Modulation of Epithelial Morphology, Monolayer Permeability, and Cell Migration by Growth Arrest Specific 3/Peripheral Myelin Protein 22

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Peripheral myelin protein 22 (PMP22) is associated with a subset of hereditary peripheral neuropathies. Although predominantly recognized as a transmembrane constituent of peripheral nerve myelin, PMP22 is localized to epithelial and endothelial cell-cell junctions, where its function remains unknown. In this report, we investigated the role of PMP22 in epithelial biology. Expression of human PMP22 (hPMP22) slows cell growth and induces a flattened morphology in Madin-Darby canine kidney (MDCK) cells. The transepithelial electrical resistance (TER) and paracellular flux of MDCK monolayers are elevated by hPMP22 expression. After calcium switch, peptides corresponding to the second, but not the first, extracellular loop of PMP22 perturb the recovery of TER and paracellular flux. Finally, subsequent to wounding, epithelial monolayers expressing hPMP22 fail to migrate normally. These results indicate that PMP22 is capable of modulating several aspects of epithelial cell biology, including junctional permeability and wound closure.

INTRODUCTION

The tetraspan glycoprotein peripheral myelin protein 22 (PMP22), also known as growth arrest specific 3 (*gas3*) gene, has proposed roles in peripheral nerve myelin formation, cell-cell interactions, and cell proliferation (Suter and Snipes, 1995). Although the highest expression levels are found in myelin-forming Schwann cells, PMP22 mRNA can be detected in a multitude of developing and mature non-neural tissues, including epithelia of the intestine (Baechner *et al.*, 1995; Taylor *et al.*, 1995; Wulf and Suter, 1999) and the choroid plexus (Roux *et al.*, 2004). The specific role of PMP22 in Schwann cells remains undefined, although it is known that altered expression is associated with heritable demyelinating peripheral neuropathies (Naef and Suter, 1998). Similarly, the function of the protein at nonneural locations remains undetermined.

In vitro studies have identified a role for PMP22 in the regulation of cell proliferation and morphology. In Schwann cells, elevated expression delays the transition from G0/G1 to the S phase of the cell cycle (Zoidl *et al.*, 1995), and can lead to apoptosis in some instances (Fabbretti *et al.*, 1995; Zoidl *et al.*, 1997). Conversely, reduced PMP22 mRNA levels are associated with enhanced DNA synthesis and entry into the S + G2/M phases (Zoidl *et al.*, 1995). In NIH 3T3 fibroblasts, PMP22 overexpression regulates cell spreading, an effect that is dependent on the Rho-GTPase pathway (Brancolini *et al.*, 1999). Recent studies have detected exogenous

PMP22 in ADP-ribosylation factor 6 (Arf-6)-positive plasma membrane-endosomal recycling vacuoles before apoptosis or changes in cell shape (Chies *et al.*, 2003). This pathway is known to be involved in modulating the actin cytoskeleton, cell polarity, adhesion, and migration (Donaldson, 2003). Together, these findings support the notion that PMP22 has a significant role in basic cellular processes, extending beyond an involvement in Schwann cell myelination.

We previously described PMP22 as a constituent of apical intercellular junctions in epithelial and endothelial cells (Notterpek *et al.*, 2001; Roux *et al.*, 2004). Although PMP22 shares significant amino acid homology with members of the claudin superfamily, overexpression of the protein in L-fibroblasts (Notterpek *et al.*, 2001) or C6 glioma cells (Takeda *et al.*, 2001) did not induce tight junction strands. Nonetheless, PMP22 might function in the establishment and maintenance of ion-selective paracellular barriers. Transmembrane proteins of the apical junctional complex such as the claudins, occludin, and the junctional adhesion molecules (González-Mariscal *et al.*, 2003) all participate in the regulation of junctional permeability. In addition, based on the findings of Brancolini and colleagues (Brancolini *et al.*, 1999, 2000; Chies *et al.*, 2003), PMP22 might be involved in the regulation of epithelial proliferation and/or cell migration, dynamic processes that involve changes in cell adhesion and morphology. In support of this possibility, PMP22 contains the carbohydrate L2/HNK1 adhesion/recognition epitope in the first extracellular loop (Snipes *et al.*, 1993; Schachner *et al.*, 1995).

Here, we examined the role of PMP22 in several facets of epithelial cell biology, including proliferation, cell shape, and migration. An elevated level of PMP22 slows the migration of epithelia and reduces the formation of lamellipodial protrusions. The transepithelial electrical resistance (TER) and paracellular flux also are affected in these cultures. Application of a PMP22 peptide perturbs the TER and permeability of Madin-Darby canine kidney (MDCK) mono-

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Abbreviations used: PMP22, peripheral myelin protein 22; SF, scatter factor; TER, transepithelial electrical resistance; TX-100, Triton X-100; ZO-1, zonula occludens-1.

layers. Together, these results indicate that PMP22 plays a role in modulating growth, morphology, migration, and paracellular permeability in epithelial cells.

MATERIALS AND METHODS

Cell Culture

MDCK type I (high-resistance) and type II (low-resistance) cells were grown in Eagle's minimum essential medium supplemented with 5% fetal calf serum (FCS) or DMEM with 10% FCS, respectively. Cells were cultured on Costar Transwell filters (0.4- μ m pores) (Corning, Acton, MA) or on tissue culture dishes and maintained at 37°C and 5% CO₂. For TER or paracellular flux studies (see below), MDCK II monolayers were grown on filters (3×10^5 cells/cm²) in low Ca²⁺ media (see below) for 48 h to ensure confluence before Ca²⁺ addition. TER measurements were recorded every 24 h after Ca²⁺ addition until 6 d postplating, at which time TER levels had reached a steady state. After plating MDCK I cells on filters (3×10^5 cells/cm²), the medium was replaced every 24 h until the sixth day when TER levels had reached a steady state. For the Ca²⁺-switch assay (Gumbiner and Simons, 1986), cells were treated with EDTA (4 mM) containing media for 4 h (Notterpek *et al.*, 2001) or for 18 h in Ca²⁺- and Mg²⁺-free media with 5% Chelex (Sigma-Aldrich, St. Louis, MO)-treated FCS.

