.3). Mutation of residues

that are different between human and mouse Tspan-2 might be helpful to resolve this question.

Differences in banding patterns on immunoblots also occurred when comparing expression between two cell lines: hamster fibroblasts (BHK) and human epithelial cells (293) (Fig. 3.2*B*). The major difference was the appearance of an additional band of ~26 kDa in both human and mouse proteins when transfected in 293 cells. Also, the migration of putative full-length Tspan-2 seemed slightly increased in 293 cells (see also Fig. 3.3 and 3.4). The migration of Tspan-2 remained unaltered under reducing conditions although there was a decrease in overall detection (Fig. 3.2*A*).

Human Tspan-2 is N-glycosylated at residue 139

The presence of two major bands in the case of mouse Tspan-2 led us to hypothesize that they may be glycosylated and unglycosylated forms of Tspan-2. Indeed, sequence analysis identified a putative N-glycosylation site at asparagine 139 in both human and mouse homologs (Fig. 3.1). Treatment with the glycosidase PNGase F resulted in a decrease of Tspan-2 protein size as detected by αV5 immunoblotting (Fig. 3.3*A*). However, the 27-kDa band of mTspan-2 seen in untreated cells ran slightly faster than unglycosylated full-length mTspan-2, and it was also PNGase F-sensitive, suggesting it is not unglycosylated mTspan-2 as first hypothesized. Mouse Tspan-2 might undergo a different type of maturation process in addition to N-glycosylation.

The location of the glycosylation site to asparagine 139 was confirmed by substitution to a glutamine (construct N139Q) in the human Tspan-2 protein. N139Q protein migrated at a similar level relative to unglycosylated hTspan-2 (~30 kDa; compare Fig. 3.3*A* and *B*). Interestingly, when expressed in 293 cells, the ~26 kDa band seen in wild-type hTspan-2 was not visible for the N139Q mutant, suggesting that glycosylation of hTspan-2 might affect protein processing.

Association of human Tspan-2 with CD81 is N-glycosylation-independent

To begin examining the participation of hTspan-2 in the tetraspanin web, we tested its association with human CD81 by co-immunoprecipitation assays. As expected, we found that hTspan-2 was able to bind CD81 under conditions that do not disrupt the tetraspanin web (Brij-99) (Fig. 3.4). Strikingly, no hTspan-2 was precipitated by CD81-

8C, a CD81 mutant protein lacking eight juxtamembrane cysteines—potential sites for palmitoylation (Fig. 3.4*A*). However, no hTspan-2 could be detected in the unbound fraction (supernatant). We think this is due to a low efficiency in Tspan-2 transfection; the small amount of Tspan-2 protein in cell lysates might be too dilute to be detected (only a small aliquot of the supernatant is used for immunoblotting). On the other hand, Tspan-2 protein in the immunoprecipitated samples is very concentrated and therefore, easier to detect. Repeating this experiment with more concentrated protein and/or more antibody will be necessary to confirm these results. Palmitoylation is an important post-translational modification for the assembly of the tetraspanin web. Our result is in agreement with previous reports showing a decrease in tetraspanin-tetraspanin associations when palmitoylation sites were mutated[168, 170, 172, 173]. Nevertheless, a complete inhibition of these associations has not been reported, emphasizing the necessity to repeat this experiment with appropriate controls.

Interestingly, binding of hTspan-2 to endogenous CD81 from 293 cells was not inhibited by a lack of N-glycosylation: N139Q was able to bind CD81 as efficiently as wild-type hTspan-2 (Fig. 3.4*B*). Additionally, we did not observe the lower ~ 26 kDa hTspan-2 band (usually present in 293 cell lysates) in the material precipitated by CD81 (Fig. 3.4*B*). To our knowledge, this is the first report that examined the role of glycosylation in a tetraspanin-tetraspanin association. Surprisingly, although most tetraspanins have putative N-glycosylation sites, few reports have been focused on the influence this post-translational modification can exert on tetraspanin function. CD82 N-glycosylation was reported to impair CD82 interaction with integrin α_5[162]. Our findings suggest that glycosylation of a tetraspanin may not be required for binding to other tetraspanins. Future experiments with other tetraspanins will confirm whether or not this property can be extrapolated to the rest of the superfamily. The tetraspanin LEL is not always necessary for tetraspanin-tetraspanin associations; instead, it appears that the transmembrane and cytoplasmic (via palmitoylation) domains can facilitate this binding[25]. This would explain why the addition of glycans in the LEL of Tspan-2 did not affect its binding to CD81. On the other side, the association between tetraspanins and specific binding partners relies more often on the proper structure of the LEL (as may be the case for the CD82/α_5 interaction). Thus, the presence of bulky carbohydrate groups

