Tight Junction–associated MARVEL Proteins MarvelD3, Tricellulin, and Occludin Have Distinct but Overlapping Functions

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In vitro studies have demonstrated that occludin and tricellulin are important for tight junction barrier function, but in vivo data suggest that loss of these proteins can be overcome. The presence of a heretofore unknown, yet related, protein could explain these observations. Here, we report marvelD3, a novel tight junction protein that, like occludin and tricellulin, contains a conserved four-transmembrane MARVEL (MAL and related proteins for vesicle trafficking and membrane link) domain. Phylogenetic tree reconstruction; analysis of RNA and protein tissue distribution; immunofluorescent and electron microscopic examination of subcellular localization; characterization of intracellular trafficking, protein interactions, dynamic behavior, and siRNA knockdown effects; and description of remodeling after in vivo immune activation show that marvelID3, occludin, and tricellulin have distinct but overlapping functions at the tight junction. Although marvelD3 is able to partially compensate for occludin or tricellulin loss, it cannot fully restore function. We conclude that marvelID3, occludin, and tricellulin define the tight junction–associated MARVEL protein family. The data further suggest that these proteins are best considered as a group with both redundant and unique contributions to epithelial function and tight junction regulation.

INTRODUCTION

The tight junction is a network of transmembrane and peripheral proteins that form a semipermeable barrier to paraacellular flux (Farquhar and Palade, 1965; Claude and Goodenough, 1975). The functions of some of these proteins have been defined in recent years, including roles for the peripheral membrane proteins ZO-1 and -2 in recruiting transmembrane claudin proteins to the tight junction and the critical responsibilities of the latter in defining ion selectivity (Itoh et al., 1999; Umeda et al., 2006). However, the specific contributions of most tight junction–associated proteins remain enigmatic. Foremost among these is occludin, the first transmembrane protein to be associated with the tight junction and the critical responsibilities of the latter in defining ion selectivity (Itoh et al., 1999; Umeda et al., 2006). However, the specific contributions of most tight junction–associated proteins remain enigmatic. Foremost among these is occludin, the first transmembrane protein to be associated with the tight junction (Furuse et al., 1993; Balda et al., 1996; McCarthy et al., 1996; Wong and Gumbiner, 1997; Yu et al., 2005). Moreover, both in vitro and in vivo studies have associated occludin endocytosis with pathophysiological and pharmacological tight junction barrier loss (Massoumi and Sjolander, 2001; Clayburgh et al., 2005; Shen and Turner, 2005; Utech et al., 2005; Schwarz et al., 2007). These data are difficult to reconcile with the observations that occludin knockout mice are viable, fail to display defective epidermal, respiratory, renal, or intestinal barrier function, and do not develop spontaneous skin, airway, kidney, or gut disease (Saitou et al., 2000). As a result, many have concluded that occludin is unnecessary for normal epithelial tight junction function. However, occludin knockout mice do have significant pathologies, including small size, testicular atrophy, male infertility, salivary gland dysfunction, atrophic gastritis, thinning of compact bone, and brain calcifications. Thus, two potential explanations must be considered. First, occludin may contribute to epithelial activities other than development of basal barrier function, and second, that other tight junction proteins may partially compensate for occludin loss.

The occludin-related protein tricellulin has recently been reported (Ikenouchi et al., 2005). Both occludin and tricellulin contain the tetra-spanning MARVEL (MAL and related proteins for vesicle trafficking and membrane link) domain that is present in proteins involved in membrane apposition and concentrated in cholesterol-rich microdomains (Sanchez-Pulido et al., 2002). Like occludin (McCarthy et al., 1996; Yu et al.,
2005), tricellulin knockdown interferes with tight junction assembly and enhances solute diffusion across cultured epithelial monolayers (Ikenouchi et al., 2005), while tricellulin overexpression enhances barrier function (Krug et al., 2009). Human tricellulin mutations are associated with nonsyndromic hearing loss, but with no other pathologies (Riazuddin et al., 2006; Chishti et al., 2008). Tricellulin is, therefore, not essential for function of epidermal, respiratory, renal, or intestinal tight junctions. Given that both occludin and tricellulin are MARVEL domain–containing proteins, we sought additional members of this family that participate in tight junction function.

To comprehensively define the members of the MARVEL domain–containing protein family present at the tight junction, we performed bioinformatic analyses. These identified the previously uncharacterized protein marvelD3 as a potential tight junction component. Analyses of evolutionary conserved regions distributed to subcellular localization, trafficking, protein interactions, dynamic behavior, siRNA knockdown, and in vivo responses to immune activation were used to characterize marvelD3 as well as tricellulin and occludin. The data define marvelD3, occludin, and tricellulin as the only members of the tight junction–associated MARVEL protein (TAMP) family and show that although marvelD3 may partially compensate for occludin or tricellulin loss, it is insufficient to fully restore function in the absence of other TAMPs. We conclude that these proteins are best considered as a group with parallel, but nonredundant, functions.

MATERIALS AND METHODS

Bioinformatic Analyses

As MARVEL domain–containing protein sequences are highly divergent, alignment was performed using the E-INS-i algorithm in the MAFFT package (Katoh and Toh, 2008), one of the most accurate algorithms for alignment of sequences with conserved residues scattered across an extended region. Transmembrane topology for the alignment was established using PolyPhobius (Kall et al., 2005) and the TMHMM program (Sonnholm et al., 1999), which generated similar predictions. However, as the former algorithm is known to produce more reliable results for transmembrane topology, especially for eukaryotic proteins (Jones, 2007), PolyPhobius predictions are showed.

