A Novel Cysteine Cross-linking Method Reveals a Direct Association between Claudin-1 and Tetraspanin CD9⋯

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Tetraspanins serve as molecular organizers of multiprotein microdomains in cell membranes. Hence to understand functions of tetraspanin proteins, it is critical to identify laterally interacting partner proteins. Here we used a novel technical approach involving exposure and cross-linking of membrane-proximal cysteines coupled with LC-MS/MS protein identification. In this manner we identified nine potential tetraspanin CD9 partners, including claudin-1. Chemical cross-linking yielded a CD9-claudin-1 heterodimer, thus confirming direct association and adding claudin-1 to the short list of proteins that can directly associate with CD9. Interaction of CD9 (and other tetraspanins) with claudin-1 was supported by subcellular colocalization and was confirmed in multiple cell lines, although other claudins (claudin-2, -3, -4, -5, and -7) associated to a much lesser extent. Moreover claudin-1 was distributed very similarly to CD9 in sucrose gradients and, like CD9, was released from A431 and A549 cells upon cholesterol depletion. These biochemical features of claudin-1 are characteristic of tetraspanin microdomain proteins. Although claudins are major structural components of intercellular tight junctions, CD9-claudin-1 complexes did not reside in tight junctions, and depletion of key tetraspanins (CD9 and CD151) by small interfering RNA had no effect on paracellular permeability. However, tetraspanin depletion did cause a marked decrease in the stability of newly synthesized claudin-1. In conclusion, these results (a) validate a technical approach that appears to be particularly well suited for identifying protein partners directly associated with tetraspanins or with other proteins that contain membrane-proximal cysteines and (b) provide insight into how non-junctional claudins may be regulated in the context of tetraspanin-enriched microdomains. Molecular & Cellular Proteomics 6: 1855–1867, 2007.

Tetraspanin proteins regulate cell fusion, invasion, migration, and differentiation thereby affecting a variety of physiological processes in the brain, eye, skin, immune system, developing embryo, blood vessels, tumor cells, and elsewhere (1–5). Genetic evidence points to critical roles for tetraspanins in mammals, insects, worms, and fungi (4, 6–11). Although tetraspanins are transmembrane proteins that typically reside on the cell surface, they do not generally function as ligands or receptors. Rather they assemble with themselves and other proteins, together with gangliosides and cholesterol, to form tetraspanin-enriched microdomains (TEMs) (4, 5, 12, 13). Hence to understand how tetraspanin proteins function, it is necessary to identify their partner proteins. Partner proteins for tetraspanins include integrins, Ig superfamily proteins, growth factors and their receptors, G-protein coupled receptors, signaling enzymes, and various other molecules (4, 14–16). However, for many of the 33 mammalian tetraspanins, no partner proteins have been yet identified. A recent strategy for discovering partner proteins has been to lyse cells in relatively mild detergent (e.g. Brij 96/97), collect tetraspanin complexes, fractionate tetraspanin-associated proteins by SDS-PAGE, and then to identify them using nanoscale LC-MS/MS (17–20). Although this approach can be quite effective, the use of mild detergents increases the number of nonspecific and indirectly associated proteins that are identified.

Covalent chemical cross-linking has been a useful tool for demonstrating direct tetraspanin protein-protein interactions. For example, it has been used to demonstrate direct associations of tetraspanins UP1a, UP1b, CD151, CD9, and CD81 with respective partner proteins UPII, UPIII, α3β1 integrin, EWI-F, and EWI-2 (18, 21–23). For CD9 and other tetraspanin partners, inhibition of protein palmitoylation leads to exposure of membrane-proximal cysteines, which then can be cross-linked (24). In this manner, CD9 was shown to form homodimers, -trimers, and -tetramers (24–26). Nearly all tetraspanins undergo palmitoylation and contain multiple intracellular cysteine palmitoylation sites with proximity to...
transmembrane domains 1, 2, 3, and/or 4 (27–30). Furthermore, many tetraspanin partner proteins are also palmitoylated. For example CD9 partners (CD36, n3 and n6 integrins, EWI-2, and EWI-F) all contain membrane-proximal cysteines and are known to undergo palmitoylation (31, 32). Palmitoylation of tetraspanins and their partners helps to stabilize tetraspanin-enriched microdomains (27–29, 32). To identify more efficiently the proteins that directly associate with tetraspanins, we developed a novel strategy that involves (i) partial inhibition of protein palmitoylation to expose membrane-proximal cysteines, (ii) covalent cross-linking of exposed cysteines, (iii) cell lysis and immunosolation of tetraspanin complexes, and (iv) protein identification by LC-MS/MS. Using this approach, we discovered a direct protein–protein interaction between tetraspanin CD9 and claudin-1.

Tetraspanin CD9 plays a major role during sperm–egg fusion (33–35), other types of cell–cell fusion (36–39), cell migration, and tumor suppression (40, 41). It also affects paracellular junction formation in the peripheral nervous system (42) and signaling by membrane-bound agonists for the epidermal growth factor (EGF) receptor (43–45). In this regard, CD9 not only associates directly with Ig superfamily proteins (EWI-2 and EWI-F) but also may directly contact membrane-bound growth factor HB-EGF (46). Several new CD9 partners have been identified recently (20) but were not shown to associate directly. Like tetraspanins, claudins also contain four transmembrane domains, but the sequences and functions of these two families of proteins are quite distinct. Claudins, including claudin-1, are key components of epithelial and endothelial tight junctions (TJs), which act as a barrier to paracellular flux of water and solutes and transmigration of other cells (47, 48). Within tight junctions, claudins on apposing cells form homophilic and heterophilic complexes (49). Claudins also associate with other TJ proteins, including occludin and ZO-1 (50). Like tetraspanins, claudins possess juxtamembrane cysteine residues, and claudin-14 was shown to undergo palmitoylation, which is required for proper TJ localization and barrier function (51). In this study, we demonstrate that tetraspanins (such as CD9) can associate with and stabilize the expression of claudins when they are not in tight junctions.

