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Density-enhanced Phosphatase 1 Regulates Phosphorylation of Tight Junction Proteins and Enhances Barrier Function of Epithelial Cells*

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Cell-cell adhesion is a dynamic process that can activate multiple signaling pathways. These signaling pathways can be regulated through reversible tyrosine phosphorylation events. The level of tyrosine phosphorylation of junctional proteins reflects the balance between protein-tyrosine kinase and protein-tyrosine phosphatase activity. The receptor-tyrosine phosphatase DEP-1 (CD148/PTP-η) has been implicated in cell growth and differentiation as well as in regulating phosphorylation of junctional proteins. However, the role of DEP-1 in regulating tight junction phosphorylation and the integrity of cell-cell junctions is still under investigation. In this study, we used a catalytically dead substrate-trapping mutant of DEP-1 to identify potential substrates at cell-cell junctions. We have shown that in epithelial cells the trapping mutant of DEP-1 interacts with the tight junction proteins occludin and ZO-1 in a tyrosine phosphorylation-dependent manner. In contrast, PTP-PEST, Shp2, and PTPμ did not interact with these proteins, suggesting that the interaction of DEP-1 with occludin and ZO-1 is specific. In addition, occludin and ZO-1 were dephosphorylated by DEP-1 but not these other phosphatases *in vitro*. Overexpression of DEP-1 increased barrier function as measured by transepithelial electrical resistance and also reduced paracellular flux of fluorescein isothiocyanate-dextran following a calcium switch. Reduced DEP-1 expression by small interfering RNA had a small but significant increase in junction permeability. These data suggest that DEP-1 can modify the phosphorylation state of tight junction proteins and play a role in regulating permeability.

Tight junctions are the most apical of junctions formed by epithelia and provide a regulated barrier to paracellular transport of ions, solutes, macromolecules, and even other cells. In addition, tight junctions act as a "fence" within the plane of the membrane, dividing the apical and basolateral domains of polarized epithelial cells. These junctions play an important role in the regulation of multiple cellular processes including cell differentiation, proliferation, and polarity (for reviews see Refs. 1 and 2). Functional tight junctions are characterized by the presence of membrane spanning proteins (claudins, occludin, and JAMs), which interact with cytoplasmic proteins (AF-6 and ZO-1, -2, -3), regulating assembly and maintenance of tight junctions. Occludin spans the membrane four times and was the first transmembrane component of the tight junction to be identified (3). It has two extracellular regions, an intracellular loop, as well as both an N- and C-terminal cytoplasmic tail (3). The C-terminal tail of occludin binds directly to the ZO family of proteins, which link the protein complex to the actin cytoskeleton (4–8). The long C-terminal domain is rich in serine, threonine, and tyrosine residues (9). In fact, several kinases and phosphatases interact with and modulate the phosphorylation state of tight junction proteins (10–14). Serine and threonine phosphorylation of occludin is abundant in epithelia with intact junctions, whereas tyrosine phosphorylation is undetectable (15). However, tyrosine phosphorylation of occludin is associated with a decrease in transepithelial electrical resistance (TER)[2] (16, 17) and loss of protein localization at the tight junction (18). Increases in tyrosine phosphorylation of occludin and ZO-1 result in the dissociation of the occludin-ZO-1 complex and reduces the localization at the tight junction of these proteins (12, 19). These data suggest that the phosphorylation state of tight junction proteins can affect the integrity of the tight junction complex and therefore the integrity of the tight junction itself. Both serine and threonine kinases and phosphatases bind to and act on TJ proteins (reviewed in Ref. 20). Previous studies have identified that c-Src and c-Yes are protein-tyrosine kinases, which act on the TJ, however, to date no protein-tyrosine phosphatases have been specifically characterized as acting on TJ proteins (11–13, 21).

