Tight junctions are multiprotein complexes that form the fundamental physiologic and anatomic barrier between epithelial and endothelial cells. This structure encircles the apical end of the lateral membrane of epithelial cells and can be visualized by transmission electron microscopic analysis as a series of close membrane appositions. However, the fine structure of tight junctions is best revealed by freeze fracture electron microscopy, where the sites of membrane apposition appear as a network of anastomosing transmembrane protein strands that interact between adjacent cells. In unfixed tissue, these continuous strands appear as rows of 10 nm particles (reviewed in Ref. 1), suggesting that the transmembrane protein components of the tight junction are organized into large repeating protein complexes. Defining the nature of these complexes is critical to understanding how the selective barrier properties of the tight junction are created, regulated, and altered in disease.

Although several tight junction transmembrane proteins have been identified, including the members of the Marvel domain-containing family (occludin (2), tricellulin (3), and Marvel D3 (4, 5)) and the JAM IgG family (6), only members of the claudin family (7) are able to recapitulate the tight junction strands seen in freeze fracture EM when expressed in tight junction-null cells. It is presumed that these strands represent claudin oligomers, with other proteins like occludin recruited to the strands by an interaction with claudins (8). Despite considerable effort, however, only a few studies have been able to show biochemical evidence for claudin oligomers (9–13), and in general, these interactions have been demonstrated using overexpressed proteins in fibroblastic cells.

Blue native-PAGE (BN-PAGE)3 has been successfully used to identify membrane protein-protein interactions and complexes (14). Mild neutral detergents are used for initial solubilization, and the anionic dye Coomassie Blue G-250 is used instead of SDS to introduce a negative charge to the protein complex for electrophoretic separation. Although this method has been used most extensively to analyze components of mitochondrial protein complexes (15), it has also been used to study aquaporin oligomerization (16–18), cadherin interactions (19), and as a tool for proteomic analysis of multiprotein plasma membrane complexes (20, 21).

In this study, we use BN-PAGE to demonstrate that in MDCK II cells a homodimer is the favored oligomeric unit for claudin-2 (cldn2) but that in the same cell line both claudin-4 (cldn4) and occludin are monomers. Formation of the cldn2 dimer is not dependent on extracellular domains or on the cytoplasmic carboxyl-terminal domain, suggesting that dimerization is mediated by one or more of the transmembrane domains. Despite considerable effort, however, only a few studies have been able to show biochemical evidence for claudin oligomers (9–13), and in general, these interactions have been demonstrated using overexpressed proteins in fibroblastic cells.

Claudin-2 Forms Homodimers and Is a Component of a High Molecular Weight Protein Complex

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with at least cldn1 and occludin, is present in a high molecular weight protein complex.

**EXPERIMENTAL PROCEDURES**

Antibodies, Constructs, and Cell Lines—Antibodies were purchased from Zymed Laboratories Inc./Invitrogen, except the M2 anti-FLAG monoclonal antibody (Sigma), the ZO-1 rat monoclonal R40.76 (gift from Bruce Stevenson, (22)) and VSV-g monoclonal antibody (gift from Thomas Kreis). Tet-Off MDCK II cells and inducible cell lines expressing cldn2, cldn4, and chimeras have been described previously (23–25). Amino-terminally His6- and FLAG-tagged cldn2 constructs were generated using PCR and cloned into pTRE (Clontech) and pcDNA 3.1 (Clontech); primers used were for His6 tag, 5′-ctctcgcccc-3′, and for FLAG tag, 5′-ctctgtggtgggcatgagaagcaccgtgtt-3′. Cldn2 mutants were generated using the Stratagene QuickChange site-directed mutagenesis kit; the cldn2 A3 mutants were made by replacing the highly conserved 49GLW51 in the first extracellular domain with AA using the primers 5′-attgtgacggttggcttcccttggtggcgt-3′ and 5′-gaattcgatctacccatggactac-3′. Cldn2 mutants were prepared for BN-PAGE sample buffer (Invitrogen) and incubated on ice for 15 min. Insoluble material was removed by centrifugation at 100,000 × g for 10 min in a TLA100 rotor (Beckman Coulter), and 0.1 volume native-PAGE 5% G-250 sample additive (Invitrogen) was added.