EXPERIMENTAL PROCEDURES

Cell Culture—Cell lines used were human A431 (epidermoid carcinoma), A549 (lung carcinoma), MDA-MB-231 and MCF7 (breast adenocarcinoma), C2BBe1 (a derivative of Caco-2 colorectal adenocarcinoma), and Madin-Darby canine kidney (MDCK) cells. Cells were obtained from ATCC (Manassas, VA) and then were grown in DMEM supplemented with 10% fetal bovine serum, 20 mM HEPES, 2 mM L-glutamine, and antibiotics (all from Invitrogen). For calcium depletion experiments, C2BBe1 cells were incubated overnight in calcium-free minimum essential medium modified for suspension (S-MEM) with 10% dialyzed fetal bovine serum (Invitrogen), and then 1 mM EGTA was added for 45 min.

Antibodies, Protein Immunoprecipitation, Solubility, and Cross-linking—Polyclonal and monoclonal antibodies against C-terminal intracellular tails of human claudin-1, -2, -3, -4, -5, and -7; occludin; and ZO-1 proteins were from Zymed Laboratories Inc. (currently Invitrogen). Monoclonal anti-tetraspanin antibodies (CD9, Alb6 and MM2/57; CD81, M38; and CD151, 5C11 and 1A5) were referenced previously (32). Other antibodies used were anti-CD71 (polyclonal antibody C-20, Santa Cruz Biotechnology), anti-CD98 (mAb 4F2), anti-caveolin-1 (mAb C060), anti-E-cadherin (mAb clone 36), anti-β-catenin (mAb clone 14, all from BD Biosciences), anti-β-actin (mAb clone AC-15, Sigma), and anti-β1 integrin (polyclonal antibody, Chemicon). For immunoprecipitation, cells were washed twice with HBSE buffer (20 mM HEPES-NaOH, pH 7.2, 150 mM NaCl, 1 mM EDTA) and lysed in HBSE buffer containing either 1% Brij 96, 1% Triton X-100, or RIPA (1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) supplemented with Complete protease inhibitor mixture (Roche Diagnostics) for 1 h at 4 °C. Cell lysate was centrifuged (14,000 × g for 20 min), precleared with protein G-agarose beads containing specific antibodies (0.5–2 μg/ml), and then immune complexes were collected on protein G-agarose beads (1–12 h). For protein solubility experiments, cell lysate were lysed in 1% Brij 96, 1% Triton X-100, or RIPA as described above. Centrifugation (14,000 × g for 1 h) yielded the “detergent-soluble” supernatant. The insoluble pellet was rinsed twice with lysis buffer and then incubated in SDS-PAGE loading buffer containing 1% SDS with repeated heating and vortexing for 20–30 min. Additional centrifugation yielded supernatant designated as the “detergent-insoluble” fraction. For chemical cross-linking, A431 cells were treated with 50 μM 2-bromopalmitate (2-BP) for 18–20 h and then incubated on ice with cysteine cross-linker (0.2 mg/ml diethylmaleimidoethane (DTME)) for 45 min as described previously (52). Cells were then processed for immunoprecipitation as described above.

Cholesterol Depletion and Protein Release—As described previously (53), cells were incubated (45–60 min at 37 °C) in serum-free DMEM containing 10 mM methyl-β-cyclodextrin (MβCD; Sigma). Then, cell-free medium was either precipitated using 10% trichloroacetic acid (to yield total released proteins) or supplemented with 1% Brij 96, protease inhibitors, and 1 mM EDTA (for subsequent immunoprecipitation experiments). MβCD-treated cells were lysed in HBSE buffer containing 1% Brij 96, and proteins were immunoprecipitated as described above.

Purification of Tetraspanin Complexes and Mass Spectrometry—To prepare CD9 affinity beads, mAb Alb6 (200 μg) was incubated with 250 μl of protein G-agarose beads in PBS followed by covalent coupling with dimethyl pimelimidate (Sigma). A431 cells (2 × 10⁶) were incubated with 2-BP (to expose cysteines) and then DTME (to cross-link cysteines) as mentioned above and then lysed in HBSE buffer containing 1% Triton X-100 and protease inhibitors. The lysate was centrifuged at 14,000 × g for 30 min and then precleared successively (each at 4 °C for 1 h) with Sepharose 6B beads (one time), protein G-agarose beads (three times, 150 μl of beads each time), and mouse IgG-agarose beads (three times, 150 μl of beads each time). Lysate was then incubated with CD9 affinity beads for 2 h at 4 °C followed by extensive washing of beads (in minicolumns) with lysis buffer (at 25 °C). Bound proteins were eluted with 100 mM glycine, pH 2.4, and concentrated using TCA precipitation. Eluted proteins were analyzed by SDS-PAGE in the presence of a reducing agent to cleave the disulfide bond in the cross-linker. The 15–35-kDa region of the gel, containing ~10 Coomasie Blue stained-bands, was excised and submitted to the Taplin Biological Mass Spectrometry Facility (Harvard Medical School). A similar protocol was used to isolate uncross-linked CD9 and CD151 protein complexes except that 2-BP and DTME treatments were omitted, and 1% Brij 96 was omitted.

2 O. V. Kovalenko, T. V. Kolesnikova, X. H. Yang, and M. E. Hemler, unpublished observations.
Claudin-1 Associated with Tetraspanin CD9

Many proteins have been suggested to associate with CD9 (4, 20), but only two (EVI-2 and EVI-F/CD9-P1) have been shown definitively to associate directly with CD9 (18, 23). Results in Tables I and II (row I) suggested the possibility that CD9 could associate directly with claudin-1. To confirm this, we performed chemical cross-linking experiments to capture CD9-claudin-1 complexes. As in Table II (row I), A431 cells were pretreated with 2-BP to expose membrane-proximal cysteines (present in

RESULTS

Identification of Claudins as Tetraspanin Partners—Human A431 epidermoid carcinoma cells were pretreated with 2-bromopalmitate to expose membrane proximal cysteines, and then intact cells were incubated with the homobifunctional cysteine cross-linking agent DTME. Hence CD9 complexes with partner proteins were stabilized, enabling the use of stringent lysis conditions (1% Triton X-100). CD9 complexes were then resolved by SDS-PAGE under reducing conditions to cleave the DTME cross-linker and separate CD9 from its partners. A total of nine membrane-associated or membrane-spanning proteins were identified, besides CD9 itself, using LC-MS/MS analysis of tryptic peptides (Table I). Among the proteins identified, most are known to undergo palmitoylation or to express membrane-proximal cysteines that could potentially be palmitoylated (see Table I legend). CD9 association with claudin-1 was confirmed and studied further (see below). CD9 association with α6 integrins, CD44, Tspan-14, and MHC class I was documented previously (67, 58). For the remaining proteins, proximity to CD9, extent of association, and functional implications will be evaluated more fully elsewhere.