Establishment of Stable Transgene-expressing Cells

The human PMP22 (kind gift of Dr. Clare Huxley, Imperial College School of Science, Technology, and Medicine, London, United Kingdom) and human occludin (Van Itallie and Anderson, 1997) open reading frames were inserted into the pLNCX-II retroviral vector (BD Biosciences Clontech, Palo Alto, CA), under the control of the cytomegalovirus (CMV) promoter. After transient transfection with LipofectAMINE Plus reagent (Invitrogen, Carlsbad, CA), MDCK cells were treated with 1.1 mg/ml Geneticin (G418 sulfate) (Invitrogen) for 4 wk. Subclones of the stably expressing MDCK II cells were monitored for transgene expression by immunoblotting as described below. Three subclones were analyzed for each construct. Where indicated, 2.5 mM sodium butyrate was added for 20 h to enhance transgene expression under the CMV promoter (Gorman *et al.*, 1983).

Primary Antibodies

Monoclonal mouse antibodies to occludin, ZO-1 (Zymed Laboratories, South San Francisco, CA), β -catenin (BD Transduction Laboratories, Lexington, KY), E-cadherin clone rrl (Developmental Studies Hybridoma Bank, Iowa City, IA), α -tubulin, actin (Sigma-Aldrich), GP-135 (kind gift from Dr. George Ojakian, SUNY Downstate Medical Center, Brooklyn, NY), rat anti-E-cadherin (Zymed Laboratories), and rabbit polyclonal anti-occludin (Zymed Laboratories), anti-PMP22, and affinity-purified anti-PMP22 antibodies (Notterpek *et al.*, 2001; Roux *et al.*, 2004) were used.

Immunofluorescence Microscopy

MDCK cells plated on glass coverslips or 12-mm Transwells were grown to confluence and fixed with either 3% paraformaldehyde (PFA) followed by a 1-min incubation in 100% acetone at -20°C (for PMP22 detection) or 1% PFA followed by permeabilization with 0.2% Triton X-100 (TX-100) (junction staining). For z-plane imaging of confluent monolayers, filters were fixed with 4% PFA followed by 5-min incubation in 100% methanol at -20°C and then frozen in liquid nitrogen-cooled N-methylbutane before cryosectioning filters along the z-plane (Notterpek *et al.*, 2001). After blocking and incubation with primary antibodies, Alexa anti-mouse IgG fluorescein isothiocyanate (FITC)- and anti-rabbit IgG Texas Red-conjugated antibodies were added (Molecular Probes, Eugene, OR). Nuclei were stained with Hoechst dye (10 μ g/ml) (Molecular Probes). Actin filaments were visualized with FITC-conjugated phalloidin (Molecular Probes). Coverslips were mounted by using the Pro-Long Antifade kit (Molecular Probes). Samples were imaged with a Spot camera attached to a Nikon Eclipse 800 microscope and formatted for printing by using Adobe Photoshop 5.0. Images were measured using Spot Advanced 3.5.

Cell Cycle Proliferation Studies

The DNA synthesis rate of subconfluent MDCK II cells plated on glass coverslips was analyzed using a bromodeoxyuridine (BrdU) labeling and detection kit (Roche Diagnostics, Indianapolis, IN). The percentage of BrdU-positive cells was determined by counting in four random fields (0.8 mm²) and comparing with the total number of nuclei (Hoechst). More than 500 cells were counted for each condition. The percentage was calculated from the ratio between BrdU-positive and total cells. Cell cycle analysis was performed on exponentially growing MDCK cells by using the CycleTEST PLUS kit (BD Biosciences, San Jose, CA). The data were collected by fluorescence-activated cell sorter (FACSscan; BD Biosciences) and analyzed using Modfit version 2.0.

Epithelial Cyst Formation

To generate MDCK II cysts, 5×10^4 cells/ml were suspended in 2 mg/ml rat tail collagen type I (Sigma-Aldrich) on 6.5-mm Transwells (0.4 μ m) (Pollack *et al.*, 1998). After the collagen gel solidified at 37°C, medium was added to the apical and basal chambers and was replaced every 48 h. Eight days after plating, the collagen gels were fixed in 4% PFA before freezing in liquid nitrogen-cooled N-methylbutane. Cryosections (7 μ m) were processed for immunolabeling as described above.

Cell Surface Biotinylation and Western Blotting

For Western blotting, monolayers of confluent MDCK cells were lysed (Notterpek *et al.*, 2001), and where indicated, treated with N-glycosidase (PNGase F) (Pareek *et al.*, 1997). Samples were separated by SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA) before immunoblotting. Bound horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (Sigma-Aldrich) were detected using ECL reagents (PerkinElmer Life and Analytical Sciences, Boston, MA).

For the detection of PMP22 at the cell surface, confluent monolayers of stably expressing MDCK II cells (6-cm dish) were biotinylated with biocytin hydrazide (Lisanti *et al.*, 1989; Prince *et al.*, 1993). Biotinylated monolayers were lysed for affinity precipitation in NP-40 buffer (25 mM HEPES/NaOH, pH 7.4, 150 mM NaCl, 4 mM EDTA, 25 mM NaF, 1% NP-40, 1 mM Na₃VO₄, 1 $\times$ Complete protease inhibitor [Roche Diagnostics]) as reported previously (Sakakibara *et al.*, 1997). Streptavidin beads (Pierce Chemical, Rockford, IL) suspended in lysis buffer, were added to the cell lysate and gently rocked for 2 h at 4°C. Streptavidin beads were washed four times with NP-40 buffer, boiled in SDS gel sample buffer (Notterpek *et al.*, 1997), and treated with PNGase F. Samples were processed for immunoblotting with anti-PMP22 antibodies.