attached to the LEL is likely to influence these protein-protein interactions. Binding partners specific to Tspan-2 are yet to be discovered; nevertheless, this hypothesis can be tested by mutating putative N-glycosylation sites of well-known tetraspanins and examine how they interact with binding partners.

Conclusion

In summary, we showed that the migration of Tspan-2 on SDS-PAGE could vary depending on species (human vs. mouse Tspan-2) and cell type, suggesting changes in the maturation of Tspan-2. Interestingly, the electrophoretic migration of CD82—a tetraspanin with three N-glycosylation sites—also depends on the cell type studied[120]. This is thought to be due to a differential glycosylation of CD82. We confirmed that N-glycosylation of Tspan-2 occurs at a single site in the LEL: asparagine 139. More importantly, we found that the Tspan-2 association with another tetraspanin, CD81, appeared to be independent of Tspan-2 glycosylation status. Therefore, these findings provide a starting ground for a more complete characterization of Tspan-2.

Figures

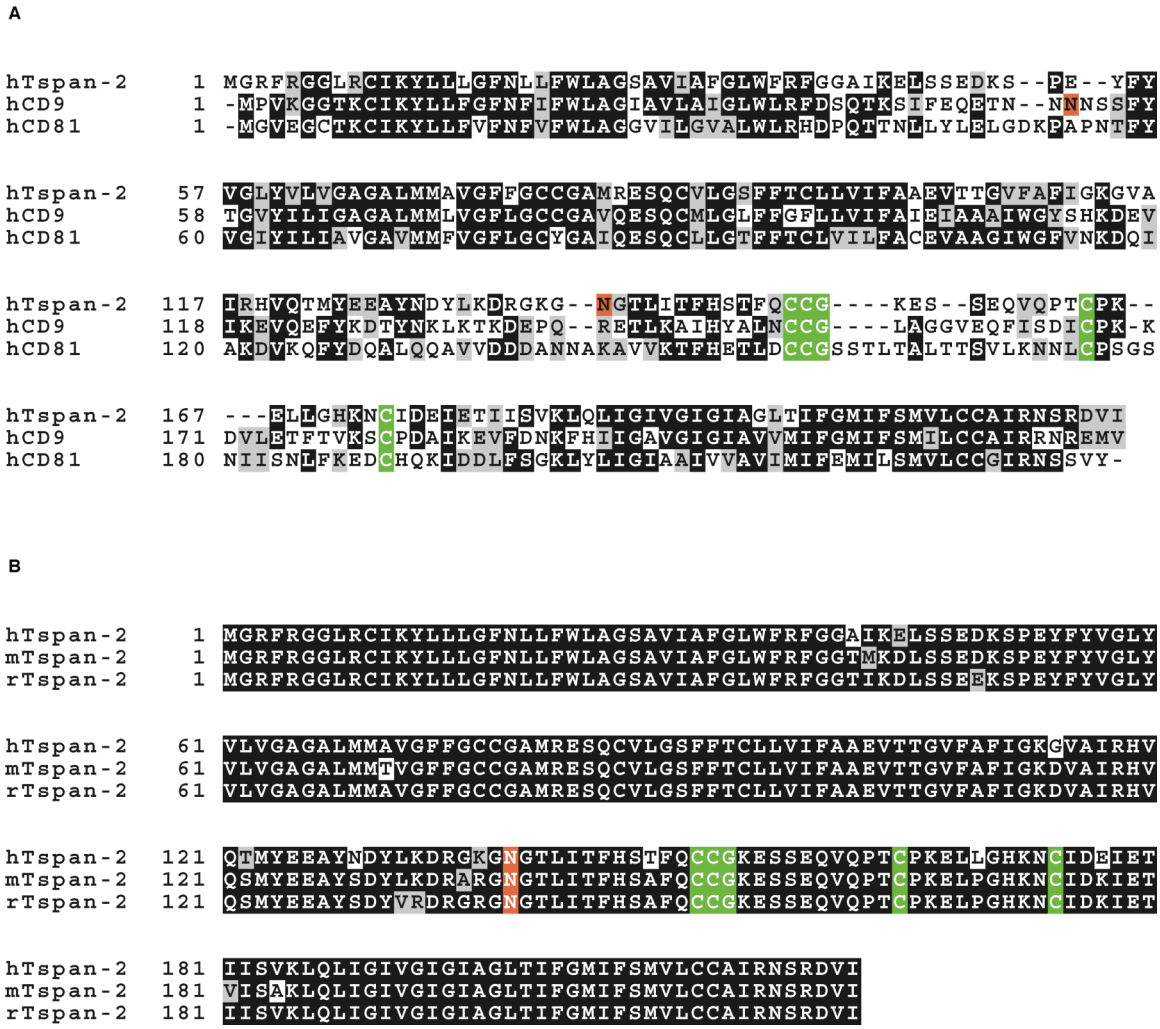


Figure 3.1 Sequence alignment of mammalian Tspan-2 proteins

The predicted amino acid sequence of human Tspan-2 was aligned with those of human CD9 and CD81 (*A*), or with mouse and rat Tspan-2 (*B*). Alignments were obtained by using ClustalW and BoxShade (www.ch.embnet.org). Grey and black boxes correspond to similar and identical amino acids, respectively. Green boxes indicate the conserved cysteine residues that form disulfide bonds in the large extracellular loop. The orange box represents a putative N-glycosylation site.