Preparation of MDCK and HEK 293T Cells Expressing Transgenes—Cells were washed with PBS or HBSS and resuspended in 1% DDM, 20 mM Tris, pH 7.5, 50 mM NaCl, incubated on ice for 20 min, and insoluble material was removed by centrifugation at 15,000 × g for 20 min, and the supernatants were prepared for BN-PAGE. When tagged claudins were purified by affinity chromatography, cells were resuspended in 1% DDM, 20 mM Hepes, pH 7.4, 50 mM NaCl and clarified as above before incubation with FLAG affinity resin (Sigma) or Talon resin (Clontech) according to the manufacturers’ directions. Tagged claudins were eluted from affinity resin using either FLAG peptide (Sigma) or 250 mM imidazole as appropriate.

Cross-linking with Photo-activated Cross-linkers—His-tagged cldn2 was eluted from Talon resin and incubated with 2 mM NHS-diazirine (SDA), NHS-LC-diazirine or Sulfo-NHS-diazirine (S-SDAD) (Thermo Scientific) for 30 min. Samples were quenched with 0.1 m Tris, pH 7.5, dialyzed against four changes of 20 mM Hepes, pH 7.4, 50 mM NaCl 0.3% DDM in Slide-a-Lyzer mini dialysis cups (Thermo Scientific), and 15 μl transferred to glass slides; drops were constrained within circles drawn with a wax pencil. Cross-linking was performed by exposing drops to UV light at 350 nm for the indicated times. Equal volumes of SDS sample buffer were added to terminate the cross-linking, and samples were subjected to SDS-PAGE, transferred, and immunoblotted as described below.

Cross-linking with Glutaraldehyde—His-tagged cldn2 and cldn4 were eluted from Talon resin and incubated with 100 μM glutaraldehyde for the indicated times (27); SDS was added to one sample before addition of glutaraldehyde to disrupt oligomeric organization. Reactions were quenched by the addition of 0.1 m Tris, pH 7.5, and samples were processed for SDS-PAGE and immunoblotting as described below.

Cysteine Cross-linking—MDCK II cells induced to express VSV-g-tagged cldn2 with and without mutation of perimembrane cysteines to serines were treated overnight with the palmitoylation inhibitor, 2-bromopalmitate, as described previously (28), washed twice with Dulbecco’s PBS, and incubated with 2 mg/ml dithiobismaleimidoethane (DTME, Thermo Scientific) for 30 min at 4 °C. Cross-linker was quenched with 2 mM cysteine in Dulbecco’s PBS, and cells were extracted with SDS sample buffer lacking reducing agents. Samples subjected to SDS-PAGE and immunoblotted as described below.

Claudin-containing Oligomers

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Claudin-containing Oligomers

Preparation of a High Molecular Weight Complex from Mouse Liver—Mouse liver membranes were prepared with a simplified method based on that of Goodenough and co-workers (29). One mouse liver was minced with razor blades and incubated with 1 mM NaHCO₃ supplemented with protease inhibitors for 10 min on ice and homogenized using a glass/Teflon homogenizer. The resulting homogenate was filtered through four layers of cotton gauze and centrifuged at 2000 × g for 15 min. The supernatant was discarded and the soft pellet made 50% sucrose by addition of 3 volumes of 67% sucrose in 1 mM NaHCO₃. Aliquots of 0.8 ml were placed at the bottom of each of four 2-ml ultracentrifuge tubes and overlaid with 0.8 ml of 42.9% sucrose and 0.5 ml of 20% sucrose. Samples were centrifuged in a Beckman Coulter TLS-55 rotor at 100,000 × g for 60 min, the upper interfaces collected, combined, and diluted 10-fold with 1 mM NaHCO₃, and the diluted material centrifuged at 4000 × g for 30 min. The resulting pellet was resuspended in 200 µl of 1% dodecyl maltoside in 10 mM Tris, pH 7.5, 50 mM NaCl and incubated on ice for 20 min. Resuspended pellets were centrifuged at 100,000 × g for 10 min to remove insoluble material, and the resulting supernatant prepared for BN-PAGE. Samples were electrophoresed on duplicate gels; one was stored at 4 °C, and the second was transferred and immunoblotted for cldn1 as described previously. The position of the immunoreactive band was determined and the corresponding region excised from the duplicate BN gel; the excised band was forced three times through a 30-gauge needle and electroeluted overnight in 25 mM Tricine, 3.75 mM imidazole, 5 mM aminocaproic acid, pH 7.0, for 16 h at 100 V using an Elutrap (Schleicher & Schüll). Following brief voltage reversal (200 V, 20 s), the electroeluted material was removed, precipitated with 20% trichloroacetic acid, washed three times with cold acetone, and air-dried. The pellet was resuspended in 4 × SDS/Laemmli sample buffer, incubated at 37 °C for 10 min, and briefly sonicated. Aliquots were electrophoresed in SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with cldn1, cldn2, and occludin antibodies.