Similarly, the other major junction of epithelia, the adherens junction (AJ), is also regulated by tyrosine phosphorylation. Increased tyrosine phosphorylation of the AJ decreases the stability of the cadherin-catenin complex, disrupting the association with the cytoskeleton and reducing junctional integrity (22–24). Therefore, these studies suggest that maintenance of junctional integrity for both the TJ and AJ is regulated in part by reversible tyrosine phosphorylation that results from a competing balance of protein-tyrosine kinase and protein-tyrosine

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[2] The abbreviations used are: TER, transepithelial electrical resistance; DEP-1, density-enhanced phosphatase 1; ZO-1, zonula occludens-1; GFP, green fluorescent protein; PTP, protein-tyrosine phosphatase; MDCK, Marin-Darby canine kidney; HEK, human embryonic kidney; EGF, epidermal growth factor; FITC, fluorescein isothiocyanate; AJ, adherens junction; TJ, tight junction; DMEM, Dulbecco's modified Eagle's medium; WT, wild type; GST, glutathione *S*-transferase; siRNA, small interfering RNA.

phosphatase (PTP) activity. Several PTPs have been localized to AJs and shown to bind components of the cadherin-catenin complex. The PTPs in AJs include receptor-PTPs (PTPμ, DEP-1, and vascular endothelial-PTP), as well as cytosolic PTPs (PTP1B and Shp-2) (25–30). The high concentration of PTPs at cell-cell junctions indicates the importance of maintaining low levels of tyrosine phosphorylation except when the junctions need to be remodeled or disassembled.

DEP-1 (density-enhanced phosphatase-1) is a receptor PTP that was first cloned from a human cDNA library and named based on the observation that its expression was elevated with increasing cell density (31). Also known as PTP-η, PTPRJ, and CD148, DEP-1 is comprised of an extracellular domain of eight fibronectin type III repeats, a transmembrane domain, and a single cytoplasmic catalytic domain. The protein is ubiquitously expressed (32), indicating its potential involvement in a large number of diverse signaling pathways. DEP-1 is involved in regulating the differentiation of epithelial cells (33–36), as well as controlling cell growth and adhesion (33, 34). In addition, DEP-1 is able to attenuate the cellular response to growth factors through the preferential dephosphorylation of several growth factor receptors, suggesting that DEP-1 can selectively dephosphorylate certain tyrosines to more finely control signaling (37–41).

In addition to its role in proliferation and differentiation, DEP-1 localizes to areas of cell-cell adhesion in endothelial and epithelial cells, overlapping with the AJ marker vascular endothelial-cadherin in endothelia (42). Interaction with p120 catenin as well as other members of the catenin family also supports the hypothesis that DEP-1 plays a role in regulating AJ protein phosphorylation (27, 40). In the current study, we investigated whether adjacent tight junction proteins are also substrates of DEP-1. We now demonstrate that the substrate-trapping mutant of DEP-1 interacts with the tight junction proteins occludin and ZO-1. The association of DEP-1 with occludin and ZO-1 is specific to DEP-1 and not other phosphatases tested. In addition, DEP-1 is able to dephosphorylate occludin and ZO-1 indicating that these tight junction proteins are substrates of DEP-1. Furthermore, increased expression of DEP-1 enhances barrier function as junctions reform following a calcium switch and loss of DEP-1 levels increased the permeability of a stable epithelial monolayer. Together these results indicate that ZO-1 and occludin are substrates of DEP-1 and imply a role for DEP-1 in influencing the phosphorylation state of tight junction proteins and junction permeability.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections

MCF10A cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium supplemented with 5% horse serum, 20 ng/ml epidermal growth factor (EGF), 0.01 mg/ml insulin, 500 ng/ml hydrocortisone, 100 ng/ml cholera toxin and antibiotics. MDCK II, A431, and HEK 293 cells were cultured in Dulbecco's modified Eagle's medium-high glucose enriched with 10% fetal bovine serum and antibiotics. Cells were transfected with Lipofectamine 2000 (Invitrogen) or FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions. RNA interference oligos were transfected using TransIT-siQUEST (Mirus).