ProTest was used to fit models to the alignment (Abascal et al., 2005), and Blossum62 with site heterogeneity and empirical residue frequency was found to be the optimal phylogenetic model for tree reconstruction. Toward this goal, PhyML (Guindon and Gascuel, 2003) with 800 bootstrap replicates and MrBayes (Huelsenbeck et al., 2001) with a single Markov chain for 1 million generations were each conducted. Both algorithms yielded highly convergent trees, but the latter typically yields a more accurate result and is presented in Figure 1.

Gene conservation data were extracted using the PhastCons track from the UCSC Genome Browser (Kuhn et al., 2009). This hidden Markov model-based analysis, which considers both individual alignment columns as well as flanking sequences, approximates the likelihood that a nucleotide belongs to a conserved region based on a multispecies alignment (Siepel et al., 2005).

Using the updated annotation file for the Affymetrix Mouse Genome 430 2.0 Array, Mouse4302_Mm_ENSG (Dai et al., 2005), we reanalyzed the tissue expression profile database GEO series GSE1896 (Barrett et al., 2007) using GCRMA and Biocor (Gentleman et al., 2004; Wu and Irizarry, 2004) and residues 13–26, RPRERDPGRRPHPD; and residues 174–187, EVEYQSEAEGLLE (in combination (YenZym, South San Francisco, CA). These peptides, which share 89% homology between mouse and human sequences, are common to both splice variants, and were selected based on predicted immunogenicity. Immune responses were assessed by ELISA. Polyclonal antisera were affinity-purified.

Immunoblotting

Lyse of mouse jejunal enterocytes and Caco-2 cells were separated by SDS-PAGE and transferred to PVDF membranes. Sample protein concentration was determined by dye-binding protein assay (Bio-Rad, Hercules, CA), and equal quantities of protein were loaded in each lane. Immunoblots were performed using the previously described antibodies, as well as GAPDH (Invitrogen), VSVG, and b-actin (Sigma-Aldrich, St. Louis, MO), followed by either HRP– (Cell Signaling Technology, Boston, MA) or infrared dye-conjugated (Li-Cor, Lincoln, NE) secondary antibodies. Protein was detected either by chemiluminescence or using an Odyssey System (Li-Cor). Densitometry of immunoblot data were performed using ImageJ software (Abramoff et al., 2004).

Animals

Seven- to 10-wk-old C57BL/6 mice were used for all studies, which were conducted in an AAALAC-accredited facility under protocols approved by The University of Chicago IACUC.

Epithelial Isolation

Mouse jejunum was opened lengthwise, and epithelial cells were isolated at 4°C as described previously (Clayburgh et al., 2005). Cells were resuspended and lysed in Laemmli sample buffer or buffer RLT (Qiagen, Valencia, CA) for protein or RNA analysis, respectively.

Immunoelectron Microscopy

Seven-week-old C57BL/6 mice were perfused with 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 10 min, and jejuna were fixed in 1% paraformaldehyde for 20 min at 4°C. Immunelectron microscopy using ultrathin cryosections was performed as described previously (Tokuyasu, 1980). Ultrathin sections were blocked with 10% normal goat serum in PBS for 30 min at room temperature, incubated with anti-marvelD3 polyclonal antisera at 1:100 for 17 h at 4°C, rinsed, and then incubated with 10-nm gold conjugated goat anti-rabbit IgG (BB International, Llanishen, Cardiff, United Kingdom) for 1 h at room temperature. Samples were examined with an H-7500 transmission electron microscope (Hitachi, Tokyo, Japan) at an acceleration voltage of 100 kV.
RESULTS

Occludin and Tricellulin Are Members of a MARVEL Subfamily That Includes MarvelD3

The tight junction proteins occludin and tricellulin each contain MARVEL domains (Sanchez-Pulido et al., 2002). To identify additional tight junction–associated MARVEL domain–containing proteins, a minimally redundant collection of human MARVEL domain protein sequences was obtained from the EMBL-EBI InterPro database. Alignment of these sequences (Figure 1A) and tree reconstruction (Figure 1B) suggested the existence of four subfamilies within the greater MARVEL family. One subfamily was composed of the tight junction proteins occludin and tricellulin and two splice variants of marvelD3, a previously uncharacterized protein. MarvelD3 splice variants 1 and 2 have identical amino-terminal cytoplasmic tails, but due to translation of either exon 4 or 3, respectively, differ in the sequence of their cytoplasmic carboxy-terminal regions and contain identical MARVEL domains.

Gene conservation analysis of marvelD3, tricellulin, and occludin across vertebrate species demonstrated a higher degree of evolutionary conservation within exons encoding MARVEL domains than in those encoding cytoplasmic tails or within noncoding genomic sequences, suggesting that the MARVEL domain itself significantly influences the function of these proteins (Figure 1C). Moreover, analysis of murine tissue transcript profiles revealed a highly correlated (p < 0.001), although nonredundant, pattern of marvelD3, tricellulin, and occludin expression, consistent with shared functions among members of this MARVEL subfamily (Figure 1D). The analysis also indicated enrichment of these proteins in epithelial organs, including lung, stomach, kidney, and liver. In sum, these results suggest that marvelD3, tricellulin, and occludin represent a functionally unique subfamily within the broader family of MARVEL domain–containing proteins.