CD9 association with claudin-1 (Table II, row I) was confirmed in additional mass spectrometry experiments not involving covalent cross-linking. In each experiment, cells were lysed in less stringent detergent conditions (1% Brij 96), CD9 complexes were isolated using beads coated with anti-CD9 antibody, CD9-associated proteins were resolved by non-reducing SDS-PAGE, and then proteins in the 15–35-kDa range were identified by mass spectrometry. In this manner, claudin-1 was again identified as a CD9 partner in A431 cells (Table II, row II) and in MCF10A cells (Table II, row III). In a similar experiment, tetraspanin CD151 complexes were isolated from 1% Brij 96 lysates of human breast carcinoma cells (MDA-MB-231 and MCF10A), leading to identification of claudin-1 (Table II, row IV). Repeated detection of claudin-1 in tetraspanin protein complexes prompted us to address further the specificity and significance of this association with particular attention to CD9-claudin-1 interaction.

CD9–Claudin-1 Association Is Direct—Many proteins have been suggested to associate with CD9 (4, 20), but only two (EVI-2 and EVI-F/CD9-P1) have been shown definitively to associate directly with CD9 (18, 23). Results in Tables I and II (row I) suggested the possibility that CD9 could associate directly with claudin-1. To confirm this, we performed chemical cross-linking experiments to capture CD9-claudin-1 complexes. As in Table II (row I), A431 cells were pretreated with 2-BP to expose membrane-proximal cysteines (present in

used for lysis and subsequent bead washes. The mAb SC1, coupled to beads, was used for isolation of CD151 complexes. For protein identification, excised SDS-polyacrylamide gel bands were chopped into 1-mm³ pieces, and in-gel digestion with trypsin was performed as described previously (54).

Mass spectrometry data were acquired by nanoscale microcapillary liquid chromatography coupled to a linear ion trap mass spectrometer (ThermoElectron, San Jose, CA) as described previously (55). Peak lists were created as .dta files using the ExtractMass program from Bioworks Version 3.2. Database searching was carried out using the program TurboSEQUEST Version 27 (revision 12). Data (3,360 MS/MS spectra) were searched using no enzyme specificity and filtered to select for only peptides with two tryptic termini with XCorr values of greater than 1.5 for +1 and +2 charged peptides and 3.0 for +3 charged peptides. These parameters resulted in an estimated false positive rate of <1% based on the target-decoy database searching strategy (56). In addition, all spectra corresponding to proteins with less than three peptides/protein were manually examined for correctness. For simplification, proteins identified by less than one peptide/protein were not included in the final list, which removed all known (reversed sequence) false positive identifications. Protein entries are from the Advanced Biomedical Computing Center (ABCC) Non-Redundant Protein Database available at ftp.ncifcrf.gov/pub/nonredund/protein, nrdb.README. This database was filtered to contain only human entries. At the time of the searches there were 250,755 entries in the database. Protein matches from the database were examined with respect to redundancy to ensure correct identification. For example, among the 10 proteins listed in Table I, the two proteins listed in Table II, and the multiple peptides listed for each protein, manual inspection revealed that there are no redundancies.

Sucrose Gradient Centrifugation—A431 cells were lysed in HBSE buffer containing 1% Brij 96, and then 2 ml of lysate, adjusted to contain 45% (w/w) sucrose, was placed on the bottom of an ultracentrifuge tube and overlaid with 2 ml of 35% sucrose and 1 ml of 5% sucrose gradient. The tube was centrifuged for 18 h at 45,000 rpm in a Beckman T55 rotor. Twelve fractions (0.4 ml) were collected from the top of the gradient and analyzed by SDS-PAGE and immunoblotting.

siRNA Transfection and Pulse-Chase Labeling—siRNA duplex oligonucleotides (Dharmacon, Chicago, IL) were used to target human CD9 (CCAGAAGAGCTGACTGTTG) and CD151 (TCCAGAGCTG- GGCGAGAC). Control siRNA, targeting p21 protein of human immunodeficiency virus, was kindly provided by Dr. Carl Novina (Dana-Farber Cancer Institute, Boston, MA). A431 cells were transfected with siRNAs using Lipofectamine 2000 reagent (Invitrogen) and re- plated 2 and 4 days post-transfection. At day 6, cells (~80–90% confluent) were analyzed for protein knockdown efficiency by Western blotting and for pulse-chase labeling. For the latter, cells were incubated (1 h) in methionine- and cysteine-free DMEM, labeled (2 h) in the same medium supplemented with 5% dialyzed fetal bovine serum and 0.4 mCi/ml [35S]methionine/cysteine mixture (PerkinElmer Life Sciences) and chased with complete growth medium supplemented with 10% FBS and 1 mM L-cysteine for 3–9 h. Cells were lysed in buffer containing 1% Triton X-100 and analyzed by immunoprecipitation as described above. Proteins were resolved by SDS-PAGE, transferred to PVDF membrane (Millipore, Bedford, MA), and exposed to Eastman Kodak Co. Biomax MR film at −80 °C with an intensifying screen. The membrane was subsequently probed with specific antibodies (e.g. to claudin-1, claudin-2, and caveolin-1) to assess protein loading. Band intensities from autoradiography and Western blotting were analyzed using GeneTools software from Syngene (Frederick, MD).

Immunofluorescent Staining—Monolayers of A431 cells were fixed in 4% (w/v) paraformaldehyde (25 °C for 15 min), permeabilized with 0.25% Triton X-100 for 15 min, and blocked with 1% BSA for 1 h. Antibodies to CD9 (mAb MM2/57) and claudin-1 (rabbit polyclonal) were added (5 μg/ml for 1 h), and then secondary antibodies (Alexa-488-conjugated goat anti-mouse and Alexa-549-conjugated goat anti-rabbit; Molecular Probes, Eugene, OR) were added (2 μg/ml for 45 min at 25 °C). Slides were mounted in n-propyl gallate:glycerol (1:1) and analyzed by a Nikon Eclipse TE300 fluorescence microscope.