Measurements of Junctional Permeability

TER was measured in 37°C culture media by using an EVOM epithelial voltammeter with an STX-2 electrode (WPI, Sarasota, FL). The TER values were calculated by subtracting the background TER of blank filters and normalized by the area of the monolayer. Steady-state TER measurements ($n = 9$ wells/construct) were detected 6 d after cell plating under the described culture conditions. To measure nonionic paracellular flux, FITC-dextran of 3 and 40 kDa (Molecular Probes) was dissolved in P-buffer (Balda *et al.*, 1996) at a concentration of 10 mg/ml. Apical and basolateral compartments of cells cultured on Transwell filters ($n = 3-4$ wells/construct) were rinsed with P-buffer and allowed to equilibrate for 10 min. The FITC-dextran stock solution (25 and 50 μ g/100 μ l, 3 and 40 kDa, respectively) was added to the apical chamber, and the cells were incubated at 37°C for 30 min. By sampling the basal media, the amount of FITC-dextran diffusion from the apical to the basal chamber was measured in a VersaFluor fluorometer (Bio-Rad). A standard curve was used to convert relative fluorescent units to the concentration of dextran in solution.

Peptide Perturbation

High-performance liquid chromatography-purified peptides, corresponding to a portion of the first (aa 45-63) (NH₂-SALGAVQHCVSSSVSEWLQ-COOH) (PMP22-1st) and the entire second loop (aa 117-132) (NH₂-YTVRHE-WHVNVDYSY-COOH) (PMP22-2nd) of murine PMP22 were chosen (United Biochemical Research, Seattle, WA). A scrambled peptide using the same amino acids as the second loop of PMP22 (NH₂-HDEYVSNTHWYRSYTV-COOH) (scrambled-2nd) served as a control. A 44-aa peptide corresponding to the second extracellular loop of the chicken occludin (Occ-2nd) was used as a positive control (Wong and Gumbiner, 1997). Peptides were dissolved in dimethyl sulfoxide (DMSO) as 10 mM stocks and added to Ca²⁺-containing media at the indicated concentrations. Monolayers ($n = 3-4$ wells/condition) were fed every 24 h with fresh peptide-containing medium.

Wound Migration Studies

Wound assays using MDCK cells have been reported previously (Fenteany *et al.*, 2000; Sabo *et al.*, 2001). Highly confluent MDCK II monolayers on either glass coverslips or tissue culture plastic were wounded with a 200- μ l pipette tip (Sabo *et al.*, 2001). Long scratches and short wounds were made before rinsing the monolayer in fresh medium to remove detached cells. At the indicated time points after wounding, wound areas were imaged with a Nikon DS camera attached to a Nikon Eclipse TS100 inverted microscope. To ensure that identical areas were imaged between time points, multiple positioning marks were made at the center of the denuded surface with a small needle. Relative wound areas ($n = 3$ /construct) were measured with the NIH Image analysis program. Alternatively, the mean distance migrated along the wound edge ($n = 6$, measurements in two separate fields) was determined using Adobe Photoshop 5.0. The Rho-kinase inhibitor Y-27632 (10 μ M) (Calbiochem, San Diego, CA) was applied to MDCK monolayers 2 h after wounding, and monolayers were fixed for staining after an additional 3 h (Omelenko *et al.*, 2003). Scatter factor (SF) was obtained by culturing highly confluent NIH 3T3 fibroblasts in DMEM with 1% FCS for 72 h, followed by

Figure 1. PMP22 alters epithelial cell proliferation and morphology. (A) Total lysates (T), TX-100-soluble (S) and -insoluble (I) fractions (30 μ g/lane) of stable, neo, and human PMP22 (hPMP22)-expressing MDCK II cells were analyzed with (+) and without (–) PNGase F digestion. The glycosylated hPMP22 is detected at ~26 kDa (arrowhead) and after PNGase F treatment becomes readily visible, migrating at ~18 kDa (arrow). Compared with total lysates, hPMP22 is enriched in the detergent-insoluble pellet. (B) Plasma membrane targeting of hPMP22 was determined by cell surface biotinylation, followed by PNGase F treatment and immunoblotting with anti-hPMP22 antibodies. The majority of biotinylated hPMP22 is detected in the 1% NP-40-insoluble (I) fraction (arrow). Molecular mass, in kilodaltons. (C) As measured by BrdU incorporation in subconfluent cultures, compared with neo cells, hPMP22 expression reduces DNA synthesis by $32.9 \pm 9.5\%$, ($*p < 0.004$). (D) In confluent hPMP22-MDCK monolayers, the cell density is $51.7 \pm 8.4\%$ of the neo cultures ($*p < 0.004$). (E) The reduced cell density in confluent PMP22 monolayers is in agreement with an increase in nuclear area. Hoechst staining of representative cultures is shown. Bar, 15 μ m. Quantification of nuclei reveals ~1.5-fold increase in nuclear dimension of hPMP22-MDCK cells compared with neo controls ($*p < 0.0001$). (F) The apical area of the hPMP22-MDCK cells, outlined by ZO-1 immunostaining, is significantly larger than in neo cells ($*p < 0.0004$). Error bars in C–F show means $\pm$ SD. *p* values were determined by *t* test.

0.45- μ m filtration. Before use, SF was tested confirming its ability to induce the dispersion of small colonies of neo-MDCK cells (Stoker *et al.*, 1987).