BN-PAGE, SDS-PAGE, Immunoblotting, and Immunofluorescence—BN electrophoresis was performed using native-PAGE Novex BisTris gels (Invitrogen) according to the manufacturer’s directions; the protocols are based on techniques developed by Schagger and von Jagow (15). Gels were transferred for 60 min at 30 V to PVDF membrane (Immobilon P, Millipore) in Tris-glycine buffer containing 20% methanol and 0.1% SDS. Membranes were fixed in 8% acetic acid for 15 min, air-dried, destained in 100% methanol for 20 s, washed with H₂O, and incubated in blocking buffer (10% NFDM in PBS) for 60 min. Immunoblotting for BN-polyacrylamide gels was performed as described previously (30), except HRP-coupled secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were used, and detection was performed using SuperSignal West chemiluminescent substrate (Thermo Scientific). SDS-PAGE, immunoblotting, IR detection, and immunofluorescence were all performed as described previously (30). Recombinant aquaporin Z was extracted from bacterial pellets with 1% DDM, purified on Talon resin, dialyzed, electrophoresed on BN-PAGE, and stained with Coomassie Brilliant Blue; aquaporin Z plasmid construct was generously provided by Dr. Michael Wiener, University of Virginia.

RESULTS

Some Claudins Form Discrete Oligomers in MDCK Cells—To analyze the oligomeric organization of endogenous claudins in cultured epithelial cells (MDCK II), we used sucrose density fractionation to enrich tight junction-containing membranes and extracted these fractions with 4 M urea to remove peripheral membrane proteins. Protein pellets were dissolved in the nonionic detergent DDM, and samples were subjected to BN-PAGE. Immunoblot analysis of BN-polyacrylamide gels transferred to PVDF membranes revealed that cldn1, -2, -7 and occludin were easily detectable (Fig. 1A). When compared with soluble molecular weight markers, cldn2 migrated at ~112 kDa. There are considerable pitfalls in molecular weight estimation using BN-PAGE (31) especially for small membrane proteins (32), partially because unit of mass includes the protein, variable amounts of bound Coo-
Claudin-containing Oligomers

massie dye, and an unknown amount of lipid and partly be-
cause electrophoresis of soluble protein markers variably de-
viates from that of membrane proteins of known molecular
weight in BN-PAGE. We applied a correction factor to obtain
the protein alone based on an estimate of dye binding of 0.56
g/g protein (32) (observed range is about 0.35–0.6 g/g (31));
after this correction, the approximate molecular mass for the
protein component of the complex was ∼62 kDa. This size is
consistent with either a dimer or trimer of cldn2. Similar
analysis suggested cldn1 and cldn7 were either dimers or tri-
mers, whereas endogenous occludin (correction: 130 ×
0.56 = 65 kDa) appeared to migrate as a monomer. These
relative molecular weight assumptions were substantiated by
comparison with that of aquaporin Z electrophoresed in par-
allel in BN-PAGE. Aquaporin Z (monomer = 25 kDa) is
known from crystallographic studies to assemble as a tet-
ramer (33); its apparent molecular mass in BN-PAGE was 185
kDa; correction for the bound Coomassie dye (185 × 0.56 =
103 kDa) resulted in a calculated molecular mass consistent
with a protein tetramer containing four 25-kDa monomers
(supplemental Fig. 1). The various claudins had slightly differ-
ent electrophoretic mobility; the reason for this is unclear but
may be result from differences in protein molecular weights,
isoelectric points, and associated lipids or due to difference in
protein interactions (homo- or hetero-oligomerization). The
extraction and BN-PAGE was repeated using different non-
ionic detergents with similar results, although use of digitonin
resulted in slightly higher molecular weight complexes (data
not shown), consistent with other reports that digitonin re-
moves less associated lipid from membrane protein com-
plexes than does DDM (34). Despite several attempts, we
were unable to detect endogenous cldn4 after BN-PAGE from
wild-type MDCK II cells.