Monolayers of A431 cells were fixed in 4% (w/v) paraformaldehyde (25 °C for 15 min), and permeabilized with 1 mM L-Met and 1 mM L-Cys for 3–9 h. Cells were lysed in buffer containing 1% Triton X-100 and analyzed by immunoprecipitation as described above. Proteins were resolved by SDS-PAGE, transferred to PVDF membrane (Millipore, Bedford, MA), and exposed to Eastman Kodak Co. Biomax MR film at −80 °C with an intensifying screen. The membrane was subsequently probed with specific antibodies (e.g. to claudin-1, claudin-2, and caveolin-1) to assess protein loading. Band intensities from autoradiography and Western blotting were analyzed using GeneTools software from Syngene (Frederick, MD).
Both CD9 and Claudin-1, intact cells were incubated with 2-bromopalmitate and cross-linked, and CD9-associated proteins were identified as described under “Experimental Procedures.” A total of 45 proteins were identified by LC-MS/MS based on data from two or more distinct peptides/protein. Listed are all proteins obtained (and Swiss-Prot accession numbers) that are either membrane-spanning or membrane-associated.

| Name (accession number) | Peptides | Description |
|-------------------------|----------|-------------|
| CD9 (P21926)            | 5        | Tetraspanin CD9 (p24, MRp-1, Tspan-29) |
| VAMP3 (Q15836)          | 4        | VAMP3 (Synaptobrevin-3, cellubrevin, CEB) |
| TF (P13726)             | 3        | Tissue factor (TF, coagulation factor III, thromboplastin, CD142 antigen) |
| CLD1 (O95832)           | 2        | Claudin-1 (SEMP1) |
| 1B07 (PO1889)           | 2        | MHC class I antigen B7 (HLA-B) |
| ITA6 (P23229)           | 2        | Integrin a6 (VLA-6, CD49f) |
| 1A01 (P30443)           | 2        | MHC class I antigen A1 (HLA-A) |
| Q9BOE6 (Q9BOE6)         | 2        | Q9BOE6 (MGC2477 protein) |
| TSN14 (Q8NG11)          | 2        | Claudin-1 (Tspan-14, DC-TM4F2) |
| CD44 (P16070)           | 2        | CD44 antigen (PGP-1, ECMR-III, GP90 lymphocyte homing receptor, hyaluronate receptor, heparan sulfate proteoglycan) |

Additional information regarding peptides and protein identification is in Supplemental Table 1.

Peptides obtained from Claudin-1 represent either 7.1% (for one peptide) or 14.7% (for two peptides) of sequence coverage.

| Cell line | Tetraspanin “bait” | Detergent | Claudin | Claudin peptide(s) |
|-----------|--------------------|-----------|---------|--------------------|
| I         | A431               | CD9       | Triton X-100 | Claudin-1 (a) K ↓ VFSLLNLSTIQATR ↓ A |
| II        | A431               | CD9       | Brij 96   | Claudin-1 (b) R ↓ IVQEFDPMPVRNAR ↓ Y |
| III       | MCF10A             | CD9       | Brij 96   | Claudin-1 (c) K ↓ VFSLLNLSTIQATR ↓ A |
| IV        | MCF10A             | CD151     | Brij 96   | Claudin-1 (d) R ↓ IVQEFDPMPVRNAR ↓ Y |

As expected, cross-linked products were much less obvious if 2-BP pretreatment was omitted (middle panel, lane 1). Again reducing conditions caused Claudin-1 to appear mostly as a monomer (bottom panel, lanes 1 and 2).

To identify more definitively the multimeric bands seen in Fig. 1A, A431 cells were treated with 2-BP and DTME and lysed in Triton X-100, and then reciprocal CD9 and Claudin-1 immunoprecipitations were carried out. Careful alignment of non-reduced multimers (Fig. 1B) revealed bands of identical size in both lane 2 (Claudin-1 immunoprecipitation (IP) → CD9 blot) and lane 3 (CD9 IP → Claudin-1 blot). As expected for a CD9-claudin-1 heterodimer, its mobility lies between CD9-CD9 (lane 1) and Claudin-1-claudin-1 (lane 4) homodimers.
Fig. 1. Chemical cross-linking of CD9 and claudin-1 proteins. A, A431 cells were cultured, treated with or without 50 μM 2-BP for 18 h, and then lysed in buffer containing 1% Brij 96 or 1% Triton X-100. For one cell sample, cells cultured with 2-BP were also treated with 0.2 mg/ml thiol-specific cross-linker DTME prior to Triton X-100 lysis. CD9 immunoprecipitates were analyzed by Western blotting for CD9 and claudin-1 using non-reducing (NR) conditions and for claudin-1 using reducing conditions in which the DTME cross-linker is cleaved. Note that the size of CD9 decreases due to loss of palmitoylation when 2-BP is added. B, A431 cells were cultured in the presence of 2-BP, cross-linked with DTME, and lysed in Triton X-100 (as in A). CD9 and claudin-1 immunoprecipitates were then analyzed by Western blotting for CD9 and claudin-1 (using non-reducing conditions). Samples shown are from the same gel processed in an identical manner. For unknown reasons, a small amount of monomeric CD9 appears to be slightly larger than the bulk of CD9 monomer.