Constructs and Antibodies

pMT2 DEP-1 WT, pMT2 DEP-1 D/A, and pMT2 DEP-1 C/S (GenBank^{TM} accession number U10886) were kindly provided by Nicholas Tonks (Cold Spring Harbor Laboratory). GFP DEP-1 WT was generated by subcloning the pMT2 construct into pEGFP-N2 (Clontech). DEP-1 cytoplasmic domain constructs were generated using the pMT2 DEP-1 wild type, D1205A, or C1239S point mutants as templates. A 5′ primer introduced an EcoRI site before the DEP-1 cytoplasmic sequence at nucleotide 3338 and a 3′ primer introduced a XhoI site after the DEP-1 stop codon. The resulting PCR fragments (nucleotides 3338–4362) were cloned into the EcoRI/XhoI sites of the pGEX-4T-1 vector (GE Healthcare), creating wild type and point mutants D1205A and C1239S pGEX-DEP-1 constructs. GST-PTP-PEST wild type and the substrate-trapping mutant were obtained from Dr. Sarita Sastry (University of Texas, Galveston, TX), and GST-PTPμ wild type and substrate-trapping mutant were kind gifts from Dr. Susann Brady-Kalnay (Case Western). Full-length occludin was provided by Alan Fanning (University of North Carolina, Chapel Hill, NC). For adenovirus, the full-length cDNA of DEP-1 WT or DEP-1 D/A was cloned into pENTR1D (Invitrogen). The entry clone was recombined with the pAd/CMV/V5 destination vector via LR clonase II. The ViraPower Adenviral expression vector (Invitrogen) was linearized and then transfected into 293A cells via Lipofectamine 2000 to generate adenovirus encoding V5-tagged DEP-1 WT and DEP-1 D/A. The virus particles were used to infect MDCK II cells. RNA interference oligos were ON-TARGETplus SMARTpool siRNA for DEP-1 or ON-TARGETplus Control siRNA 1 (Dharmacon).

Mouse anti-occludin, rabbit anti-ZO-1, and mouse anti-ZO-1 antibodies were purchased from Zymed Laboratories Inc.. Mouse anti-human CD148 (DEP-1) antibody was purchased from BIOSOURCE. GFP monoclonal antibody was from Roche. Monoclonal antibodies to E-cadherin, p120-catenin, and moesin were purchased from BD Bioscience, anti-Src was purchased from Millipore, AF-6 rabbit polyclonal was from Novus Biological, and monoclonal PY-20 was purchased from Santa Cruz Biotechnology.

GST Fusion Proteins

Expression of the fusion proteins in *Escherichia coli* was induced with 100 μM isopropyl β-D-thiogalactopyranoside for 16 h at room temperature. Bacterial cells were lysed in buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 1% Triton X-100, 5 mM $MgCl_2$, 1 mM dithiothreitol, 10 μg/ml each of aprotinin and leupeptin, and 1 mM phenylmethylsulfonyl fluoride and the fusion proteins were purified by incubation with glutathione-Sepharose 4B beads (GE Healthcare) at 4 °C. Catalytic activity of the fusion proteins was checked by PTP activity assays using *p*-nitrophenyl phosphate (Sigma).

Substrate Trapping Pulldown

MCF10A cells were either left untreated or treated with 100 μM pervanadate (phosphatase inhibitor) for 15 min prior to lysis. Cells were rinsed twice in phosphate-buffered saline and lysed in a modified RIPA buffer (1% Triton X-100, 0.5% deoxycholate, 0.2% SDS, 150 mM NaCl, 20 mM Hepes, pH 7.4, 2 mM EDTA, 10 μg/ml each aprotinin and leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Insoluble material was removed by centrifugation. MCF10A lysates (1 mg) were incubated with 10 μg of GST proteins for 1 h at 4 °C unless otherwise noted. Beads were washed in the modified RIPA buffer, resuspended in Laemmli sample buffer, and analyzed by SDS-PAGE and Western blotting on polyvinylidene difluoride membranes (Millipore).

To determine whether the substrates were binding DEP-1 D/A at the PTP active site, we tested the effect of orthovanadate on complex formation. GST fusion proteins bound to glutathione-Sepharose were preincubated in a Hepes lysis buffer (1% Triton X-100, 150 mM NaCl, 20 mM Hepes, pH 7.5, 5 mM $MgCl_2$), with or without 2 mM orthovanadate. MCF10A cells were treated with 100 μM pervanadate for 15 min and also lysed in Hepes lysis buffer with or without 2 mM orthovanadate. Lysates (500 μg to 1 mg) were incubated with 2 μg of GST-DEP-1 or GST-DEP-1 D/A for 1.5 h at 4 °C. Beads were washed in the Hepes lysis buffer, resuspended in Laemmli sample buffer, and analyzed by SDS-PAGE and Western blotting on polyvinylidene difluoride membranes. Because ZO-1 interaction was difficult to detect with low concentrations of recombinant DEP-1 D/A, 50 μg of GST-DEP-1 D/A was incubated with lysates prepared with or without 10 mM orthovanadate for 1.5 h at 4 °C to efficiently compete for the substrate binding.