To determine whether induced cldn2 in stably transfected
MDCK cells showed the same oligomeric organization as the
endogenous cldn2, we next performed BN-PAGE on DDM-
extracted MDCK Tet-Off cells induced to express mouse
cldn2 by removal of doxycyline. To distinguish cross-linking
of subunits within a complex versus freely diffusing uncom-
plicated proteins, duplicate samples were first treated with 1%
SDS to denature and thus dissociate oligomers (Fig. 1B, left
panel). When induced mouse cldn2 was extracted in DDM, it
migrated at approximately the same molecular mass as en-
dogenous canine cldn2, whereas SDS-treated cldn2 migrated
at ∼55 kDa, and when corrected for bound detergent (55 ×
0.56 = 31 kDa) approximates a monomer, suggesting that the
112-kDa band most likely represents a cldn2 dimer and not
trimer. Because of the high level of transgene induction, the
endogenous cldn2 was not detected at the exposure times
employed (data not shown). In contrast to the results seen
with cldn2, BN-PAGE analysis of induced human cldn4 re-
sulted in identical migration behavior for DDM- and SDS-
treated samples (Fig. 1B, right panel), suggesting that in
MDCK cells, cldn4 exists as a monomer.

To test if the cldn2 complex visualized by BN-PAGE likely
represented a homodimer or a complex containing cldn2 and a
different protein, HEK cells were transiently transfected
with Hisc-tagged cldn2 and FLAG-tagged cldn2 separately or
together (Fig. 1C). FLAG- and His-tagged cldn2 migrated
identically to wild-type cldn2 in BN-PAGE when expressed
either transiently or stably (data not shown). All transfected
HEK cells showed approximately equal levels of cldn2 expres-
sion (Fig. 1C, lysates, top left); untransfected HEK cells do not
express endogenous claudins (data not shown). The FLAG
antibody did not recognize His-tagged cldn2 (Fig. 1C, lane 2)
in cells transfected with His-tagged cldn2 alone (lysates, bot-
tom left). DDM extract of transfected cells was purified on
metal affinity resin; only extracts from cells transfected with
His-tagged cldn2 were specifically eluted with imidazole (Fig.
1C, upper right panel). Only when FLAG-tagged cldn2 was
co-transfected with His-tagged cldn2 did it specifically elute
from metal affinity resin (Fig. 1C, lane 3, lower right panel),
suggesting that the FLAG-tagged protein was retained on the
resin via an interaction with the His-tagged protein (lane 3).
In contrast, there was no apparent interaction between His-
and FLAG-tagged cldn2 when separately transfected cells
were co-cultured (Fig. 1C, lane 4) or supernatants of sepa-
rate transfected cells were mixed before incubation with
affinity resin (lane 5). These results suggest that dimerization
is not due to transcellular interactions between proteins ex-
pressed in apposing cells or as an artifactual interaction
formed during isolation. Because FLAG-His dimers depend
on co-transfection, it is possible that dimers form during syn-
thesis or trafficking.

Chemical Cross-linking of cldn2 Results in Formation of
Dimers but Not Higher Molecular Weight Forms—Chemical
cross-linking is a commonly used method to define protein-
protein interactions within oligomers. We used two separate
approaches to determine whether chemical cross-linking
could confirm the existence of cldn2 dimers in DDM extracts
and perhaps identify other higher molecular weight com-
plexes. First, we used a series of photoactivatable succinimidy-
est diazirine (SDA) cross-linkers to probe for cross-linking
between His-tagged cldn2, which had been induced in MDCK
II cells, extracted in DDM, and purified by metal affinity resin
chromatography (Fig. 2A). As reported previously, we variably
observed a low background of SDS-resistant dimer even in the
absence of cross-linking (12). Incubation of purified cldn2
with SDA, NHS-LC-diazirine, or S-SAD resulted in in-
creased levels of dimeric cldn2, with only very minor appear-
ance of higher molecular weight bands. Of note, although in
particular after S-SAD, as much as 15–20% of cldn2 appears
dimer, most cldn2 migrates as monomer; the reason for
this limited cross-linking is unclear. There was little time-de-
pendent increase in UV cross-linking; it seems likely that the
rate-limiting step is the first amine-dependent cross-linking
and that the UV-dependent cross-linking is relatively efficient
and rapid.