Statistical Analysis

Where indicated, means and SDs were calculated, and statistical significance was determined by unpaired two-tail *t* test by using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA).

RESULTS

PMP22 Overexpression Alters Epithelial Cell Proliferation and Morphology

To investigate the role of PMP22 in epithelial biology, human PMP22 (hPMP22) was overexpressed in the pLNCX-2 vector under the control of the CMV promoter in MDCK II cells (hPMP22-MDCK), a frequently used model of polarized epithelia (Stevenson *et al.*, 1988). In total cell lysates (T), by using an antibody optimized to detect the human protein, hPMP22 is faintly observed at ~26 kDa (Figure 1A, arrowhead). On PNGase F treatment, the protein is detected more prominently at 18 kDa (Figure 1A, arrow). Enhanced immunoreactivity of PMP22 after endoglycosidase treatment has been observed previously (Fabbretti *et al.*, 1995). When the cell lysate is fractionated into detergent-soluble (S) and detergent-insoluble fractions (I), the overexpressed hPMP22 protein is highly enriched in the detergent-insoluble pool. Similar detergent solubility profiles have been observed for both the endogenous canine and exogenous myc-tagged rat PMP22 in MDCK cells (Notterpek *et al.*, 2001). Vector-only cells (neo) are not immunoreactive with the anti-human PMP22 antibody (Figure 1A).

We next examined the targeting of hPMP22 to the cell surface, by using biotinylation and subsequent streptavidin precipitation (Ryan *et al.*, 2002) (Figure 1B). Endoglycosidase treatment of the precipitated protein revealed hPMP22 at the plasma membrane. Because the majority of hPMP22 is found in the detergent-insoluble fraction (arrow), the cell surface protein is likely accumulated at apical intercellular junctions and/or, as reported in Schwann cells, possibly in lipid rafts (Erne *et al.*, 2002; Hasse *et al.*, 2002).

Because PMP22 is known to modulate cell cycle progression (Schneider *et al.*, 1988; Manfioletti *et al.*, 1990; Zoidl *et al.*, 1995, 1997; Karlsson *et al.*, 1999), we next examined how hPMP22 might affect epithelial proliferation. In subconfluent cultures, elevated levels of PMP22 lead to a ~33% reduction in BrdU incorporation, compared with neo cells (Figure 1C) or human occludin (Occ-MDCK)-expressing cells (our unpublished data). Furthermore, cell cycle analysis identified

Table 1. Cell cycle analysis of hPMP22-MDCK cells

	% G0/G1	% S	% G2/M
neo-MDCK	54.08 $\pm$ 0.18	28.79 $\pm$ 0.11	17.13 $\pm$ 0.20
hPMP22-MDCK	65.52 $\pm$ 0.59	22.73 $\pm$ 0.79	12.75 $\pm$ 0.23

Asynchronously growing MDCK cells were subjected to flow cytometric analysis to determine cell cycle distribution. hPMP22 expression results in a significant increase in cells in G0/G1 and a decrease in S and G2/M.

Figure 2. Protein polarity and junctional constituents in PMP22-MDCK monolayers. (A) Confluent, filter-grown neo- and hPMP22-MDCK monolayers were immunostained and examined by z-plane cryosectioning. In neo- and hPMP22-expressing monolayers, the protein polarity of GP-135 (green) and E-cadherin (red) are apical and basolateral, respectively. The flattened morphology of the PMP22 cells is apparent. Bar, 10 μm . (B) In a 3-D collagen matrix polarization model, both cultures form multicellular cysts with apical GP-135 (green) oriented toward the center. Nuclei are visualized by Hoechst dye (blue). Bar, 15 μm . (C) Compared with neo cultures, an increased level of PMP22 immunoreactivity is detected at intercellular junctions of hPMP22-MDCK cells, when the images are taken at a constant exposure time. In parallel monolayers, the localization of a representative tight and adherens junction constituent, occludin and β -catenin, respectively, seem comparable. Bar, 20 μm . (D) Immunoblotting of 0.5% TX-100-soluble (S) and -insoluble (I) fractions (20 μg /lane) reveals comparable levels and detergent solubilities for claudin-4, occludin, β -catenin, as well as actin, between confluent neo- and hPMP22-MDCK monolayers. Soluble claudin-4 is slightly decreased and insoluble occludin slightly elevated in the hPMP22-MDCK sample.

an increase in the percentage of PMP22 cells in G0/G1 (Table 1). At confluence, there were $51.7 \pm 8.4\%$ fewer cells in the hPMP22-MDCK cultures, compared with controls (Figure 1D). The lower cell density of confluent hPMP22 monolayers is readily visible by Hoechst imaging of nuclei (Figure 1E). The hPMP22 cell nuclei look larger, and when quantified, they reveal an ~ 1.5 -fold increase in area (Figure 1E, graph), whereas the Occ-MDCK cells were morphologically similar to neo cells ($100.4 \pm 2.4\%$). As predicted from a confluent monolayer with reduced cell density, the apical surface area of the hPMP22 cells, as determined by ZO-1 immunostaining (see below), is ~ 1.9 -fold larger than that of neo (Figure 1F) or occludin (our unpublished data) controls. These morphological characteristics of hPMP22-MDCK cultures suggest that the monolayer consists of fewer cells with a flattened morphology.