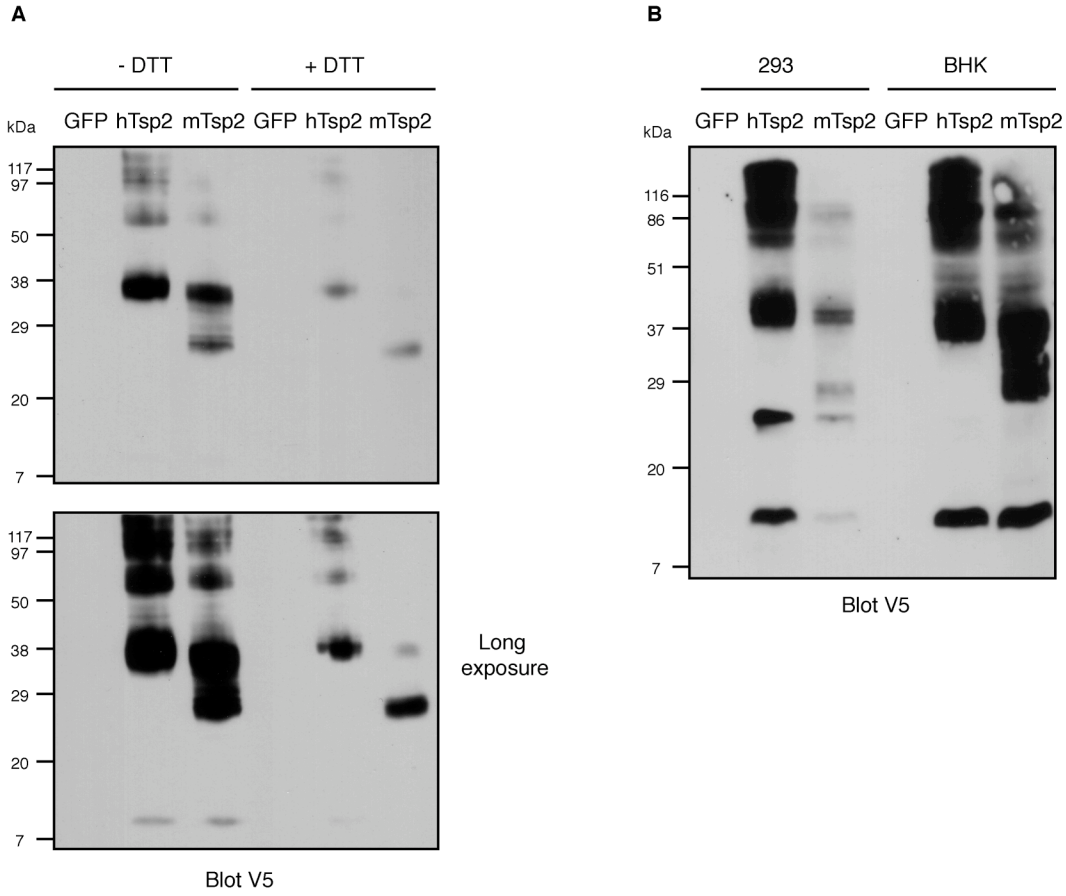


Figure 3.2 Expression of human and mouse Tspan-2

BHK cells transfected with control GFP, human Tspan-2 (hTsp2), or mouse Tspan-2 (mTsp2) were lysed with 1% Triton X-100 and analyzed under non-reducing (- DTT) and reducing (+ DTT) conditions for Tspan-2 expression by αV5 immunoblotting (*A*). Human and mouse Tspan-2 were expressed in BHK and 293 cells and analyzed as in panel *A* (non-reducing conditions) to determine differences in expression patterns between the two cell lines (*B*). In panel *A*, a longer exposure is shown below.