Glutaraldehyde-dependent cross-linking has often been
used to obtain information about subunit number or proxim-
ity, and although highly efficient, its lack of chemical specific-
ity can at times produce oligomers of questionable biologic
relevance (35). However, despite this lack of specificity, the
efficiency of glutaraldehyde cross-linking suggested that it
might provide a useful approach to identify potential higher
order cldn2 oligomers from DDM-extracted MDCK cells (Fig.
Claudin-containing Oligomers

A

| Time | SDA | LC-SDA | S-SDAD |
|------|-----|--------|--------|
| 0    | -   | +      | +      |
| 1    | +   | +      | +      |
| 5    | +   | +      | +      |
| 15   | +   | +      | +      |

Crosslinker: - (none), + (cross-linked)

Time (min)

250
200
150
100
50
0

Percent Cldn2 as Dimer

+100 μM glut
+100 μM glut + 1% SDS

B

Cldn2

Cldn4

0
2
10
30
0
30
30 min
+ SDS

75kDa
50kDa
37kDa
25kDa
20kDa
15kDa

dimer
monomer

FIGURE 2. Chemical cross-linking of cldn2 results in formation of dimers but not higher molecular weight forms. A, His-tagged cldn2 was extracted from induced MDCK II cells with 1% DDM and purified on Talon resin and cross-linked using photoactivated cross-linkers, electrophoresed using SDS-PAGE, and immunoblotted with cldn2 antibody. Some SDS-resistant cldn2 dimer is evident in the absence of cross-linker (−); addition of three cross-linkers of different lengths all resulted in an increase in dimer formation with only minor increases in higher molecular weight bands. Water-insoluble SDA and NHS-LC-diazirine showed maximal cross-linking at shortest period (1 min) of UV exposure; in no case was cross-linked cld2 more than 20% water-soluble S-SDAD showed maximal cross-linking at shortest period (1 min) of UV exposure; in no case was cross-linked cld2 more than 20% water-soluble S-SDAD showed maximal cross-linking at shortest period (1 min) of UV exposure; in no case was cross-linked cld2 more than 20% water-soluble S-SDAD showed maximal cross-linking at shortest period (1 min) of UV exposure; in no case was cross-linked cld2 more than 20% water-soluble S-SDAD showed maximal cross-linking at shortest period (1 min) of UV exposure; in no case was cross-linked cld2 more than 20%

Dimers Form within a Single Cell—Highly conserved residues in the first extracellular domain of claudins (GLW) have been implicated in proper claudin localization and in the formation of claudin-dependent cell contacts (36, 37). To test if claudin dimer formation was dependent on intercellular contacts, we mutated GLW residues of this conserved region to alanines; the location of these mutations is shown in the diagram in Fig. 4A. Immunofluorescent analysis of induced wild-type cldn2, tagged at the carboxyl terminus with a VSV-g motif, showed that it co-localized with the tight junction cytoplasmic protein ZO-1 but was also present in intracellular vesicles as reported previously (Fig. 4B, top right) (25). Localization of cldn2 with the triple alanine mutation (A3) was almost exclusively intracellular, and it did not co-localize with 2B, panel a, top left panel). Time-dependent glutaraldehyde cross-linking of purified cldn2 resulted in conversion of most cldn2 to a dimer with no increase in any specific higher molecular weight species; preincubation of DDM-extracted purified cldn2 with 1% SDS prior to cross-linking prevented cross-linking to a dimer. There was, however, loss of some fraction of the immunoreactive cldn2 signal after extended incubation in glutaraldehyde, consistent with nonspecific cross-linking to nonclaudin protein contaminants, to insoluble aggregates, or to sizes exceeding resolution on the gel. In contrast to glutaraldehyde-dimerized cldn2, purified DDM-extracted cldn4 inducibly expressed in MDCK cells was not cross-linked by glutaraldehyde (Fig. 2B, top right panel). The conversion of cldn2 monomer to dimer is presented graphically in Fig. 2B, panel b, bottom panel, supporting a kinetic plateau of cross-linking at a dimer. Together these cross-linking studies support a dimer as the fundamental oligomeric state of cldn2.