PMP22 Does Not Alter Epithelial Polarity or the Localization of Junctional Proteins

To further evaluate the altered epithelial morphology induced by hPMP22 overexpression, confluent filter-grown monolayers were double immunostained with anti-E-cadherin and anti-GP135 antibodies after z-plane cryosectioning. As expected, in neo-MDCK cultures, GP135 is apical (Ojakian and Schwimmer, 1988) and E-cadherin labels the lateral borders (Figure 2A). Although the hPMP22-MDCK cells are flattened, the protein polarity is similar to that of the neo controls. We also examined protein polarity in a three-dimensional (3-D) model of epithelial cysts in which MDCK cells form a polarized multicellular structure with the apical surface facing the lumen (Ojakian and Schwimmer, 1994). After sectioning and immunostaining for GP135, similar to neo cells, hPMP22 cells formed cysts with normal polarity (Figure 2B).

Because PMP22 is a constituent of apical intercellular junctions (Notterpek *et al.*, 2001; Roux *et al.*, 2004), we next investigated how hPMP22 overexpression affects the localization and detergent solubility properties of tight and ad-

herens junction molecules (Figure 2, C and D). Compared with neo cells, an elevated level of PMP22-like immunoreactivity is associated with the cell-cell contacts of hPMP22-MDCK monolayers (Figure 2C). The expression and localization of a representative tight junction-associated protein, such as occludin, as well as the adherens junction-associated β -catenin, seem similar between neo- and hPMP22-MDCK samples (Figure 2C). Likewise, the distribution of claudin-1, ZO-1, and E-cadherin was unaltered by PMP22 (our unpublished data). As described above (Figure 1F), the larger apical dimensions of the hPMP22 cells are apparent by the junctional immunostaining. To further examine the levels and detergent-solubility characteristics of junction-associated proteins, parallel samples were processed for immunoblotting with the indicated antibodies (Figure 2D). Four examined proteins, claudin-4, occludin, β -catenin, and actin, show similar expression levels and detergent-solubility properties between the neo- and hPMP22-MDCK cells, with a slight decrease in the soluble claudin-4 and an increase in insoluble occludin in the hPMP22 samples. Thus, the overexpression of hPMP22 does not alter epithelial protein polarity nor drastically change the levels or localization of representative tight and adherens junction molecules.

Paracellular Permeability Is Altered by PMP22 Expression

Overexpression of occludin in MDCK cells revealed a role for this protein in regulating paracellular resistance (Balda *et al.*, 1996, 2000; McCarthy *et al.*, 1996, 2000). Because PMP22 is similarly localized to intercellular junctions of MDCK cells (Notterpek *et al.*, 2001), we investigated whether the TER of the hPMP22-expressing epithelial monolayers is altered compared with neo controls. Two days after the addition of Ca^{2+} , hPMP22- and occludin-expressing cells have a high TER level indicative of newly confluent monolayers, after which the TER decreases to steady state by day 4 (Figure 3A). Occ-MDCK monolayers, used as a positive control, have TERs slightly higher than the hPMP22 cells. By 4 d after

Figure 3. Altered paracellular permeability of epithelial monolayers by hPMP22 expression. (A) TER recordings beginning 2 d after the addition of Ca^{2+} , from newly confluent MDCK monolayers are shown. Three different clones of neo, hPMP22, and Occ cells ($n = 3$ filters/clone) were used for the quantification. Compared with neo cultures, the TER is elevated in hPMP22 and Occ cells. (B) At steady state, 4 d after plating, hPMP22- and Occ-MDCK monolayers have increased TER, compared with neo cells (* $p < 0.0001$). (C) Treatment with sodium butyrate (20 h) further enhances the differences in TER values between control and hPMP22, as well as Occ monolayers (* $p < 0.0001$). (D) The paracellular flux of 3-kDa nonionic FITC-dextran by hPMP22- and Occ-MDCK monolayers is significantly elevated (* $p < 0.0001$), compared with neo cells. Error bars show means $\pm$ SD. p values were determined by t test.

Ca^{2+} addition, the monolayers reach a steady-state level of confluence, and the TER of the hPMP22 and occludin cultures is ~ 1.6 -fold higher than the neo control (Figure 3B). To enhance transgene expression, confluent monolayers were treated with sodium butyrate for 20 h (Gorman *et al.*, 1983), leading to a 5.8- and 1.6-fold increase in hPMP22 and Occ protein levels, respectively. As indicated in Figure 3C, butyrate-treated PMP22- and Occ-MDCK monolayers exhibit a 3.2- and 4.1-fold increase in TER, respectively, compared with butyrate-treated neo cells. A similar phenomenon has been observed previously in epithelial monolayers overexpressing occludin (Balda *et al.*, 1996, 2000; McCarthy *et al.*, 1996, 2000). Because hPMP22 alters MDCK cell morphology, TER values were normalized to the relative linear junctional length for each culture. After correction for junctional length, the differences in TER values between neo control and PMP22- or occludin-overexpressing cells remained highly significant ($p < 0.001$), with or without butyrate treatment. Expression levels of junctional proteins can alter paracellular flow of nonionic molecules (Balda *et al.*, 1996, 2000; McCarthy *et al.*, 1996, 2000); therefore, we performed a dextran flux assay on confluent hPMP22-MDCK monolayers. Compared with neo controls, the flux of a 3-kDa nonionic FITC-labeled dextran is elevated ~ 17 -fold in both the hPMP22- and Occ-MDCK monolayers (Figure 3D). These results indicate that elevated expression of hPMP22 results in altered permeability of MDCK monolayers.

Figure 4. PMP22 peptides perturb the TER of epithelial monolayers. (A) Peptides corresponding to a portion of the first (1) and the entire second (2) extracellular loops of murine PMP22, a scrambled second loop, and the second extracellular loop of chicken occludin were applied to confluent MDCK monolayers after a Ca^{2+} switch. (B) Twenty hours after the addition of the peptides, the TER of PMP22-2nd peptide-treated monolayers (32 μ M) remains low compared with naive, DMSO-, PMP22-1st-, or scrambled-2nd-treated cells (** $p < 0.0001$). TER reformation by monolayers exposed to Occ-2nd peptide (16 μ M) is also significantly perturbed (* $p < 0.0006$). (C) Dosage curve for PMP22-2nd peptide reveals an effective concentration range for TER disruption between 8 and 32 μ M. Error bars show means $\pm$ SD. p values were determined by t test. (D) After a 20-h PMP22-2nd peptide treatment, and subsequent washout, the monolayers regain a TER similar to untreated samples.