MarvelD3, Tricellulin, and Occludin Are the Only Members of the Tight Junction–associated MARVEL Protein Subfamily

Previous analyses have identified MARVEL family members within cellular structures that include intracellular transport vesicles, the apical plasma membrane, and the endoplasmic reticulum (Zacchetti et al., 1995; Haass et al., 1996; Breitwieser et al., 1997). Although occludin and tricellulin are the only MARVEL domain–containing proteins reported to concentrate at the tight junction (Furuse et al., 1993; Ikenouchi et al., 2005), our evolutionary analyses suggest that marvelD3 may also be associated with tight junctions. To test this hypothesis, marvelD3, tricellulin, occludin, and representative proteins from each of the three additional MARVEL subfamilies (Figure 1B) were expressed as enhanced green fluorescent protein (EGFP)-fusion constructs in Caco-2 human intestinal epithelial cells. EGFP-tricellulin and EGFP-occludin were present at junctions, where they colocalized with ZO-1 (Figure 1E), and were concentrated at tricellular (where three cells meet) and bicellular (where two cells meet) junction regions, respectively (Furuse et al., 1993; Ikenouchi et al., 2005). Consistent with the evolutionary relationship between marvelD3, tricellulin, and occludin suggested by MARVEL domain alignment and tree reconstruction, EGFP fusion proteins of both marvelD3 splice variants also colocalized with ZO-1 at junctions and were primarily localized
Figure 1. MarvelD3, tricellulin, and occludin are the only members of the tight junction–associated MARVEL protein (TAMP) subfamily. (A) MARVEL domain alignment was ordered after model fitting and tree reconstruction and shaded within each of the four MARVEL protein lineages according to the Blossum62 score. Amino acids are shaded according to physicochemical properties as indicated in the figure. The characteristic acidic residue in the third transmembrane domain (Sanchez-Pulido et al., 2002) is indicated by an asterisk (*). (B) The alignment was used for tree reconstruction, and disparities were calculated according to the fraction of mutated residues. Posterior values are shown as 100-fold original predictions, implying that a bootstrap value of 60 is highly reliable. (C) Gene conservation analysis demonstrates that exons encoding MARVEL domains (*) show the highest degree of evolutionary conservation within the marvelD3, tricellulin, and occludin genes. Data are presented as a base-by-base conservation measurement, with 0 indicating a neutral region and 1 indicating perfect conservation, from 28 vertebrate species including marsupial, monotreme, and placentals mammals, as well as reptile, amphibian, bird, and fish clades. (D) Analysis of transcript expression in the Affymetrix GeneChip Mouse Genome 430 2.0 Array (Santa Clara, CA) reveals a highly correlated (p < 0.001), though nonredundant, pattern of expression for marvelD3, tricellulin, and occludin within epithelial organs. (E) EGFP-MARVEL domain–containing fusion proteins (green) were transfected into Caco-2 human intestinal epithelial cells. Polarized monolayers were grown on Transwell supports, and ZO-1 (red) was detected by immunofluorescent staining. Three-dimensional reconstruction shows that EGFP-marvelD3 splice variants 1 and 2, EGFP-tricellulin, and EGFP-occludin localize to tight junctions and intracellular vesicles. Both EGFP-marvelD3 splice variants are enriched along bicellular tight junctions similar to occludin, whereas tricellulin is specifically concentrated at tricellular regions. MARVEL proteins from other lineages were found to decorate the apical plasma membrane and intracellular transport vesicles, but not the tight junction. Images are representative of ≥3 experiments, all with similar results. Bar, 10 μm.
to bicellular regions (Figure 1E). In contrast, EGFP-MAL concentrated at apical membranes, whereas proteolipid protein 2 and pantophysin predominantly localized to cytoplasmic vesicles (Figure 1E), consistent with previous studies of each protein (Zacchetti et al., 1995; Haass et al., 1996; Breitwieser et al., 1997). All three members of a single subfamily of MARVEL domain-containing proteins are, therefore, concentrated at the tight junction, whereas representative members of other subfamilies are not. These data indicate that marvelD3, tricellulin, and occludin constitute the complete tight junction–associated MARVEL protein (TAMP) family. Moreover, they support the hypothesis that marvelD3 is a functional component of the tight junction.

**TAMPs Are Differentially Expressed at the Tight Junction across a Wide Variety of Epithelial Tissues**

Occludin and tricellulin are expressed in a variety of epithelial tissues (Ikenouchi et al., 2005), and in silico analysis of transcript profiles (Figure 1D) suggests that marvelD3 expression may be similar. Semiquantitative RT-PCR was used to assess marvelD3, occludin, and tricellulin mRNA content in Caco-2 monolayers, where all TAMP transcripts were found to be expressed, but at different levels (Figure 2A). In particular, marvelD3 variant 2 message was less abundant than marvelD3 variant 1, tricellulin, and occludin transcripts. TAMP transcripts were also found to be differentially expressed throughout mouse intestine, liver, and kidney (Figure 3A). MarvelD3 variant 1 message was more abundant within mouse liver, marvelD3 splice variant 2 transcript predominated in mouse kidney, and expression of the two forms was comparable in small intestine and colon.

Polyclonal antisera against peptides derived from cytoplasmic regions common to both marvelD3 splice variants were developed. SDS-PAGE immunoblot of Caco-2 cell lysates using these antisera demonstrated two bands consistent with the predicted molecular mass of human marvelD3 splice variants 1 and 2 of 46 and 45 kDa, respectively (Figure 2B). The antisera were specific, because preincubation with the peptides used as immunogens eliminated these bands (Figure 2B). Similarly, SDS-PAGE immunoblot analysis of murine jejunal epithelium using marvelD3 polyclonal antisera produced two bands consistent with the predicted molecular mass of mouse marvelD3 splice variants 1 and 2 at 42 and 44 kDa, respectively (Figure 3B). Detection of both bands was eliminated by preincubation of antisera with peptide immunogens (Figure 3B). Along with immunoblots using commercially available antisera to tricellulin and occludin (Figure 2C), these data show that all three TAMPs are expressed in Caco-2 cell monolayers as well as within various murine tissues including jejunal and colonic epithelial cells, stomach, small intestine, colon, liver, kidney, and lungs (Figure 3C). In contrast, expression of marvelD3, tricellulin, and occludin was limited or absent in organs lacking significant epithelial tissue, such as lymph nodes and spleen.