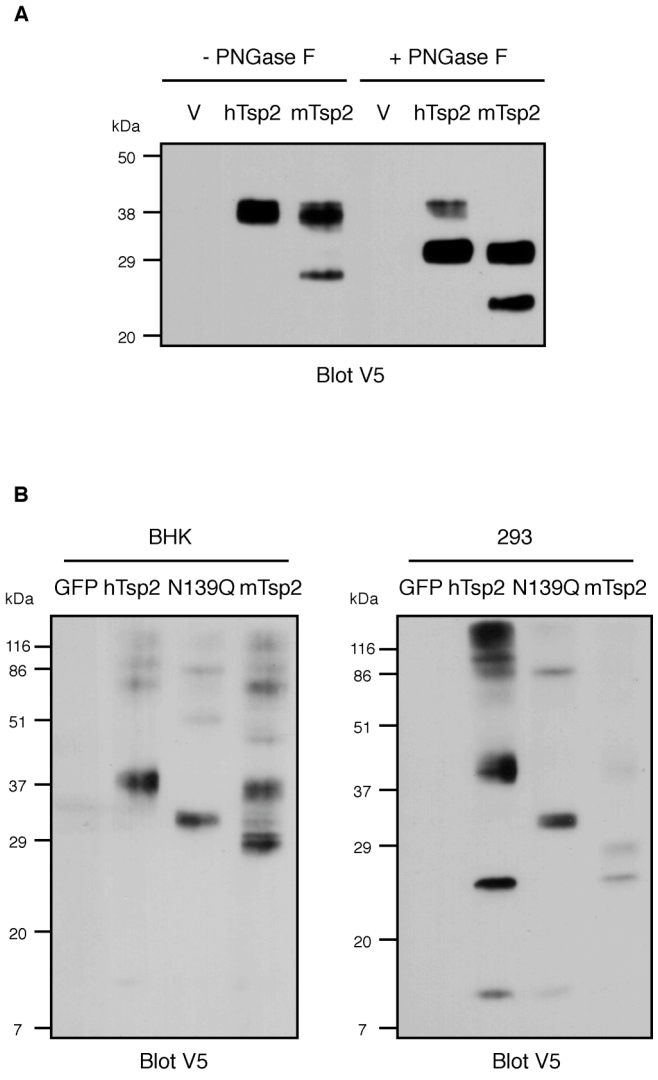


Figure 3.3 N-glycosylation of human and mouse Tspan-2

BHK cells transfected with human Tspan-2 (hTsp2), mouse Tspan-2 (mTsp2), or vector control (V) were treated with PNGase F and analyzed for Tspan-2 expression by αV5 immunoblotting (*A*). The expression pattern of the N139Q protein was compared to hTsp2 and mTsp2 in BHK and 293 cells lysed with 1% Brij-99 by αV5 immunoblotting (*B*).