Epithelial Monolayer Permeability Is Perturbed by PMP22 Peptides

An alternative approach to elucidate the role of junctional proteins is to apply peptides that correspond to extracellular domains of endogenous proteins (Wong, 1997; Wong and Gumbiner, 1997; Lacaz-Vieira *et al.*, 1999; Vietor *et al.*, 2001; Tavelin *et al.*, 2003; Lee *et al.*, 2004). Because the canine PMP22 has not been cloned, we designed peptides representing portions of the first and second extracellular domains of the mouse protein (Figure 4A). Although PMP22 has homology to some members of the claudin family, the proteins are less similar in the extracellular domains (Align software; our unpublished data). Confluent MDCK I monolayers were treated with the indicated peptides, after a Ca^{2+} switch. Twenty hours after the readdition of Ca^{2+} with the corresponding peptides (PMP22-1st, -2nd, PMP22-scrambled-2nd, or Occ-2nd), the TER of the monolayers was recorded (Figure 4B). As expected based on the literature (Wong, 1997; Wong and Gumbiner, 1997; Vietor *et al.*, 2001), the Occ-2nd loop peptide inhibited TER recovery by $50.4 \pm 8.7\%$. Similarly, the PMP22-2nd loop peptide diminished TER recovery by $91.3 \pm 0.4\%$. Application of vehicle (DMSO), PMP22-1st, or scrambled-2nd peptides had no significant effect on the TER compared with naive cells (Figure 4B). The application of PMP22-2nd peptide to intact monolayers had no effect on TER values (our unpublished data). A concentration curve for PMP22-2nd peptide identified an effective range of TER disruption between 8 and 32 μ M (Figure 4C). After 60 h of PMP22-2nd treatment, MDCK

Figure 5. PMP22 peptides increase the paracellular flux of epithelial monolayers. (A) MDCK monolayers were treated with the indicated peptides and the paracellular flux of 3-kDa, and 40-kDa FITC-labeled nonionic dextrans were determined. In PMP22-2nd (***p* < 0.0001) and Occ-2nd-treated monolayers (**p* < 0.002), the paracellular flow of the 3-kDa, but not the 40-kDa, dextran is significantly elevated. Error bars show means $\pm$ SD. *p* values were determined by *t* test. (B) In PMP22-2nd and scrambled-2nd peptide-treated monolayers (32 μ M), the localization of ZO-1 and E-cadherin remain comparable. Bar, 20 μ m.

monolayers remained unable to restore TER (our unpublished data). The washout of PMP22-2nd peptide from MDCK monolayers results in the restoration of the TER to control levels (Figure 4D). Therefore, the disruptive effect of the PMP22-2nd loop peptide on the monolayer TER is reversible.

Next, we examined the paracellular flux and the morphology of peptide-treated monolayers (Figure 5). The flux of the 3-kDa, but not the 40-kDa FITC-dextran is significantly elevated in both the PMP22-2nd- and Occ-2nd-loop-treated monolayers, indicating a size selective disturbance of paracellular permeability (Figure 5A). The observed increase in the flow of the 3-kDa dextran in Occ-2nd-treated cultures is in agreement with previous reports (Wong and Gumbiner, 1997). To determine whether the PMP22-2nd peptide treatment alters the distribution of junctional constituents, the localization of ZO-1 and E-cadherin was examined in parallel peptide-treated monolayers (Figure 5B). Both ZO-1 and E-cadherin seem unaltered after treatment with PMP22-2nd or scrambled-2nd peptides. These findings indicate that the PMP22-2nd peptide reduces monolayer permeability with-

out radically altering major constituents of apical intercellular junctions.

PMP22 Expression Slows the Migration of Epithelial Monolayers

Injured epithelial monolayers display signs of tight junction remodeling before successful wound closure as judged by the down-regulation of ZO-1 and occludin mRNAs (Cao *et al.*, 2002). Because the expression of hPMP22 alters the morphology and permeability of epithelial monolayers, we hypothesized that the protein might modulate the dynamic processes involved in epithelial migration. To investigate this, hPMP22-overexpressing epithelial cells were tested in a two-dimensional wound-migration assay (Figure 6A) (Fenteany *et al.*, 2000). By phase microscopy, 24 h after wounding, the neo cultures nearly close the denuded area (Figure 6A, top). In comparison, the rate of monolayer closure is visibly reduced in hPMP22-MDCK cells (Figure 6A, bottom), which do not cover the denuded area until ~48 h postwounding. Indeed, within a 24-h period, hPMP22 monolayers exhibit a $60.3 \pm 6\%$ reduction in wound closure compared with neo cells (Figure 6B). At higher magnification of Hoechst-stained samples, the neo monolayers display a characteristic wave of migrating cells at the wound edge that look flattened and spread out (Scheffers *et al.*, 2003; Matsubayashi *et al.*, 2004) (Figure 6C). In contrast, the nuclei of hPMP22-expressing cells are more compact at the wound edge and are uniformly spaced throughout the monolayer. Wound closure is unaffected by the overexpression of occludin (our unpublished data).