Fig. 2. Tetraspanin-claudin specificity. A, A431 cells were lysed in buffer containing 1% Brij 96; immunoprecipitations were carried out using antibodies to the indicated proteins, and gel-fractionated samples were blotted for CD9 and claudin-1. mIgG, total mouse IgG. CD71 and CD98 proteins are both highly expressed at levels greater than or equal to CD9 in A431 cells and are readily extracted in 1% Brij 96. B, Brij 96 lysates of A431 cells were subjected to two sequential rounds of IP with CD9 antibody and then IP and lysate samples were analyzed by blotting using the following relative cell equivalents: CD9 blot: lysate, 0.08, IP, 0.5; claudin-1 blot: lysate, 0.08; IP, 1.0; /H9251 blot: lysate, 0.08; IP, 1.0; /H9252 blot: lysate, 0.13; IP, 0.75. Band intensities were quantitated by densitometry, and percentage of total protein precipitated was calculated. C, Brij 96 lysates of A431 cells were subjected to two sequential rounds of IP with CD9 antibody and then IP and lysate samples were analyzed by blotting using the following relative cell equivalents: CD9 blot: lysate, 0.08, IP, 0.5; claudin-1 blot: lysate, 0.08; IP, 1.0; /H9251 blot: lysate, 0.13; IP, 0.75. Band intensities were quantitated by densitometry, and percentage of total protein precipitated was calculated. C, Brij 96 lysates of A459 cells were immunoprecipitated using antibodies to indicated proteins. Relative cell equivalents used for immunoblotting (IB) were: lysates, 0.27; IPs, 1.0. Quantitation was done as in B. D, MCF7 cells were lysed in Brij 96, indicated proteins were immunoprecipitated, and claudins were blotted. E, Brij 96 lysate and immunoprecipitations from A431 and MCF7 cells were lysed in Brij 96, and then lysates (0.33) and immunoprecipitates (1.0 cell eq) were analyzed by Western blotting. exp., exposure; E-cadherin; Cav., caveolin.
Claudin-1 Associated with Tetraspanin CD9

Fig. 3. Cholesterol depletion effects on tetraspanin and claudin proteins. A, A431 cells were incubated with or without 10 mM MβCD in serum-free medium for 60 min at 37 °C. Cell supernatants were then analyzed by immunoprecipitation (after addition of 1% Brij 96, protease inhibitors, and 1 mM EDTA) and Western blotting. Cells were lysed in 1% Brij 96 and analyzed likewise. Relative amounts of each fraction loaded on the blots are indicated. ~75% of total CD9 and ~37% of total claudin-1 are released from the cells upon MβCD treatment. B, A431 cells were incubated with or without MβCD as in A, and cell-free supernatants ("S") and cell lysates ("C") were analyzed by Western blotting. 7/8 of total supernatant and 1⁄70 of total lysate were loaded for each immunoblot. Bands were analyzed by densitometry, and fractions of specific proteins present in supernatant (relative to total supernatant + cell lysate) were calculated. C, MCF7 cells were treated as in B. Sup., supernatant; int., integrin; E-Cad., E-cadherin.

selves associate with CD9 (Fig. 2A, top panel), it is possible that they are linked to claudin-1 indirectly through CD9. To assess the extent of CD9-claudin-1 association in A431 cells, we performed two rounds of CD9 immunoprecipitation and then quantitated claudin-1 by densitometry. As indicated (Fig. 2B), CD9 immunoprecipitation yielded ~11% of total claudin-1 in the Brij 96 lysate. By contrast, established partners for CD9, integrin β1 and α3 subunits (15), were recovered to a lesser extent (~1.9 and 7.2% of the total, respectively). Recovery of CD9 itself was ~58%. Thus, by extrapolation, up to 20% of total claudin-1 could be associated with total CD9 protein in A431 cells. Whereas CD9 yielded 11% of total claudin-1 (Fig. 2B), a combination of anti-CD9, anti-CD81, and anti-CD151 antibodies yielded only ~10–15% of total claudin-1 from A431 cell lysates (data not shown). This result, together with data in Fig. 2A, suggests that of the three tetraspanins examined CD9 may be the preferred binding partner of claudin-1. In conditions such as those used in Fig. 2B (which are different from Fig. 1B), immunoprecipitation of claudin-1 did not yield CD9 (not shown). We suspect that the C terminus of claudin-1 (to which polyclonal antibody is directed) is not accessible within Brij 96-solublized CD9-claudin-1 complexes.

From human lung carcinoma A549 cell lysate, claudin-1 was again readily co-precipitated with CD9 and to a small extent with CD151 (Fig. 2C). Approximately 7.9% of total claudin-1 was co-precipitated with CD9 with ~53% of total CD9 itself being precipitated; thus, the extent of CD9-claudin-1 association in A549 cells is similar to that in A431 cells. Despite its abundance, claudin-2 was not detected in either CD9 or CD151 immunoprecipitates in this experiment. Antibodies to caveolin and E-cadherin did not yield CD9, CD151, claudin-1, or claudin-2 (Fig. 2C). Our subline of human breast adenocarcinoma MCF7 expresses claudin-3, -4, -5, and -7 but not -1 or -2. Immunoprecipitations of tetraspanins CD9 and CD81 yielded only small amounts of claudin-3 (<1% of total in lysate) and no detectable claudin-4 or -5 (Fig. 2D). Claudin-7 was barely detectable in CD9 immunoprecipitations from MCF7 cells and was not detectable at all from A431 cells (Fig. 2E). In a control experiment, no E-cadherin was associated with CD9 or CD151. From results in Figs. 1 and 2 we conclude that claudin-1 is the preferred claudin partner for tetraspanin CD9 with ~15–20% of total claudin-1 potentially complexing with CD9 in A431 and A549 cell lines.

Co-release of CD9 and Claudin-1 upon Cholesterol Depletion—Depletion of cellular cholesterol by MβCD treatment causes release of CD9 and other tetraspanins from intact cells while retaining homo- and heterotypic CD9 associations (53). Upon treatment of A431 cells with 10 mM MβCD for 45 min, ~75% of total CD9 and ~37% of total claudin-1 were detected in the cell supernatant. Neither protein was present in cell supernatants in the absence of MβCD treatment (Fig. 3A). Moreover claudin-1 remained associated with CD9 and CD81 in cell supernatants (after addition of 1% Brij 96). Whereas ~3–4% of cellular claudin-1 was co-precipitated with CD9 from cell lysates (from either MβCD-treated or untreated cells), ~7–8% of released claudin-1 (from +MβCD cell supernatant) was co-precipitated with released CD9. Thus, association of claudin-1 with CD9 was retained and perhaps even enriched in the supernatant fraction.
In another experiment with A431 cells (Fig. 3B), cholesterol depletion again caused substantial release of CD9 (~64%) and claudin-1 (~49%) as well as CD9 partner β1 integrin (~26%). By contrast, neither E-cadherin nor cytoplasmic protein β-tubulin was appreciably released. When A549 cells were treated with MβCD, claudin-1 was again substantially released (~33%), but other proteins, such as occludin, ZO-1, and E-cadherin, showed very little release (<2% for each; not shown). When MCF7 cells were analyzed, CD9 again showed substantial release (22.9%), whereas claudin-3, -4, and -5, as well as E-cadherin and β-tubulin, showed minimal release (Fig. 3C). These experiments show that proteins associating with CD9, such as claudin-1, are released together with CD9 upon cholesterol depletion.