Immunofluorescent microscopy showed that endogenous marvelD3 was concentrated at the apical junctional complex of polarized Caco-2 cell monolayers and was also present within intracellular vesicles (Figure 2D). Like occludin, marvelD3 at the apical junctional complex was predominantly at bicellular regions, whereas tricellulin was concentrated at tricellular regions. The subcellular distributions of marvelD3, tricellulin, and occludin within murine jejunal enterocytes were similar to those in Caco-2 monolayers (Figure 3D), and all three TAMPs were associated with hepatocyte junctional complexes (located at the interface between the apical canalicular and basolateral sinusoidal plasma membrane domains; Figure 3D). MarvelD3, tricellulin, and occludin were also all expressed in renal tubular epithelium, where they concentrated at the apical junctional complex. In contrast, marvelD3 and tricellulin were expressed in glomerular epithelium, but occludin was not (Figure 3D).

Although the immunofluorescence analyses above suggest that marvelD3 localizes to the tight junction, it is possible that these fluorescent signals emanate from other apical

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**Figure 2.** TAMPs are differentially expressed at intercellular junctions of Caco-2 human intestinal epithelial cell monolayers. (A) Analysis of TAMP transcript expression by RT-PCR reveals differential expression of marvelD3 splice variants, tricellulin, and occludin in Caco-2 cells. (B) MarvelD3 proteins (arrowheads) were detected in Caco-2 cells as determined by SDS-PAGE immunoblot analysis. Preincubation of polyclonal antisera with peptides used as immunogens eliminated the marvelD3 bands. (C) Tricellulin and occludin proteins (arrowheads) were also detected in Caco-2 cells by SDS-PAGE immunoblot. (D) Immunofluorescence of polarized Caco-2 monolayers demonstrates junctional and vesicular localization of all TAMP family members. MarvelD3 (green) staining was abrogated following preincubation of antisera with competing peptides. Tricellulin (green) specifically localized to tricellular junctions (arrowheads). Occludin (red) is shown for orientation in each image. Bar, 10 μm. Imaging along the z-axis at the indicated position (dashed line) reveals colocalization of marvelD3 and tricellulin with occludin at apical intercellular junctions. Bar, 5 μm.
Figure 3. TAMPs are differentially expressed across epithelial tissues. (A) Analysis of TAMP transcript expression by semiquantitative RT-PCR reveals differential expression of marvelD3 splice variants, tricellulin, and occludin message in epithelial-rich mouse organs. (B) MarvelD3 splice variants (arrowheads) were each detected in mouse jejunal epithelial samples as determined by SDS-PAGE immunoblot analysis. Preincubation of polyclonal antisera with peptides used as immunogens eliminated the marvelD3 bands. These data indicate that occludin and marvelD3 interact, but suggest that these proteins are common (Figure 4A). In contrast, occludin and marvelD3 were able to pull down VSVG-ZO-1-GuK. Data are representative of ≥2 experiments, each in duplicate, with similar results.

Figure 4. TAMPs demonstrate unique interactions with each other and ZO-1. (A) Coimmunoprecipitation of TAMPs from Caco-2 lysates reveals interactions among marvelD3, tricellulin, and occludin. The data are consistent with a model of TAMP interaction, where marvelD3 physically associates with both occludin and tricellulin, but the latter two TAMPs do not interact. (B) TAMP cytoplasmic tails were expressed as GST fusion constructs, and equal quantities of each were loaded onto GST-agarose, as confirmed by the Coomassie blue–stained gel. Pulldown experiments using VSVG-ZO-1-GuK as prey demonstrate interaction with the carboxy-terminal tail of occludin (immunoblot). In contrast, neither cytoplasmic tails of tricellulin nor marvelD3 were able to pull down VSVG-ZO-1-GuK. Data are representative of ≥2 experiments, each in duplicate, with similar results.

TAMPs Demonstrate Unique Interactions with Each Other and ZO-1

The overlapping morphological location of TAMP family members suggests that marvelD3, tricellulin, and occludin may physically associate with one another. To determine whether TAMPs engage in heterotypic interactions, marvelD3, tricellulin, and occludin were immunoprecipitated from Caco-2 lysates. Tricellulin and marvelD3 coimmunoprecipitated with one another, suggesting that interactions between these proteins are common (Figure 4A). In contrast, occludin immunoprecipitates contained marvelD3, but marvelD3 immunoprecipitates did not contain occludin. These data indicate that occludin and marvelD3 interact, but suggest that the fraction of the total marvelD3 pool bound to occludin may be greater than the fraction of the total occludin pool images (BB, brush border; TJ, tight junction; L, lumen; BLM, basolateral membrane; N, nucleus; BC, Bowman’s capsule; M, mesenchyme; GT, glomerular tuft; US, urinary space) are shown for orientation. Bars, 10 μm. (B) Transmission immunoelectron microscopy of mouse jejunal duodena demonstrates specific localization of marvelD3 to the tight junction in sagittal and en face orientations (arrowheads). Immunofluorescence insets with marvelD3 (green) and actin (red) are shown for orientation. Gray boxes approximate electron microscopic fields. White bars, 10 μm; black bars, 100 nm. Data are representative of ≥2 experiments, all with similar results.
bound to marvelD3. Tricellulin and occludin did not coimmunoprecipitate, suggesting that these TAMP family members do not physically interact or that associations are either unstable or uncommon. These data lend themselves to a model of TAMP interaction at the tight junction, whereby marvelD3 associates with both occludin and tricellulin, whereas the latter TAMPs do not interact directly.