In response to monolayer wounding, migrating MDCK cells maintain cell-cell contacts, form an actin purse-string along the wound edge and pull multiple cell rows forward, by Rac-dependent lamellipodial crawling (Fenteany *et al.*, 2000). Lamellipodial protrusion by leader cells, but not the formation of an actin purse-string, is required by MDCK monolayers to close a wound (Fenteany *et al.*, 2000). Therefore, we examined these two structures in hPMP22-MDCK cells (Figure 7A). As expected, 24 h after monolayer wounding, neo cells (top row) have an actin-belt along the wound edge; with periodic breaks (arrowhead on the right) representative of migrating cells. Leader cell lamellipodia are observed in the neo cultures by tubulin and E-cadherin labeling (arrowheads). An actin purse-string is continuous along the migrating edge of hPMP22 monolayers (Figure 7A, bottom); however, breaks in this actin-belt are largely absent. Additionally, the tubulin and E-cadherin immunoreactivities seem concentrated along the leading edge in hPMP22 cells of the wound, and the monolayers have fewer cells extending lamellipodia.

Because lamellipodial protrusion is crucial for MDCK monolayer migration, we examined whether inhibition of Rho-kinase, known to induce lamellipodial expansion in wounded epithelial monolayers (Omelchenko *et al.*, 2003), could overcome the effects of hPMP22 overexpression (Figure 7B). As expected, a 3-h treatment of neo-MDCK cells with a Rho-kinase inhibitor (Y-27632) leads to increased lamellipodial-like cell protrusion, visualized by actin-phalloidin (Figure 7B, arrows). In comparison, hPMP22-MDCK cells seem resistant to the formation of lamellipodia, with few cell protrusions apparent along the wound edge (arrow). Thus, the expression of PMP22 results in the reduced migration of MDCK cells after wounding.

Fibroblast-derived SF has been shown to induce an epithelial to mesenchymal transition (EMT) in MDCK cells (Stoker *et al.*, 1987). Therefore, we investigated whether SF is capable of overriding the inhibitory effect of hPMP22 expres-

Figure 6. PMP22 alters wound healing by epithelial monolayers. (A) Confluent neo- and PMP22-MDCK monolayers were wounded with a pipette tip, and the migration of the cells into the wound area (borders outlined in black) was evaluated at 2 and 24 h. The neo-MDCK monolayers nearly close the wound by 24 h, whereas hPMP22-MDCK cells are unable to similarly reduce the wound area. Bar, 800 μm . (B) Quantification of wounding experiments reveal that compared with naïve and neo cells, hPMP22-MDCK cells are significantly less competent to migrate (* $p < 0.0003$). Error bars show means $\pm$ SD. p values determined by t test. (C) Hoechst staining of parallel samples shows dispersed nuclei along the wound edge in the neo cultures. In comparison, the nuclei of the hPMP22-MDCK cells are densely packed and uniform throughout the image. Bar, 150 μm .

Figure 7. Lamellipodial protrusion in migrating epithelial monolayers is reduced by PMP22. (A) Distribution of actin, α -tubulin, and E-cadherin was examined by fluorescence microscopy along the wound edge at 24 h postwounding. In neo cultures, an actin purse-string and lamellipodial protrusions (arrowheads) are detected (top row). In wounded PMP22 samples, the actin purse-string is uninterrupted and α -tubulin and E-cadherin seem concentrated along the wound edge. Lamellipodial protrusions into the wound space are largely absent in the PMP22-MDCK monolayers. Bar, 40 μm . (B) A 3-h treatment of wounded monolayers with Y-27632, a Rho kinase inhibitor, induces extensive lamellipodial protrusions (arrows) along the wound edge of neo cells, visualized by fluorescent labeling of actin. In PMP22-MDCK monolayers, Y-27632 is unable to bring about a similar response, as cells with lamellipodia are sparse (arrow). Bar, 50 μm . (C) Quantification of epithelial migration after wounding (5 h), in the absence and presence of SF. In normal culture medium, neo cells migrate faster than PMP22-MDCK monolayers. The addition of SF to the medium significantly increases the migration of neo- and PMP22-MDCK cells (* $p < 0.0003$; ** $p < 0.0001$, respectively). Error bars show means $\pm$ SD. p values were determined by t test.

sion on MDCK monolayer migration. When cultured in SF, neo and hPMP22 cells migrate a similar distance 5 h after a scratch wound (Figure 7C, gray bars). Compared with monolayers in normal medium (Figure 7C, white bars), wound closure in the presence of SF increases by 1.8- and 4.7-fold for the neo and hPMP22-MDCK cells, respectively. These results indicate that although hPMP22-MDCK cells are resistant to the effects of Rho kinase inhibition, they are competent to migrate after SF-induced EMT.

DISCUSSION

The described results indicate that PMP22 plays a role in several aspects of epithelial biology. The overexpression of PMP22 reduces the proliferation and final cell density of epithelial monolayers and induces flattened cell morphology. Monolayers of such cultures have increased TER and paracellular flux of nonionic dextrans. In agreement, a PMP22 peptide disrupts the reformation of paracellular resistance after Ca^{2+} switch. The migration of epithelial monolayers also is reduced by PMP22 overexpression, possibly due to a deficiency in lamellipodial-forming leader cells. These results suggest that PMP22 takes part in a pathway by which apical cell junctions regulate the proliferation and morphology of epithelial cells and modulate paracellular permeability and cell motility.