Dephosphorylation Assays

MCF10A cells were treated with 100 μM pervanadate for 15 min, washed twice with phosphate-buffered saline, and lysed in the modified RIPA buffer plus 10 μg/ml each aprotinin and leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 5 mM iodoacetic acid. After incubation of the lysates on ice for 10 min, dithiothreitol was added at a final concentration of 10 mM for another 10 min on ice to inactivate the iodoacetic acid. Lysates were clarified and 500 μg of total cell lysate was incubated with 1 μg of glutathione-Sepharose bound GST-PTPs for 20 min at room temperature. The phosphatases were removed by centrifugation, orthovanadate was added to the samples, and occludin or ZO-1 was immunoprecipitated for 2 h at 4 °C. Beads were washed in the lysis buffer and processed for SDS-PAGE. The immune complexes were analyzed by Western blotting using PY-20 antibody, occludin monoclonal antibody, or ZO-1 polyclonal antibody. Alternatively, HEK 293 cells were transfected with occludin and the pMT2.DEP-1 constructs for 48 h and lysed directly in hot 2× SDS gel sample buffer (200 mM Tris, pH 6.8, 20% glycerol, 4% SDS, 5% 2-mercaptoethanol), and boiled for 10 min. Samples were then diluted 20-fold with phospho-IP buffer (150 mM NaCl, 20 mM Tris, pH 7.6, 1% Triton X-100, 1% sodium deoxycholate, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin) to a final concentration of 0.1% SDS. One μg of occludin antibody and protein G-Sepharose were added and samples were incubated for 4 h at 4 °C. Samples were then washed in Tris-buffered saline and analyzed by Western blot using anti-PY20 antibody. Membranes were stripped and reprobed with an antibody to occludin for a loading control. For ZO-1 *in vivo* dephosphorylation, A431 cells were transfected with pMT2 constructs for empty vector, wild type, and DEP-1 C/S mutant. Cells were serum starved in DMEM + 0.5% bovine serum albumin for 4 h and EGF was added to a final concentration of 100 ng/ml for varying times. Following treatment, cells were washed with phosphate-buffered saline and lysed in the modified RIPA buffer containing 10 μg/ml each aprotinin and leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate. ZO-1 was immunoprecipitated overnight from lysates using a rabbit polyclonal anti-ZO-1 antibody (Zymed Laboratories Inc.). Immunoprecipitates were washed and analyzed by SDS-PAGE and Western blotting with PY-20 antibody and reprobed with the ZO-1 polyclonal antibody for loading control.

Immunofluorescence

MDCK II cells were fixed with 3.7% formaldehyde in phosphate-buffered saline and permeabilized in 0.5% Triton X-100. Occludin was visualized with anti-occludin monoclonal antibody (Zymed Laboratories Inc.) at 1:200 for 45 min at 37 °C followed by an anti-mouse Alexa 594-conjugated secondary antibody. Images were recorded with a Zeiss LSM510 confocal microscope.

Calcium Switch Experiments

MDCK II cells were plated at confluent density (~4×10^5 cells/cm^2) onto Transwell filters (0.4 μm pore, 12-mm diameter; Corning). Growth media was removed from cells 3–4 h after plating and replaced with DMEM containing low calcium (5 μM) overnight (~16 h). Disruption of cell-cell junctions was confirmed by TER measurements of only 2 ohms/cm^2. Junction reassembly was induced with the addition of calcium containing DMEM (1.8 mM $CaCl_2$) for the indicated length of time.

Permeability Assays

TER—MDCK II cells were infected with pAd/CMV/GFP, pAd/CMV/V5-DEP-1 WT, or pAd/CMV/V5-DEP-1 D/A. 24 h later, cells were plated at confluent density (~4×10^5 cells/cm^2) onto Transwell filters (0.4 μm pore, 12-mm diameter; Corning) and cultured for 4 days, with media replacement daily. TER was measured using an Endohm-12 Transwell chamber connected to an EVOM voltohmeter (World Precision) according to the manufacturer's instructions. For calcium switch experiments, TER was measured at the indicated time points following re-addition of calcium containing media. The resistance of the filter was subtracted from all readings.