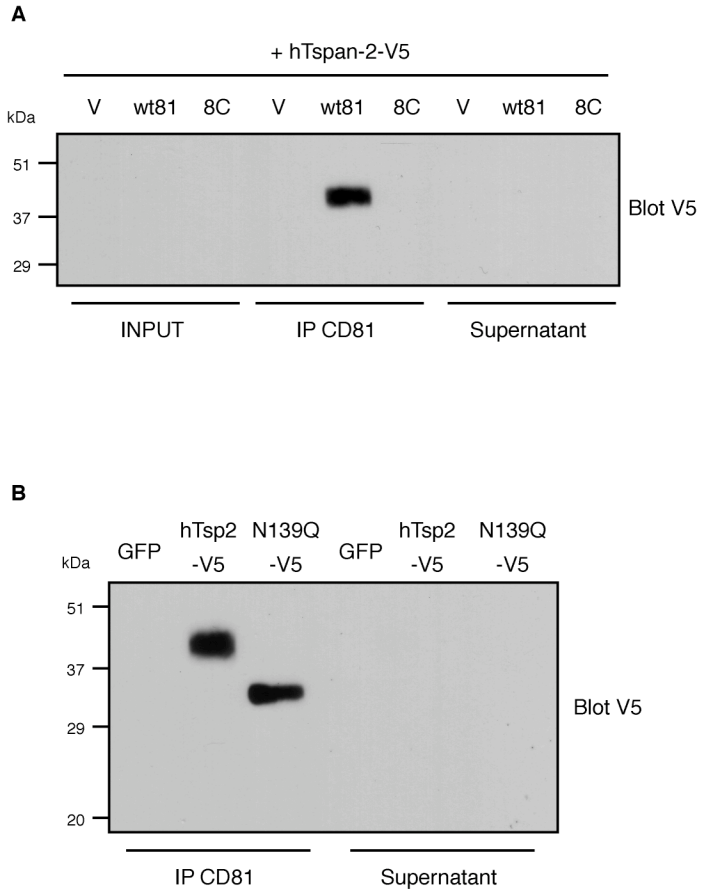


Figure 3.4 Association of human Tspan-2 and N139Q with CD81

BHK cells were co-transfected with human Tspan-2 (hTsp2) and vector (V), wild-type CD81 (wt81), or CD81-8C (8C; having eight juxtamembrane cysteines mutated), and lysed with 1% Brij-99. CD81 was precipitated using αCD81 mAb 5A6 and associated Tspan-2 was detected by αV5 immunoblot (*A*). Input and supernatant samples represent aliquots of pre-immunoprecipitation lysates and post-immunoprecipitation unbound proteins, respectively. 293 cells were transfected with GFP, hTsp2, and N139Q, lysed with 1% Brij-99, and endogenous human CD81 was precipitated using 5A6. Samples were then analyzed for Tspan-2 detection as described in *A* (*B*).

CHAPTER 4 - Conclusion

Almost two decades of continuing efforts have led to a clearer picture explaining how the tetraspanin web is organized. The field has reached the point where it can tackle the complex and exciting challenge of viewing the tetraspanin web as a highly dynamic network, and study how it can be regulated. Being able to regulate the tetraspanin web will become a powerful tool since it would have many implications as suggested by the numerous cellular functions that tetraspanins can influence. Recently, palmitoylation has emerged as a major regulatory mechanism for tetraspanins. In addition, most tetraspanins can be glycosylated, thereby providing another method to change the molecular properties of tetraspanins. In chapter two, we described the characterization of a set of CD81 mutant proteins with mutations in five, six, seven, or eight juxtamembrane cysteines—potential palmitoylation sites. Overall, the various effects observed with these mutant proteins are likely the result of a redistribution of the protein environment surrounding CD81. These changes in protein associations are consistent with previous findings from other groups. In particular, tetraspanin-tetraspanin associations are impaired when juxtamembrane cysteines are mutated. These interactions are crucial for the formation of the tetraspanin web. However, many questions remain to be answered. Interestingly, there seems to be two types of palmitoylation—constitutive and inducible—as seen with CD81 function in the B cell coreceptor. Is it possible to switch tetraspanins on or off depending on their palmitoylation levels? What is the difference between constitutive and inducible palmitoylation? Most of the studies on tetraspanin palmitoylation have used site-directed mutagenesis to substitute juxtamembrane cysteines, thus only examining constitutive palmitoylation. To investigate inducible palmitoylation, specific inhibitors—mainly 2-bromopalmitate (2-BP)—have been used. However, they present several drawbacks. First, these chemicals inhibit non-specifically the palmitoylation of all cellular proteins. Therefore, it is harder to make definite conclusions on the regulation of the protein of interest. The effect of CD81 palmitoylation in the microdomain stability of the B cell coreceptor was concluded