The cytoplasmic carboxy-terminal tail of occludin is known to interact with the GuK domain of the plaque protein ZO-1 (Furuse et al., 1994). Moreover, the carboxy-terminal tail of tricellulin has been shown to interact with the amino-terminal half of ZO-1 (Riazuddin et al., 2006) which, in addition to the GuK domain, contains three PDZ domains. To investigate the direct ZO-1 binding of TAMP family members, GST-fusion constructs of occludin (266-522) and tricellulin (364-551) carboxy-terminal, and marvelD3 amino-terminal (1-198) tails were expressed as recombinant proteins and were bound to GST-agarose (Figure 4B). These were used to assess binding of a recombinant fusion protein consisting of the VSVG epitope tag (Kreis, 1986) and the ZO-1 GuK domain (VSVG-GuK). Consistent with previous studies (Furuse et al., 1994), VSVG-GuK interacted with the carboxy-terminal domain of occludin (Figure 4B). However, the carboxy terminus of tricellulin was not able to bind VSVG-GuK, suggesting that tricellulin may interact with ZO-1 at another site. Alternatively, minor differences between the tricellulin tail constructs used here and in a previous study (Riazuddin et al., 2006) may explain the disparate results. In contrast to occludin, the GST fusion construct of the marvelD3 cytoplasmic tail failed to associate with VSVG-GuK (Figure 4B). These data highlight important distinctions between the interactions formed by ZO-1, marvelD3, tricellulin, and occludin, and again support a nonredundant model for TAMP function at the tight junction.

TAMPs Partition into Detergent-insoluble Membrane Microdomains and Claudin-based Tight Junction-like Strands

Tight junction proteins are concentrated in cholesterol and sphingolipid rich, detergent-insoluble membrane microdomains (Nusrat et al., 2000). Subcellular fractionation showed that, like occludin, marvelD3 and tricellulin are enriched in these low-density membranes (Figure 5, A and B). Tricellulin distributed across a greater density range than other TAMP family members, consistent with its distribution along lateral membranes at tricellular junction sites (Ikenouchi et al., 2005). Like occludin, members of the claudin tight junction protein family are also concentrated into detergent-insoluble membrane microdomains (Shen et al., 2006). When expressed in fibroblasts, claudin proteins are able to form tight junction-like strands (Furuse et al., 1998; Sasaki et al., 2003). In contrast, occludin and tricellulin are recruited to claudin-based strands, but are unable to form these structures in the absence of claudin family members (Furuse et al., 1998; Ikenouchi et al., 2008). To determine whether marvelD3 can form tight junction-like strands, EGFP-marvelD3 splice variants 1 and 2, EGFP-tricellulin, and EGFP-occludin localize to intracellular vesicles when expressed independently, but are recruited to tight junction-like strands (yellow) along areas of membrane overlap (arrowheads) in cells expressing claudin-1. Images are representative of ≥3 experiments, all with similar results. Bar, 10 μm.

**Figure 5.** TAMPs partition into detergent-insoluble membrane microdomains and claudin-based tight junction-like strands. (A) Caco-2 monolayers were solubilized at 4°C in Triton X-100, and lysates were separated on sucrose density gradients. OD$_{600}$, a measurement of the light scattering property of intact membranes, and protein recovery, are shown as a function of density. (B) Immunoblot analysis demonstrated that TAMPs are enriched in the light-scattering fraction of detergent-resistant membranes. (C) EGFP-TAMP constructs (green) were transfected into L929 human fibroblast cells alone or with mRFP1-claudin-1 (red). Monolayers were grown on glass coverslips, and nuclei (blue) were detected by staining with Hoechst 33342. EGFP-marvelD3 splice variants 1 and 2, EGFP-tricellulin, and EGFP-occludin localize to intracellular vesicles when expressed individually, but are recruited to tight junction-like strands (yellow) along areas of membrane overlap (arrowheads) in cells expressing claudin-1. Images are representative of ≥3 experiments, all with similar results. Bar, 10 μm.
TAMPs, including both marvelD3 splice variants, were incorporated into mRFP1–claudin-1-based, tight junction-like strands (Figure 5C). These data suggest that, much like other members of the TAMP family, marvelD3 is a component of tight junction strands but is unable to form stands independently.

**Epithelial Barrier Development Coincides with TAMP Expression and Trafficking to the Tight Junction**

To assess TAMP expression and trafficking during tight junction assembly, mRNA content, and protein expression and localization were monitored along with TER (Figure 6A). qRT-PCR analysis (Figure 6B) showed that marvelD3 splice variant 1 and tricellulin transcription increased significantly ahead of TER development (2.9 ± 0.50- and 2.3 ± 0.03-fold increases, respectively; \( p < 0.05 \) for each). In contrast, marvelD3 splice variant 2 mRNA, which was poorly expressed in mature Caco-2 monolayers (Figure 2A), was not significantly up-regulated during barrier development (1.2 ± 0.20-fold increase; \( p > 0.2 \)). Occludin and claudin-1 transcript levels also remained relatively constant during tight junction assembly (1.1 ± 0.03- and 1.0 ± 0.06-fold increases, respectively; \( p > 0.4 \) for each). Tricellulin and marvelD3 transcription was accompanied by increased protein expression, which coincided with development of peak TER 72 h after plating (Figure 6C). Consistent with qRT-PCR data, claudin-1 protein content was stable throughout monolayer maturation. In contrast, occludin protein expression increased during tight junction assembly despite constant mRNA content. These data suggest that marvelD3 and tricellulin protein expression is primarily regulated by transcriptional mechanisms during tight junction assembly, whereas expression of occludin is controlled by modifications to translation or turnover rate.