FITC-Dextran Flux—MDCK II cells were infected with DEP-1 WT, DEP-1 D/A, or GFP control adenovirus and plated the same as for the TER measurements above. For the calcium switch, calcium containing DMEM was added to the chambers for the indicated length of time; 1 ml in the outer chamber and 250 μl in the inner chamber. 10-kDa FITC-dextran (Molecular Probes) was added to the top chamber in a volume of 50 μl at a final concentration of 1 mg/ml. After 1 h, a sample was removed from the basolateral (bottom) compartments and read in a flu-

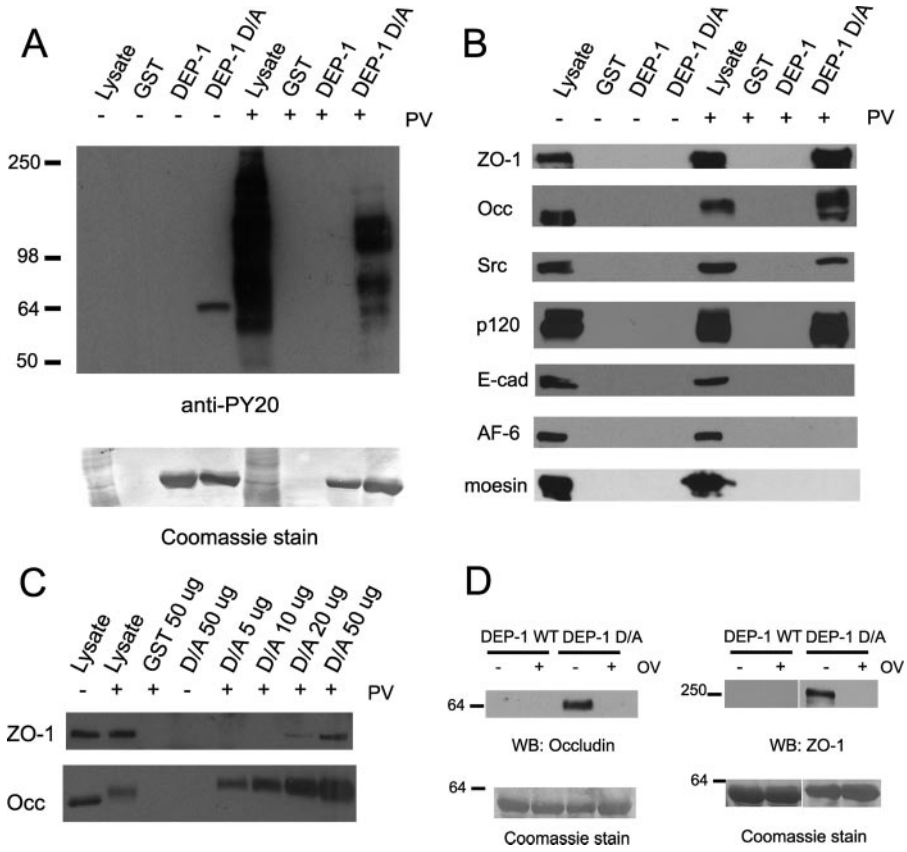

FIGURE 1. **The substrate-trapping mutant of DEP-1 binds tight junction proteins in a tyrosine phosphorylation-dependent manner.** MCF10A cells were either left untreated (−) or were treated (+) with pervanadate prior to lysis. GST alone, GST-DEP-1 WT, or GST-DEP-1 D/A fusion proteins were incubated with cell lysates and protein complexes were analyzed by SDS-PAGE and immunoblotting with: *A*, phosphotyrosine antibody (PY-20); or *B*, antibodies to ZO-1, occludin, Src, p120-catenin, E-cadherin, AF-6, and moesin. In the *lower panel* of *A*, the membrane was Coomassie stained to demonstrate equivalent amounts of GST PTPs were used. For the ZO-1 experiment in *B*, we used 100 μg of GST protein due to the reduced affinity for binding (see *C*). *C*, MCF10A cells were treated as described above. Lysates were incubated with the indicated amounts of GST proteins and protein complexes were analyzed by SDS-PAGE and immunoblotting with antibodies to ZO-1 and occludin. *D*, effects of orthovanadate on the interaction between occludin or ZO-1 and DEP-1 D/A. MCF10A cells were treated with pervanadate and lysed with (+) or without (−) sodium orthovanadate. GST-DEP-1 fusion proteins were preincubated with (+) or without (−) sodium orthovanadate and added to the lysates. Protein complexes were analyzed by SDS-PAGE and immunoblotting with occludin or ZO-1 antibodies. The membranes were stained with Coomassie Blue to confirm that equal amounts of GST-DEP-1 proteins were used.

orometer (FluroStar Optima, BMG LABTECH) (excitation 485 nm, emission 520 nm).