**Co-localization of CD9 and Claudin-1 Proteins in Cells and in Density Gradients**—By immunofluorescence microscopy, both CD9 and claudin-1 showed preferential localization to the periphery of A431 cells and to cell-cell contact sites (Fig. 4). Curiously some patches of A431 cells showed brighter CD9 staining (Fig. 4, green in the overlay panel), whereas other cells showed brighter claudin-1 staining (orange-red in the overlay panel). The physical basis for this variation in claudin-1/CD9 staining ratios remains to be determined. Unfortunately this effort is hindered by the lack of anti-claudin-1 antibodies that recognize cell surface epitopes.

Consistent with their physical association, claudin-1 and CD9 also showed similar density distribution patterns in a sucrose gradient. As indicated, both proteins (from 1% Brij 96 lysates of A431 cells) were enriched at the 5–35% sucrose gradient. As indicated, both proteins (from 1% Brij 96 lysates of A431 cells) were enriched at the 5–35% sucrose gradient. As indicated, both proteins (from 1% Brij 96 lysates of A431 cells) were enriched at the 5–35% sucrose gradient.

**Association between CD9 and Claudins in Polarized Epithelial Cells**—Results in Figs. 1–5 describe substantial association between CD9 and claudin-1 in transformed epithelial cells, such as A431, A549, and MCF7, which do not form tight junctions. To complement these results, we tested epithelial cell lines, such as C2BBe1 (a derivative of Caco-2) and MDCK cells, which form tight junctions typical of polarized epithelial sheets. Consistent with tight junction formation, the latter cell lines have much lower paracellular permeability than A431, A549, or MCF7 cells when assayed with FITC-labeled dex-trans of 4 and 40 kDa (data not shown).

CD9 and CD81 isolated from C2BBe1 cells showed very little interaction with claudin-1 and no detectable interaction with claudin-4 (Fig. 6A). Similarly CD9 in MDCK cell lysate showed minimal interaction with claudin-1 or claudin-3 (Fig. 6B). Furthermore MβCD treatment caused very little CD9 or claudin proteins to be released from MDCK cells, and CD9-claudin-1 association was absent in Brij 96 cell lysates of either MβCD-treated or untreated cells (Fig. 6B). Similar results were obtained upon treatment of C2BBe1 cells with MβCD (not shown).

We considered the possibility that in C2BBe1 and MDCK cells claudins sequestered in tight junctions could resist extraction by 1% Brij 96 and thus become less available for analysis of CD9 interaction. To address this issue, we compared protein extraction efficiencies from non-polarized cells (A549) and polarized cells (MDCK) using 1% Brij 96, 1% Triton X-100, and RIPA (Fig. 6C). In all three detergent conditions, CD9, claudin-1, occludin, and β-catenin proteins were readily extracted from both cell lines. In fact, extraction of CD9 and...
Claudin-1 Associated with Tetraspanin CD9

**Fig. 6.** Tetraspanin-claudin association and detergent solubility in polarized epithelial cell lines. A, nearly confluent C2BBe1 cells were lysed in 1% Brij 96. Cell lysates and CD9, CD81, and E-cadherin immunoprecipitates were immunoblotted for the indicated proteins. Within the CD9 blot, 3-fold more CD81 IP material was utilized compared with the other IP samples. B, nearly confluent MDCK cells were incubated with or without 10 mM MjIFCD for 1 h at 37 °C, lysed in 1% Brij 96, and subjected to immunoprecipitation and immunoblotting as indicated. C, equal amounts of A549 and MDCK cells were lysed in 1% Brij 96, 1% Triton X-100, or RIPA, and the lysates were analyzed by immunoblotting. D, C2BBe1 cells were not depleted of calcium or were incubated overnight in medium without calcium followed by 45-min incubation with 1 mM EGTA. Cells were then lysed in 1% Brij 96, and detergent soluble (“s”) and insoluble (“i”) fractions were prepared as described under “Experimental Procedures” prior to blotting. E-cad., E-cadherin; Sup, supernatant; β-cat, β-catenin; Tr-X100, Triton X-100.

Claudin-1 by Brij 96 was actually increased compared with the other detergents (Fig. 6C and data not shown). Analysis of Brij 96-insoluble pellets from C2BBe1 cells (Fig. 6D) confirmed that nearly all claudin-1 and CD9 was indeed extractable. Thus, failure to see robust CD9-claudin-1 association in polarized cells is not due to insufficient protein extraction by Brij 96. When C2BBe1 cells were incubated in calcium-free medium overnight to disrupt tight junctions, CD9 and claudin-1 remained almost entirely soluble in Brij 96 (Fig. 6D). However, there was no increase in the amount of CD9-claudin-1 complex formation (not shown). In another experiment, MDCK cells were treated for 4 days with TGFβ, which induced an EMT-like transition, while disrupting tight junctions. However, again we saw no increase in CD9-claudin-1 association (not shown). In fact, levels of both proteins were considerably diminished (not shown).