TAMPs exhibit multiple subcellular locations, including tight junction, lateral membrane, and vesicular pools. Consistent with previous studies of tricellulin and occludin (Ikenouchi et al., 2008), these proteins, as well as marvelD3, are trafficked to the tight junction in concert with TER development (Figure 6D). MarvelD3 and tricellulin accumulated at the tight junction over time, and this correlated with increased protein expression. As has been noted previously (Ikenouchi et al., 2008), tricellulin was present at bicellular as well as tricellular tight junction regions of immature monolayers (compare with Figure 2D). In contrast, occludin and claudin-1 trafficking to the tight junction during barrier development coincided with reduced intracellular pools (Figure 6D). Together with the qRT-PCR and immunoblot data, these results show that occludin and claudin-1 are redistributed from cytoplasmic stores to the tight junction during barrier development. In contrast, marvelD3 and tricellulin are delivered to the tight junction immediately after synthesis. Moreover, the correlation of increased TER with progressive enrichment of each TAMP family member at the tight junction is consistent with the hypothesis that marvelD3, like occludin, tricellulin, and claudin-1, participates in the development and maintenance of epithelial barrier function.
To directly assess the role of marvelD3 in tight junction assembly, Caco-2 cells were transfected with TAMP-targeting or control siRNAs, and TER was measured at regular intervals. siRNA transfection reduced TAMP protein expression by more than 60% at 60 h after plating (Figure 7A and B). Suppression of individual TAMPs produced modest delays in TER development, averaging 18 ± 4% (p < 0.01), relative to monolayers treated with control siRNA (Figure 7C). Similar findings have been reported for tricellulin and occludin knockdown (Ikenouchi et al., 2005; Yu et al., 2005), but this is the first demonstration of a functional role for marvelD3. Interestingly, the TER delay incurred by knockdown of any one TAMP was indistinguishable from that caused by knockdown of any other. TAMP knockdown did not induce compensatory upregulation of remaining family members. This suggests that although similar, the roles of TAMPs at the tight junction are unique. Consistent with nonredundant function, simultaneous knockdown of multiple TAMPs caused greater delays in barrier development than knockdown of any single TAMP (Figure 7D; 53 ± 12% delay; p < 0.01 relative to monolayers transfected with control siRNA; p < 0.05 relative to monolayers transfected with siRNA against a single TAMP). However, the delays for combined knockdown of any two (or all three) TAMPs were similar. Taken as a whole, these data suggest that TAMPs make both overlapping and unique contributions to tight junction assembly and barrier function.

**TAMPs Have Unique Dynamic Behaviors at Bicellular and Tricellular Tight Junction Regions**

The inability of TAMPs to compensate for loss of other subfamily members during barrier development, as well as their differential tissue expression, discrete patterns of synthesis during tight junction assembly, distinct subcellular distributions, and selective interactions all suggest that members of this MARVEL subfamily may have unique biochemical behaviors. Our previous analyses established that the tight junction is subject to continuous dynamic remodeling at steady state and that occludin is highly mobile within the tight junction and lateral membrane (Shen et al., 2008). However, the dynamic behaviors of neither marvelD3 nor tricellulin have been studied. To investigate the mobility of TAMP family members, Caco-2 epithelial cells expressing EGFP-fusion proteins were grown as polarized monolayers and protein dynamics were assessed by FRAP at bicellular and tricellular tight junction regions (Figure 8A). TAMP mobility profiles and times of half-maximal recovery at bicellular and tricellular tight junction regions spanned a broad spectrum (Table 1). Dynamic behavior of marvelD3 splice variants was a function of position within the tight junction; marvelD3 variant 1 was more stable at tricellular regions (Figure 8, B–D) whereas variant 2 was more stable at bicellular regions (Figure 8, E–G). In contrast, tricellulin and occludin mobilities were similar at bicellular and tricellular regions. The distinct dynamic behaviors of marvelD3 splice variants at bicellular and tricellular regions suggest distinct roles at each site. Moreover, the unique behaviors of each TAMP lend further support to the hypothesis that these proteins have distinct functions at the tight junction.

**TAMPs Are Differentially Expressed and Redistributed in Response to In Vivo Tumor Necrosis Factor Treatment**

We have previously shown that in vivo tumor necrosis factor (TNF)-induced diarrhea requires myosin light-chain kinase activation, tight junction reorganization, and increased paracellular permeability that is accompanied by occludin internalization (Clayburgh et al., 2005, 2006). To assess the in vivo regulation of marvelD3 and tricellulin,
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Figure 8. TAMPs have unique dynamic behaviors at bicellular and tricellular tight junction regions. (A) Caco-2 cells were transfected with EGFP-TAMP fusion proteins, and protein dynamics at bicellular (bTJ) and tricellular (tTJ) tight junction regions were assessed by FRAP. TAMPs exhibit distinct and unique dynamic behaviors at tricellular (B–D) and bicellular (E–G) areas of the tight junction. Occludin and tricellulin are highly mobile at both regions. In contrast, mobility of both marvelD3 splice variants varied between bicellular and tricellular tight junction regions (see Table 1). Data are representative of ≥3 experiments, each in triplicate, all with similar results.