Dimerization of cldn2 Is Not Mediated through Its Extracellular Domains or Its Cytoplasmic Carboxyl-terminal Domain—Because cldn2 forms dimers and cldn4 does not, we used previously generated MDCK II cell lines inducibly expressing chimeras between cldn2 and cldn4 (24, 25) to determine the region of cldn2 that was responsible for dimer formation. A diagram of the previously published chimeras is included as supplemental Fig. 2. MDCK cells expressing wild-type cldn2, cldn2 with the extracellular domains of cldn4, and cldn4 with the cytoplasmic carboxyl-terminal domain of cldn2 were extracted with DDM, electrophoresed using BN-PAGE, and immunoblotted with cldn2 antibody (Fig. 3, top left panel). We observed that wild-type cldn2 and cldn2 with the extracellular domain of cldn4 both migrate as dimers, whereas cldn4 with the tail of cldn2 migrates at a position smaller than the 66-kDa molecular mass marker, in the size range consistent with a monomer. Consistent with the lack of a role for the extracellular domains in cross-linking, cldn4 and cldn4 with the extracellular domains of cldn2 appear to be monomers, whereas cldn2 with the extracellular domain of cldn4 migrates as a dimer (Fig. 3, top right panel). When 1% SDS is added, the electrophoretic mobility is increased and similar for all transgenes (Fig. 3, bottom panels). These results suggest that cldn2 dimerization does not require the cytoplasmic or extracellular domains and is likely mediated through interaction of one or several of its four transmembrane segments.
ZO-1 (Fig. 4A, bottom right). When cells plated in parallel were extracted with DDM and analyzed by BN-PAGE (Fig. 4C), both wild-type cldn2 and the A₃ mutant primarily migrated as dimers and were similarly converted to monomers after treatment with 1% SDS. These results were consistent with the idea that the observed dimerization does not represent transcellular interaction or that it requires localization to the plasma membrane, but instead it represents cis contacts mediated by transmembrane segments.

**Targeted Cysteine Cross-linking Reveals Proximity of the Second Transmembrane Domains**—Claudins, like tetraspanins (38), are palmitoylated on perimembrane cysteines located at the cytoplasmic ends of the transmembrane segments (28). Using cldn2 sequences mutated at various perimembrane cysteines allowed us to use cross-linking to ask whether these specific cysteine positions were in close proximity within the dimer. To prevent palmitoylation and leave the cysteines free for cross-linking, cells were preincubated with 2-bromopalmitate overnight (39). Vsv-g-tagged cldn2 isolated from MDCK II cells preincubated in 2-bromopalmitate was minimally cross-linked to dimer in the absence of cysteine cross-linker (Fig. 5A, red signal), but dimer formation was significantly increased after incubation with the cysteine cross-linker DTME. The specificity of the role of the perimembrane cysteines in mediating this cross-linking is shown by the significant reduction of dimer formation in cells expressing cldn2 with mutations of four of the perimembrane cysteines to serines (C24S). Occludin (Fig. 5A, green signal) is not palmitoylated and not cross-linked to a specific product after DTME treatment; however, the decrease in occludin signal in the DTME-treated samples does suggest the formation of some occludin adducts that are not detected as specific bands. DTME cross-links cysteines at a distance of 13.3 Å. Cross-linking with a cysteine cross-linker with shorter spacer arms (bis(maleimido)ethane, 8 Å) resulted in a similar level of cross-linking (data not shown).

The perimembrane cysteines in cldn2 are located at the carboxyl-terminal ends of the second and fourth transmembrane domains; this location is conserved among claudins.
Claudin-containing Oligomers