**Effect of Tetraspanins on Claudin Stability**—Next we considered that tetraspanins might affect the biosynthesis of claudin proteins. To test this possibility, we decreased levels of CD9 and/or CD151 by using specific siRNAs and then performed 35S pulse-chase experiments. Treatment with siRNA led to a consistent decrease (by 80–95%) in total CD9 and CD151 protein levels when examined 6 days after siRNA introduction (Fig. 7A). Neither the steady-state protein level (Fig. 7A) nor the initial rate of biosynthesis (not shown) was altered for claudin-1 in siRNA-transfected cells. However, in two independent pulse-chase experiments, claudin-1 disappearance was markedly accelerated in cells in which either CD9 or CD151 was knocked down compared with cells transfected with control siRNA (Fig. 7, B and C). The half-life of claudin-1 protein decreased from ~6 h in control cells to ~3 h in CD9 or CD151 siRNA-transfected cells. In a double CD9/CD151 knockdown, claudin-1 stability was again diminished (Fig. 7D, top panel) to an extent similar to that in the single knockdowns (Figs. 7, B and C). Importantly stability of a control protein, caveolin-1, was slightly enhanced, rather than diminished, in the double CD9/CD151 knockdown (Fig. 7D, bottom panel). Thus, reducing the cellular level of tetraspanin proteins CD9 and CD151 has a destabilizing effect on claudin-1 protein.

**DISCUSSION**

**Tetraspanin-Claudin Complexes**—Here we have used a novel cysteine-cross-linking-mass spectrometry approach to identify claudin-1 as a directly associated partner for tetraspanin CD9. CD9-claudin-1 association was confirmed in multiple mass spectrometry experiments and by reciprocal co-immunoprecipitation of covalently cross-linked CD9-claudin-1 heterodimer.

Furthermore a moderate fraction of total available claudin-1 (at least 8–10%) associated with CD9 on three different cell lines. Consistent with physical association, CD9 and claudin-1 were co-localized at the cell periphery and at cell-cell contact sites as detected by immunofluorescent staining. CD9 clearly shows a preference for claudin-1 with other claudins (claudin-2, -3, and -7) associating to a much lower extent. Although claudin-1 shows 35–45% overall sequence similarity with other claudins, similarities are only 15–35% in the C-terminal tail region, which flanks the likely tetraspanin interaction site. Hence claudin-1 could easily contain a CD9 interaction site that is not shared by other claudins. In addition, other tetraspanins (e.g. CD81 and CD151) associated with claudin-1 although to a lesser extent than CD9. It remains to be determined whether claudin-1 associations with CD81 and CD151 are direct or are indirectly linked via CD9. Claudin-1 has not been shown previously to associate with tetraspanins. In fact, there has been only one prior report linking claudins with tetraspanins. In that case, Tspan-3 was re-
ported to associate with claudin-11 on oligodendrocytes (59), but covalent cross-linking was not carried out, and issues of proximity and extent of association were not addressed.

CD9 has been suggested to associate with itself (24) as well as with numerous other proteins, including integrins, Ig superfamily proteins, membrane proteases, heterodimeric G proteins, and membrane-bound EGF receptor ligands (20, 46). However, only a few CD9 partnerships (CD9-HB-EGF (46), CD9-EWI-F/CD9P-1 (23), CD9-EWI-2 (18), and CD9-CD9 (24)) have been captured by covalent cross-linking, and only CD9-EWI-F heterodimers (23) and CD9-CD9 homodimers (24) have been definitively identified. With the identification of cross-linked CD9-claudin-1 heterodimer, claudin-1 can now be added to the short list of CD9 partners for which direct association has been definitively demonstrated by covalent cross-linking.

Hallmarks of TEMs are as follows. 1) They are resistant to detergent solubilization, likely due to the presence of cholesterol and gangliosides (60, 61), which causes localization into lower density fractions in sucrose density gradients (53). 2) They can be released into the media, with protein-protein interactions intact, upon treatment of cells with cholesterol-depleting agent methyl-β-cyclodextrin (53). 3) Despite the presence of cholesterol in TEMs, tetraspanin protein-protein interactions are largely maintained upon cholesterol depletion (19, 53). With respect to these three criteria, 1) claudin-1 co-distributed with CD9 in low density fractions (at the 5–35% sucrose interface) in sucrose density gradients; 2) a large fraction of claudin-1 (37%) was released, together with CD9 (75%), upon treatment of intact cells with methyl-β-cyclodextrin; and 3) claudin-1 remained associated with CD9 after cholesterol depletion. Hence the CD9-associated subset of claudin-1 qualifies as a legitimate component of TEMs.

**Functional Implications**—Claudins have been viewed primarily as structural components of tight junctions, which provide a selective permeability barrier for both epithelial and endothelial cells (49). However, the population of claudins residing in tetraspanin microdomains is not located in tight junctions. Indeed CD9-claudin-1 complexes were most obvious in cell lines that do not form tight junctions and were minimally obvious in cell lines (C2BBe1 and MDCK) that do form tight junctions. In Brij 96, used for most of our protein-protein interaction experiments, all proteins examined were essentially completely soluble (Fig. 6, C and D). Thus, reduced CD9-claudin-1 association seen in MDCK or C2BBe1 cells is not simply due to claudin-1 residing in Brij 96-insoluble tight junctions. Furthermore CD9 did not associate with other tight junction proteins (i.e. occludin and ZO-1), and release of CD9-claudin-1 complexes upon cholesterol depletion is not characteristic of tight junction proteins. Finally although tetraspanin CD9 can sometimes be found within apical regions of polarized epithelial cells (62), tetraspanins have not been reported to localize into tight junctions. Consistent with these observations, paracellular dextran permeability was not altered in polarized epithelial cells upon knockdown of tetraspanins CD9 and/or CD151 (not shown).

However, in non-polarized epithelial cells, removal of CD9 and/or CD151 did consistently and selectively diminish the half-life of pulse-labeled claudin-1 by 3–6 h. Although there have been few, if any, studies of protein stability for non-junctional claudins, there is precedent for tetraspanins affecting the biosynthesis and/or stability of partner proteins. For example, specific heterodimer formation between tetraspanins (UPlα and

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**Fig. 7. CD9 and CD151 effects on claudin stability.** A, A549 cells were transfected with specific CD9, CD151, or control (Contr.) siRNA duplex oligonucleotides, and then after 6 days, amounts of CD9, CD151, and control proteins were analyzed by blotting. B and C, after siRNA treatment for 6 days, A549 cells were pulsed with [35S]cysteine/methionine and chased for the indicated times. Then radiolabeled claudin-1 was immunoprecipitated, quantitated, and normalized relative to claudin-1 protein loading to give a value at zero time = 100%. D, after double CD9/CD151 protein knockdown, A549 cells were analyzed for claudin-1 and caveolin-1 stability as in B and C. Supporting data for this figure are shown in Supplemental Fig. S1.
MDCK cells with TGFβ/H9252 nonspecific protein identifications. Protein solubilization while increasing the number of indirect and non-stringent detergents (e.g., Brij 96 and Brij 99) rather than more stringent detergents such as Triton X-100 (17–20). However, use of milder detergents markedly decreases protein solubilization while increasing the number of indirect and nonspecific protein identifications.