Table 1. The dynamic behavior of TAMP proteins

| TAMP          | Tight junction region | Mobile fraction (%) | t1/2 (s) |
|---------------|-----------------------|---------------------|---------|
| MarvelD3 splice variant 1 | Bicellular | 75 ± 4*† | 245 ± 39** |
| MarvelD3 splice variant 1 | Tricellular | 57 ± 1*† | 105 ± 9*† |
| MarvelD3 splice variant 2 | Bicellular | 61 ± 3*† | 149 ± 13*† |
| MarvelD3 splice variant 2 | Tricellular | 74 ± 3*† | 301 ± 7*† |
| Tricellulin   | Tricellular | 79 ± 5 | 137 ± 20 |
| Occludin      | Bicellular | 89 ± 3 | 107 ± 6* |
| Occludin      | Tricellular | 88 ± 6 | 74 ± 3* |

* Data are significantly different (p < 0.05) between bicellular and tricellular regions.
† Data are significantly different (p < 0.05) between marvelD3 splice variants at bicellular regions.
‡ Data are significantly different (p < 0.05) between marvelD3 splice variants at tricellular regions.

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Figure 9A). In contrast, occludin mRNA remained constant within 45 min of TNF exposure lead to the increased content did not change (p > 0.40). Consistent with this, expression of both marvelD3 and tricellulin transcripts increased rapidly isolated at intervals after intraperitoneal TNF injection. murine jejunal epithelial cells and intact jejunum were isolated at intervals after intraperitoneal TNF injection. MarvelD3 and tricellulin transcripts increased rapidly within enterocytes after TNF administration (p < 0.01; Figure 9A). In contrast, occludin mRNA remained constant (p > 0.40). Consistent with this, expression of both marvelD3 and tricellulin proteins increased in response to in vivo TNF treatment (p < 0.05), whereas occludin protein content did not change (p > 0.37; Figure 9B). These data suggest that increases in marvelD3 and tricellulin transcription within 45 min of TNF exposure lead to the increased translation of these TAMP family members detected after 90 min. Immunofluorescence of mouse jejunal demonstrated differential redistribution of these related tight junction members after TNF treatment. Although occludin was redistributed into cytoplasmic vesicles (Figure 9C), increased marvelD3 protein was detected at the tight junction, apical and lateral membranes, and within cytoplasmic vesicles. Similarly, tricellulin was enriched at both tricellular and bicellular tight junctions, as well as within the cytoplasm, after TNF treatment (Figure 9C). The similarity between in vivo marvelD3 and tricellulin synthesis and trafficking and that which occurred during in vitro barrier development suggests that these responses may reflect an effort to stabilize the tight junction and compensate for occludin removal. However, the fact that barrier loss still occurs (Clayburgh et al., 2005), as well as the distinct responses of each TAMP, suggest that marvelD3, tricellulin, and occludin serve unique functions in the epithelial response to proinflammatory stimuli.

DISCUSSION

As the first identified transmembrane component at the tight junction, occludin was initially thought to be essential to structure and function (Furuse et al., 1993). However, studies since that time have demonstrated that occludin is not required for tight junction barrier function (Saitou et al., 1998, 2000; Yu et al., 2005). Moreover, although occludin knockdown does reduce barrier function in vitro, it does not alter ion selectivity (Yu et al., 2005). In contrast, claudin proteins, which assemble into tight junction-like strands, are critical determinants of barrier function and paracellular ion selectivity (Simon et al., 1999; Sonoda et al., 1999; Furuse et al., 2001, 2002; Van Itallie et al., 2001; Hamazaki et al., 2002; Colegio et al., 2003; Umeda et al., 2006; Van Itallie et al., 2008). Despite the central role of the claudin protein family, in vivo analyses suggest that TAMP family members do play...
TNF treatment and remained elevated after 90 min (*p < 0.05). (C) Jejunum was frozen 120 min after TNF injection and immunostained for TAMPs (green), F-actin (red), and nuclei (blue). MarvelD3 and tricellulin protein expression increased and was enriched at the tight junction, lateral membrane, and cytoplasm. Bar, 5 μm. Data are representative of ≥3 experiments, all with similar results.

Figure 10. Model of TAMP locations and interactions at the tight junction. The figure shows the organization of proteins within a small area of a confluent monolayer (see image at the top). The bicellular tight junction is the interface between two cells, whereas the vertex where three cells meet is termed the tricellular tight junction. The tight junction strands within both bicellular and tricellular regions are composed of claudins (black spheres). All three TAMP proteins, marvelD3 (orange spheres), tricellulin (green spheres), and occludin (blue spheres) incorporate into claudin-based tight junction strands. Occludin and tricellulin are primarily found at bicellular and tricellular regions, respectively, whereas marvelD3 is present at both sites. This may explain why marvelD3 binds to both tricellulin and occludin, but tricellulin and occludin do not interact with one another. Tricellulin is unique in that it is present at the tight junction and along the lateral membrane. Only occludin interacts with the ZO-1 (red spheres) via the GuK domain. Thus, although there are similarities, each TAMP can also be defined by unique characteristics.