**FIGURE 5. Cysteine cross-linking suggests proximity of the second transmembrane domains.** A, cldn2 can be cross-linked with disulfide cross-linkers by perimembrane cysteines. MDCK cells induced to express wild-type cldn2 (C2) and cldn2 with perimembrane cysteines mutated to serines (C24S) were incubated overnight in 2-bromopalmitate to inhibit palmitoylation and then incubated without (−) and with (+) the cysteine cross-linker DTME. Cell extracts were subjected to SDS-PAGE and immunoblotted for VSVG-cldn2 (red) and occludin (green); wild-type cldn2 was cross-linked with much greater efficiency than cldn24S; cldn24S migrates as a dimer in BN-PAGE (data not shown). B, mutational analysis suggests that the second transmembrane domains may be in close proximity. MDCK cells stably transfected and induced to express VSVG-cldn2 with perimembrane cysteines mutated to serines after the second (TM2 Cys → Ser) or fourth (TM4 Cys → Ser) were incubated overnight without (−) or with (+) 2-bromopalmitate and then treated (+) with cysteine cross-linker. The only sample where cross-linking to dimer (arrow) was clearly dependent on preincubation with 2-bromopalmitate and DTME was TM4 (Cys → Ser), suggesting a critical role for perimembrane cysteines following the second transmembrane domain.

(28). To determine the relative contributions of the cysteines proximal to the second versus fourth transmembrane segments to the cysteine-dependent cross-linking, we separately mutated these cysteines to serine and performed similar cross-linking experiments as described above. Some background dimer formation was detectable in all cell lines after treatment with the cross-linking agent, perhaps due to less than complete palmitoylation of perimembrane cysteines, to incomplete oxidation of the extracellular cysteines, or to the unmutated cysteine within TM2 (see diagram in Fig. 4A). However, only in the cells expressing cldn2 where the perimembrane cysteines at the end of the fourth transmembrane domain were mutated to serines was there an appreciable increase in specific cross-linking to dimer (Fig. 5B). These data suggest that perimembrane cysteines at the end of the second, but not the fourth, transmembrane domains can participate in DTME-mediated cross-linking and thus suggest that the second transmembrane domains of two cldn2 molecules are likely to be in close proximity.

**BN-PAGE of Purified Mouse Liver Membranes Reveals That Claudins and Occludin Are Components of a High Molecular Weight Complex**—Although most cldn2 detectable by BN-PAGE of MDCK cells appears as a dimer, longer exposure of the immunoblots revealed some higher molecular weight cldn2-containing bands (Fig. 6A). Freeze fracture EM of tight junctions reveals that claudins can exist in 100-nm particle complexes, which would represent protein complexes significantly larger than dimers. We speculated that larger complexes might be more easily detected in native tissues, like liver, where the tight junction network is more developed and cell turnover rates are slower than in MDCK cells. Using a simplified protocol based on biochemical enrichment of murine hepatocyte tight junctions (29), we used sucrose density gradients to enrich junction fractions before extraction with DDM, electrophoresis in BN-PAGE, and immunoblotting. Anti-cldn1 antibody was used on tissue blots because it gave a robust signal compared with cldn2 polyclonal antibodies. The observed size of the immunoreactive complex from liver was ~1100 kDa. Correcting for bound Coomassie dye (×0.56) suggests a complex of ~600 kDa for the protein fraction (Fig. 6B). Of note, Coomassie binding should be limited to the surface of this large complex, and thus the actual protein content may be higher (31). When the corresponding area was excised from a duplicate gel, electroeluted, precipitated, electrophoresed in SDS-PAGE, and immunoblotted for cldn1, cldn2, and occludin, all of which were present in the original high molecular weight band.

**DISCUSSION**

Two significant new findings about tight junction structure emerge from our studies. First, we demonstrate that cldn2 extracted with DDM from MDCK cells and analyzed by BN-PAGE is assembled as a homodimer. Cross-linking of chimeric cldn2/cldn4 molecules reveals that dimerization is likely mediated through the transmembrane segments. Muta-
demonstration of the importance of the transmembrane segment, consistent with lateral claudin interactions (9). A more direct approach was supported by subsequent studies using FRET analysis, which also were used (40) and to some extent even on the tissue source (41). In DDM extracts from cultured cells, we were only reliably able to detect dimers, suggesting this represents the most stable or fundamental claudin oligomeric unit. In contrast, the finding that transfected cldn4 behaves as a monomer was somewhat surprising, because induction of this transgene in MDCK II cells is associated with a striking increase in the number of freeze fracture strands and a correlating increase in the physiologic barrier (23). However, we have also noted that cldn4 in MDCK II cells does not localize to tight junctions but extends all along the lateral membrane, and when expressed in fibroblasts, it does not concentrate in plaques at cell-cell junctions as do cldn1 or cldn2 (data not shown). These results suggest that it does not behave as a typical adhesive claudin and we speculate may lack some ability to self-organize; this could explain its failure to form stable dimers in BN-PAGE. In support of this possibility, a recent study (42) demonstrated that cldn4 interacted with cldn8 in a split ubiquitin yeast two-hybrid assay but did not interact with itself. These authors further showed that in cultured renal collecting duct cells, cldn4 localization required cldn8 co-expression. Unlike cldn2, cldn4 and cldn16 (see below) may participate not in homodimers but in obligate heterodimers with selected other claudins. The interesting implication is that combinatorial diversity in tight junction structure might be generated if sets of claudins follow different assembly rules.