Knockdown of DEP-1

MCF10A cells were transfected with siRNA oligos at a final concentration of 25 nM for 72 h and lysed in the modified RIPA buffer with protease inhibitors. Alternatively, cells were transfected and 24 h later plated on Transwell filters (0.4 μm pore, 12 mm diameter; Corning) for an additional 48 h and TER was measured.

Statistics

Comparison between two groups was made by the Student's *t* test for unpaired data. Comparisons of the means of the more than two groups were made by single factor analysis of variance followed by the Fisher's LSD post hoc test.

RESULTS

Identification of DEP-1 Substrates at the Tight Junction—Tyrosine phosphorylation of AJ proteins compromises the integrity of the cadherin-catenin complex as well as the junctional integrity of cell monolayers (22-24). Protein-tyrosine phosphatases, such as DEP-1, are capable of dephosphorylating AJ proteins (27, 40). Tight junction proteins can also be regulated by tyrosine phosphorylation and we sought to identify whether tight junction proteins are potential substrates of the phosphatase DEP-1. To accomplish this, we used a substrate-trapping mutant of DEP-1 in which the conserved aspartic acid in the WPD loop of the catalytic domain is mutated to an alanine (D1205A), which traps the substrate within the catalytic domain of the phosphatase (43). Cytoplasmic domains of DEP-1 wild type (DEP-1 WT) and the substrate-trapping mutant (DEP-1 D/A) were expressed as GST fusion proteins and used in pulldown assays to identify potential physiological substrates of DEP-1. MCF10A cells were treated with pervanadate to generate a pool of tyrosine-phosphorylated proteins. As seen in Fig. 1*A*, only the substrate-trapping mutant (DEP-1 D/A) was able to bind to phosphorylated proteins. Interestingly, we noticed a band at ~64 kDa that is able to bind DEP D/A without the addition of pervanadate. It is possible that this protein is the 64-kDa serine/threonine kinase found constitutively associated with DEP-1 (44).

DEP-1 is known to localize and interact with proteins located at adherens junctions (27, 40), but it is not known whether DEP-1 or any other PTPs are able to act on tight junction proteins. To identify potential substrates at the tight junction, we again performed the GST-DEP-1 pulldown assay and probed with antibodies to known junctional proteins. The tight junction proteins occludin and ZO-1 bound to DEP-1 D/A in a phosphorylation-dependent manner (Fig. 1*B*). Claudin 1 and 3 were also blotted for and did not bind DEP-1 D/A in our experiments (data not shown). The protein-tyrosine kinase Src and p120-catenin, both known substrates of DEP-1 (27, 45), were also able to bind to DEP-1 D/A (Fig. 1*B*). In contrast, no interaction was detected between DEP-1 and the junctional proteins E-cadherin and AF-6, or the cytoskeletal protein moesin (Fig. 1*B*). We can detect an interaction between occludin and DEP-1 D/A with as little as 5 μg of the fusion protein and the interaction increases in a dose-dependent manner (Fig. 1*C*). However, 20 μg of DEP-1 D/A is required to detect ZO-1 interaction perhaps indicating a difference in binding affinity (Fig. 1*C*).