Cell junction-associated proteins are known to influence cell proliferation. For example, in addition to regulating paracellular permeability (Balda and Matter, 2000; Reichert *et al.*, 2000), elevated levels of ZO-1 reduce proliferation and cell density in MDCK cells (Balda *et al.*, 2003). This effect is thought to result from sequestration of the transcription factor ZONAB, a ZO-1 binding partner, from the cell nucleus with a concomitant decrease in the level of nuclear CDK4 (Balda *et al.*, 2003). It is yet unknown by what mechanism PMP22 elicits a similar response in the MDCK model, although by quantitative Western analysis the levels of nuclear CDK4 are unchanged between PMP22 and neo control cells (our unpublished data). Thus, the PMP22-mediated effects on cell density and cell cycle do not seem to involve the ZONAB pathway. In Schwann cells and fibroblasts, overexpression of PMP22 by retroviral and transient transfection induces growth arrest, and in some instances apoptosis (Fabbretti *et al.*, 1995; Zoidl *et al.*, 1997; Brancolini *et al.*, 1999). In our stably transduced cell populations, increased apoptosis as judged by Hoechst staining was not observed, possibly due to a lower level of PMP22 expression.

In several cell types the overexpression of PMP22 affects cellular morphology (Brancolini *et al.*, 1999; 2000; Chies *et al.*, 2003). The altered cell shape observed in PMP22-MDCK monolayers is likely the consequence of reduced cell density at confluence, in which a flattened morphology is necessary for maintaining functional cell-cell junctions. An elevated TER can be the result of such a phenomenon, because confluent monolayers with reduced cell density have less linear tight junctional length (Marcial *et al.*, 1984), and paracellular junctions are more permeable than the cell itself (Stefani and Cereijido, 1983). However, after correction for junctional length, the effects of PMP22 overexpression on TER remained highly significant. In accordance, the reduced cell density reported after ZO-1 overexpression did not significantly alter the TER of the MDCK monolayers (Balda and Matter, 2000). In contrast, as shown here and by others (Balda *et al.*, 1996, 2000; McCarthy *et al.*, 1996, 2000), elevated levels of occludin increased the TER, but it was not reported to alter the cell morphology or density of the monolayers.

A role for PMP22 in modulating paracellular flow is supported by the increased ionic and nonionic permeability after exposure to the PMP22-2nd loop peptide. The reduced TER and increased flux of small dextrans may indicate that the second loop peptide disrupts homotypic interactions of PMP22. Indeed, PMP22 is known to form dimers and larger oligomers *in vivo* and *in vitro* (Tobler *et al.*, 1999, 2002). As the extracellular domains of PMP22 share no significant homology with the claudins, a potential direct effect on claudins is unlikely. However, the peptides may be perturbing the function of other, as of yet undetermined, epithelial

junction-associated proteins that are binding partners for PMP22.

The localization of exogenous and endogenous PMP22 at apical intercellular junctions (Notterpek *et al.*, 2001; Roux *et al.*, 2004), combined with the effects of elevated PMP22 expression or PMP22 peptides on paracellular permeability, supports the notion that PMP22 is a functional constituent of the apical junctional complex. Although it has not been determined ultrastructurally whether PMP22 is at tight or adherens junctions, the protein is capable of altering both the ionic and nonionic permeability of epithelial monolayers. Similar effects have been attained in studies when the expression of the tight junction protein occludin (Balda *et al.*, 1996, 2000; McCarthy *et al.*, 1996, 2000) was modulated. Because PMP22 is detected at intercellular contacts of rat neuroepithelia (Roux *et al.*, 2004), cells devoid of classical tight junctions (Mollgard *et al.*, 1987; Aaku-Saraste *et al.*, 1996), the role of the protein might not be exclusive to tight junctions. In this respect, PMP22 is similar to ZO-1, a junctional protein that in some cell types exists at sites other than the tight junctional complex (Itoh *et al.*, 1993).

Epithelial cells maintain physical contacts during wound closure, whereas they extend Rac-GTPase-dependent lamellipodia (Fenteany *et al.*, 2000). In normal epithelia, lamellipodial protrusion is promoted by inhibiting Rho kinase, likely by disrupting the actin marginal bundles along the wound edge (Omelchenko *et al.*, 2003). PMP22 expression, however, prevents the lamellipodial formation even after Y-27632 treatment. This suggests that an elevated level of the protein interferes with the signaling for lamellipodial protrusion, possibly by acting via the actin cytoskeleton directly or indirectly by modulating the Rac GTPase pathway. In comparison, the same MDCK cells are capable of migration after the application of SF, suggesting that the inhibitory action of PMP22 on wound-induced migration is likely dependent upon epithelial cell-cell contacts, because SF induces a junction-disrupting EMT (Grisendi *et al.*, 1998).

Because PMP22 is predominantly expressed in myelinating Schwann cells, what relevance do these studies in epithelia have to understanding the protein's function in the peripheral nervous system (PNS)? The growth arrest properties of PMP22 seem to occur independently of cell type, but they have yet to be directly linked to PMP22-associated disease pathology. Cell morphology is drastically altered during myelination, an event that involves extensive membrane expansion; however, PMP22 is not required for myelin wrapping (Adlkofer *et al.*, 1995). Cell migration is also crucial to proper nerve development (Lobsiger *et al.*, 2002). Nonetheless, our results show that when epithelia undergo EMT and migrate as individual cells, the overexpression of PMP22 has no inhibitory effect. Typically thought of as a component of compact myelin (Haney *et al.*, 1996), PMP22 has not yet been localized to tight junctions of PNS myelin. Based on the effects of PMP22 on epithelial paracellular permeability, the protein could have a similar role in PNS myelin at claudin-1- and -5-positive autotypic tight junctions (Poliak *et al.*, 2002). Therefore, modulating PMP22 in an epithelial model may provide some clues as to the function of the protein in PNS myelin.

In addition to identifying the participation of PMP22 in epithelial cell biology, we established an *in vitro* model that is amenable to further experimentation. Using this system, the specific activity of various PMP22 domains can be dissected by examining their effects on epithelial permeability and migration. It will be equally important to identify binding partners of PMP22 at intercellular junctions to fully

understand how the protein signals such global changes in epithelial cell biology.

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