Our finding that cldn2 formed dimers based on transmembrane interactions fits much published indirect evidence suggesting claudins interact via lateral association and that strand assembly requires cooperative cis and trans interactions. Soon after the initial identification of claudins, Tsukita and co-workers (43) demonstrated by immuno-freeze fracture electron microscopy that all claudins they tested, along with occludin, were co-localized to the same tight junction strands, despite the observation that only a subset of claudins appeared to interact across cells. These results were supported by subsequent studies using FRET analysis, which also were consistent with lateral claudin interactions (9). A more direct demonstration of the importance of the transmembrane segments in claudin interactions came from the work of Hou et al. (44). As in their analysis of cldn4, they used a split ubiquitin yeast two-hybrid assay to demonstrate that cldn19 could interact both with itself and with cldn16 and further found that a mutation in the first transmembrane domain of cldn19 could abrogate its ability to interact itself and with cldn16. The transmembrane-based interaction seems likely to happen during protein synthesis or trafficking as we observed that the intracellular A³ mutants of cldn2 formed dimers, and other studies have noted heterotypic claudin association in Golgi (42). Piontek et al. (10) recently proposed a model where claudins first form cis-oligomers in the plane of the membrane and suggested that polymerization into continuous strands depended on subsequent engagement of trans interactions across the intercellular space.

Claudin dimers are too small to account for the 10-nm tight junction particles seen in freeze fracture electron microscopic images. It has been suggested that these particles may be formed from claudin hexamers, based on analogy to the connexins, similar sized tetraspanning transmembrane proteins, which organize into hexamers and form similar sized particles freeze fracture particles (1). Unlike analysis of connexins, however, it has been difficult to demonstrate the existence of higher order claudin oligomers. Studies that suggested the existence of claudin hexamers were dependent on claudin overexpression (12, 13) and may represent nonspecific aggregation. Alternatively, these studies may demonstrate labile physiologic hexamers that are only maintained during biochemical isolation when there is an unusually high claudin concentration. Defining the composition of the 10 nm particle may depend on a better understanding of essential tight junction components, including other cellular proteins and lipids that may be recruited into a claudin-based particle.

Along with trying to understand how claudins are organized into oligomers, the approach we have used is to try to define a larger, multiprotein complex to begin to define a critical set of interacting proteins in tight junction assembly. Anatomic data suggested that claudin and occludin, at a minimum, are organized into a membrane protein complex; however, the lack of unambiguous biochemical evidence for such a complex indicates that these protein interactions might be labile or very sensitive to extraction conditions. We were able to demonstrate the existence of a high molecular weight claudin- and occludin-containing protein complex from mouse liver using BN-PAGE but not from MDCK cells despite the fact that, like liver tissue, this cell line contains cldn1, cldn2, and occludin. It is possible, as suggested above, that the minimal tight junctions of rapidly dividing MDCK cell are less stable to detergent extraction or that stabilization of the interactions of these proteins depends on unknown factors, including perhaps tissue-specific differences in protein or lipid composition, stoichiometry, or post-translational modifications. We plan to examine complex formation in Caco-2 and T84 cells after 3 weeks in culture, because at this time these cells have more elaborate tight junction organization than MDCK cells. Another possibility is that extraction of liver but not MDCK cells maintains association of cytoplasmic scaffolding proteins that might be critical in stabilizing the oligomeric organization. Further purification and charac-
Claudin-containing Oligomers
terization of the BN-PAGE purified tight junction protein complex may provide insight into these factors and should allow us to further characterize the molecular composition and organization of tight junctions.

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