Non-junctional claudin-1 has been correlated in rat epididymis (66), porcine brain capillary endothelial cells (67), and a selected subline of MDCK cells (68). However, its function outside of TJJs in these cells is not known. In some cancer cells, non-junctional claudin-1 has been correlated with tumorigenesis and/or metastasis, and claudin-1 overexpression may promote malignancy (69, 70). Hence we speculate that by stabilizing expression of non-junctional claudin-1 (as shown in Fig. 7), tetraspanins may indirectly affect claudin-1-dependent effects on tumor progression. In this regard, the tumor suppressing and promoting effects of tetranspanins CD9 and CD151 (41, 71) could involve stabilization of non-junctional claudin-1. Another possibility is that tetraspanins could keep claudin-1 away from tight junctions, thus contributing to EMT, which involves disruption of tight junctions. However, disruption of tight junctions (e.g., by depleting calcium or by treating MDCK cells with TGFβ) did not enhance CD9-claudin-1 association. Hence there must be additional unknown factors, other than simply the presence or absence of tight junctions, that determine whether claudin-1 will be more or less available for CD9 association.

Advantages of the Cysteine Cross-linking-Mass Spectrometry Approach—Large scale proteomics screening techniques have been used to produce extensive protein-protein "interactome" network maps (72, 73). However, lateral interactions among transmembrane proteins are typically not recapitulated by the two-hybrid approaches used for interactome mapping. Tetraspanin proteins, in particular, associate laterally with many other transmembrane proteins in the context of TEMs. To begin to understand how tetraspanins proteins function, it is essential to identify their major, directly interacting partners. This has not yet been done for most of the 33 mammalian tetraspanins or for any of the 22 worm tetraspanins or 36 Drosophila tetraspanins. A useful approach has been the immunofluorescence isolation of tetraspanin complexes followed by MS/MS protein identification. To maintain tetraspanin-protein interactions it has been necessary to use non-stringent detergents (e.g., Brij 96 and Brij 99) rather than a more stringent detergent such as Triton X-100 (17–20). However, use of milder detergents markedly decreases protein solubilization while increasing the number of indirect and nonspecific protein identifications.

We hypothesized that covalent cross-linking might capture tetraspanin complexes on the surface of intact cells and render them resistant to stronger detergents (e.g., Triton X-100), thereby enriching for proteins that associate directly, enabling the use of more rigorous lysing and washing conditions, and diminishing background proteins. Here we took advantage of the fact that most tetraspanins, as well as many of their partner proteins, contain one or more membrane-proximal cysteine residues. These cysteines appear to be almost entirely palmitoylated (27–29). However, as we demonstrated previously, cysteines can be exposed upon treatment of intact cells with 2-BP, which inhibits palmitoylation of newly synthesized proteins. Once exposed, these membrane-proximal cysteines can be cross-linked. Indeed this strategy was used previously to demonstrate homo- and heterodimerization of CD9 (24) and to map the CD9 dimerization interface (25). Insertion of cysteines by mutagenesis could render many other membrane proteins amenable to a similar approach. An alternative would be to use amino-reactive cross-linking to capture cell surface complexes prior to LC-MS/MS identification of associated proteins (74). However, tetraspanin extracellular loops often lack accessible lysines partly due to blocking by associated proteins (57, 75).

One potential problem with our approach is that because palmitoylation itself contributes to the assembly of tetraspanin-enriched microdomains (27–29, 32) inhibition of palmitoylation could conceivably destabilize the very same protein-protein interactions that one hopes to identify. However, although palmitoylation is important for indirect interactions, it appears not to contribute to direct protein-protein interactions involving tetranspanins (e.g., CD151 with α3β1 and EWI-2 with CD9) (26, 28). Indeed partial inhibition of palmitoylation by 2-BP did not impair CD9-claudin-1 association. It appears that 2-BP inhibition is sufficient to expose some cysteines for cross-linking while still leaving many palmitoylation sites occupied. Perhaps not surprisingly, nearly every one of the proteins listed in Table I has one or more membrane-proximal cysteines that are palmitoylated or potentially could be palmitoylated. Also of the 10 proteins identified in Table I, four have not previously been linked to CD9. Regarding these other potential CD9 partners, further experiments are needed to confirm direct association with CD9, to assess extent of association, and to determine functional implications.

Additional Insights into Claudin-1 Biochemistry—Our results indicate indirectly that claudin-1 is palmitoylated because 2-BP treatment exposed membrane-proximal cysteines for cross-linking. It remains to be determined whether claudin-1 palmitoylation is as functionally important as claudin-14 palmitoylation, which is required for proper TJ localization and barrier function (51). In addition, we demonstrated that claudin-1 in the cell membrane can form lateral cis-homodimers, which are captured by covalent cross-linking. These results were obtained on cells that do not form TJs. It remains to be determined whether cis-homodimers play a role in TJs where it is already known that claudins engage in hetero- and homoaasociations with claudins on apposing cells (49). Another insight from our results involves the claudin-tetraspanin interface. Because the DTMB cross-linker spans only 1.3 nm, membrane-proximal cysteines in CD9 and claudin-1 must be within close proximity.
In conclusion, we have developed a novel technical approach, which may be particularly well suited for identifying partners for the many transmembrane proteins, such as tetraspanins, that contain membrane-proximal cysteines. The approach has the advantage of potentially enriching for directly associated proteins while minimizing adventitious interactions.

The application of this approach to tetraspanin CD9 yielded several new potential partners, including claudin-1, that were not seen in previous mass spectrometry experiments targeting CD9 (20). Furthermore we provide strong evidence that non-junctional claudins can partition into tetraspanin-enriched microdomains, thereby regulating claudin stability. The identification of claudins as tetraspanin partners may provide an important clue toward understanding how both types of proteins can affect tumor progression.

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