because the lipid raft distribution of the B cell coreceptor regulated by CD81 and an increase in CD81 palmitoylation both occur upon coligation of the BCR with the B cell coreceptor and are inhibited by 2-BP. However, these results do not exclude the possibility that palmitoylation of additional proteins are important on the B cell coreceptor localization to lipid rafts. Second, 2-BP has been reported to have other effects aside form inhibiting protein palmitoylation[192]. Intriguingly, 2-BP treatment did not impair tetraspanin-tetraspanin interactions like palmitoylation-deficient tetraspanins. It was proposed that palmitoylation facilitates the early events of tetraspanin web assembly, and once it is established, palmitoylation is no longer required. Thus, results obtained from experiments using such inhibitors should be taken with a grain of salt.

An additional tool to study tetraspanin palmitoylation has been provided by the discovery of DHHC2 as a palmitoyl acyl transferase able to palmitoylated CD9 and CD151. More importantly, this enzyme seems specific to CD9 and CD151. Thus, it is possible to regulate the palmitoylation of these tetraspanins by overexpressing or downregulating DHHC2 expression levels. In this way, palmitoylation of CD9 and CD151 was found to be important for binding to other tetraspanins, thereby confirming the results obtained by site-directed mutagenesis. Does DHHC2 palmitoylate other tetraspanins? Are there additional palmitoyl acyl transferases responsible for tetraspanin palmitoylation? As more knowledge is gained in the field of enzymatic palmitoylation, more tools will become available to artificially disrupt the tetraspanin web and study the functional consequences. In addition, it would be interesting to identify enzymes responsible for removing palmitate from tetraspanins (palmitoyl thioesterases), and which pathways act to control the activity of such enzymes. Therefore, each approach used to investigate tetraspanin palmitoylation has its own advantages and it is the combination of all these methods that will make it possible to finally understand how the tetraspanin web dynamically orchestrates plasma membrane proteins.

Glycosylation of tetraspanins, on the other hand, has received little attention. However, the presence of multiple carbohydrates on the LEL of a tetraspanin is likely to alter its molecular interactions. More importantly, although tetraspanins share a similar structure, N-glycosylation sites do not appear to be conserved among members of the superfamily, hinting toward a possible way the cell can distinguish one tetraspanin from

another. This is illustrated by the differences in the glycans attached to tetraspanin uroplakins UP Ia and Ib, which explain why only UP Ia serves as a receptor for *Escherichia coli* infection. Similar studies need to be done on other tetraspanins. Interestingly, glycosylation of CD82 is thought to vary depending on cell type. This might provide tetraspanins a way to play different tissue-specific roles. Another question is the effect of glycosylation on protein interactions within the tetraspanin web. Does glycosylation influence tetraspanin primary, secondary, or all types of interactions? Our results presented in chapter three indicate that tetraspanin-tetraspanin associations remain unchanged. However, this needs to be confirmed by future studies.

As is often the case in science, new discoveries seem to lead to more questions than answers. Tetraspanin palmitoylation is a good example. The excitement brought by the possibility that tetraspanins could be regulated post-translationally pointed the field toward an unexpected path filled with new obstacles. More importantly, it is the power of collaboration that advances scientific knowledge by bringing different perspectives together—such as using palmitoylation-deficient proteins, treating with inhibitors, or altering DHHC2 expression to understand the role of palmitoylation. Therefore, it has been a privilege for me to add this humble contribution to the field of tetraspanins. My results represent a small puzzle piece that I hope will help in elucidating how tetraspanins undertake the incredible challenge of organizing the plasma membrane.

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