suggest a similar view of tricellulin function (Riazuddin et al., 2006; Chishti et al., 2008). Furthermore, substantial in vitro data suggest that both occludin and tricellulin are important to tight junction function (Balda et al., 1996; Chen et al., 1997; Wong and Gumbiner, 1997; Ikenouchi et al., 2005; Yu et al., 2008). Based on these observations and the presence of MARVEL domains in both occludin and tricellulin (Sanchez-Pulido et al., 2002), we hypothesized that additional TAMPs might compensate for occludin or tricellulin loss. We began with bioinformatic analyses of human MARVEL domain sequences and then developed novel tools to study these proteins. The data demonstrate that a previously uncharacterized protein, marvelD3, is a component of the tight junction and that marvelD3, occludin, and tricellulin constitute the complete TAMP subfamily of MARVEL proteins. However, the functional analyses and in vivo regulation of these proteins suggest that marvelD3, occludin, and tricellulin are not able to complement one another functionally. This is consistent with the significant structural differences outside of the MARVEL domains of each TAMP family member. Perhaps most notable is the presence of ELL domains in the cytoplasmic carboxy-terminal tails of occludin and tricellulin, but not within the short carboxy-terminus or longer amino-terminal cytoplasmic tails of either marvelD3 splice variant.

Previous unpublished and published analyses have speculated both in favor (http://www.pdg.cnb.uam.es/MARVEL/tree.gif) and against (Riazuddin et al., 2006) the existence of
an evolutionary relationship between marvelD3 and tricellulin and occludin. However, these studies were based on analyses that excluded the third transmembrane-spanning sequence of the MARVEL domain itself and used evolutionary trees that lacked posterior values at branch points. The utility of previous analyses is also limited because the approaches used were not optimized for proteins with highly divergent sequences, as seen in the MARVEL family. After narrowing the search space to include only human MARVEL domain–containing proteins, we constructed a phylogeny of complete MARVEL domains using an algorithm suitable for alignment of sequences with large, unalignable regions. The results suggest the existence of four subfamilies, one of which is composed of marvelD3, tricellulin, and occludin. Although our study has focused on the tight junction–associated MARVEL protein subfamily, it will be of interest for future studies to determine if functional themes characterize the other three MARVEL subfamilies defined here.

Our studies using antisera and EGFP-tagged proteins in murine tissues and a human intestinal epithelial cell line show that TAMP proteins concentrate at the tight junction and within cytoplasmic vesicles. Together with the relationships indicated by the evolutionary analysis, these data suggest that marvelD3, tricellulin, and occludin may fulfill similar roles in epithelial cells. This hypothesis is supported by several findings beyond our initial observation. First, TAMPs are predominantly expressed in epithelium-rich organs. Second, members of this protein subfamily interact physically (Figure 10), are concentrated in cholesterol-rich membrane microdomains of epithelial cells, and are incorporated into claudin-based tight junction-like strands between fibroblasts. Finally, the delays in development of barrier function after knockdown of any one TAMP are similar. Moreover, although more pronounced, the delays caused by knockdown of different combinations of TAMPs are comparable. It should be noted that the effect of tricellulin and occludin siRNA knockdown in the present study is different from previous published reports, which showed decreases in both baseline and peak TER (Ikenouchi et al., 2005; Yu et al., 2005; Krug et al., 2009). There are several possible explanations for the differences between our study using transient knockdown in human intestinal epithelial (Caco-2) cells and the previous studies that used stable knockdowns generated in mouse mammary (Eph4) and Madin-Darby canine kidney cells. First, cell tissue of origin may be critical. Such a hypothesis is consistent with the absence of gut pathology or intestinal epithelial barrier defects in human subjects with tricellulin mutations or in occludin knockout mice (Saitou et al., 2000; Schulzke et al., 2005). Alternatively, although our transient knockdown efficiency was more than 60% and significant knockdown was maintained for at least 6 d, we cannot exclude the possibility that residual TAMP expression or waning of the knockdown effect contributed to the barrier development that occurred in our studies. Nevertheless, the delay in barrier development demonstrates that, like the other TAMPs, marvelD3 is an important, but not essential, component of the tight junction.

The data presented here also suggest that marvelD3, tricellulin, and occludin make distinct contributions to tight junction structure and function. First, there are subtle differences between tissue distribution and subcellular localization. Moreover, deficits in tight junction assembly after knockdown of individual TAMPs cannot be compensated for by other family members. This observation might be expected for tricellulin, which has a unique affinity for cellular tight junctions and the lateral membranes beneath these regions and, perhaps as a reflection of this localization, distributes into detergent insoluble membrane fractions of higher density than do marvelD3 and occludin. However, the data also indicate differences between the roles of marvelD3 and occludin. For example, coimmunoprecipitation studies show that marvelD3 interacts with both tricellulin and occludin, but that the latter two TAMPs do not associate. This suggests that marvelD3 is present at both bicellular and tricellular tight junction regions and may play a broader organizational role than other TAMP family members. Such a role would also be consistent with the increased stability of marvelD3 at the tight junction, relative to tricellulin and occludin; the distinct behaviors of marvelD3 splice variants at bicellular versus tricellular tight junction regions; and, most strikingly, the unique responses of each TAMP to in vivo TNF exposure. As a whole, these data suggest that TAMP family members are functionally redundant and may be involved in distinct aspects of tight junction assembly, maintenance, or regulation.

In conclusion, these data demonstrate that marvelD3, tricellulin, and occludin define the TAMP family of tight junction proteins and that these proteins have overlapping, but nonredundant, expression, subcellular localization, and function. The results suggest that, as is true for claudin proteins, the role of TAMPs in paracellular barrier function is best considered in the context of a complete protein family. Finally, the identification of both the TAMP family and marvelD3 provide a new framework with which to understand the contributions of occludin and tricellulin to tight junction biology as well as tissue-specific pathologies that follow mutation or loss of individual TAMPs.

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