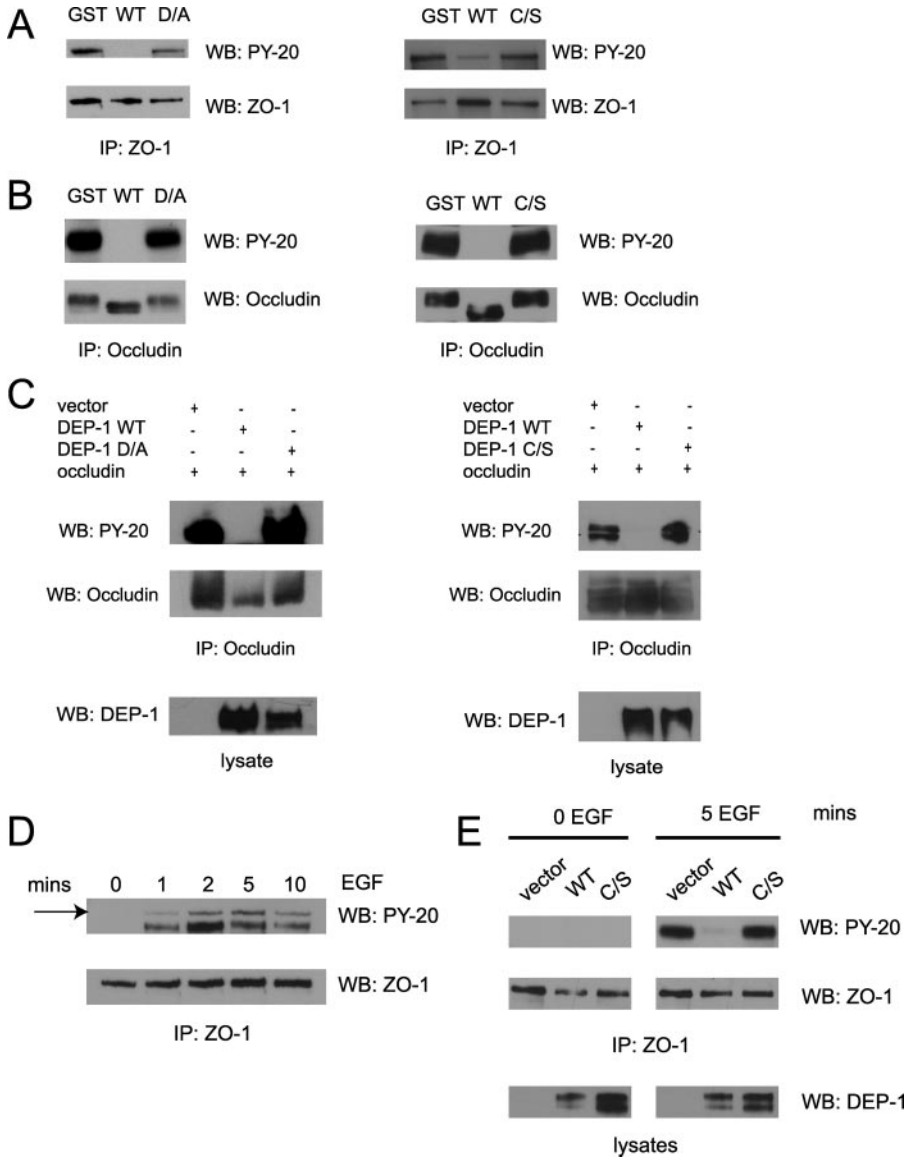

FIGURE 2. **DEP-1 dephosphorylates occludin and ZO-1.** *A* and *B*, *in vitro* dephosphorylation assay. MCF10A cells were treated with pervanadate prior to lysis. Lysates were incubated with glutathione-Sepharose bound GST or GST-DEP-1 fusion proteins, followed by the removal of the fusion proteins by centrifugation. ZO-1 (*A*) and occludin (*B*) were immunoprecipitated from the lysate supernatant. Immunocomplexes were analyzed by SDS-PAGE and immunoblotted with PY-20 antibody and reprobed with occludin or ZO-1 antibodies for the loading control. *C–E*, *in vivo* dephosphorylation assay. *C*, HEK 293 cells were transfected with occludin and empty pMT2 vector, pMT2.DEP-1 WT, pMT2.DEP-1 D/A, or pMT2.DEP-1 C/S. Cells were lysed in sample buffer, diluted in phospho-IP buffer, and immunoprecipitated (*IP*) with an anti-occludin antibody. Samples were analyzed by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody or anti-occludin antibody. Lysates are also shown to demonstrate the level of DEP-1 overexpression. *D*, A431 cells were serum starved and then treated with EGF for 0, 1, 2, 5, and 10 min. Cells were lysed and immunoprecipitated with an anti-ZO-1 antibody. Immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with a PY-20 or ZO-1 antibody. *Arrow* indicates the band for ZO-1. *E*, A431 cells were transfected with pMT2 vector, pMT2.DEP-1 WT, or pMT2.DEP-1 C/S. 24 h later cells were serum starved and left untreated (*0′*) or treated with EGF for 5 min. Cells were processed as in *D*. Lysates are shown to demonstrate the level of DEP-1 expression in the transfected cells. *WB*, Western blot.

Future experiments will need to be performed to further explore this possibility.

To determine whether ZO-1 and occludin bind to the catalytic site of DEP-1, the GST-DEP-1 proteins were preincubated with orthovanadate and tested for their ability to bind to the tight junction proteins. Orthovanadate is a competitive inhibitor that blocks the active site of DEP-1 and prevents substrate binding as well as enzymatic activity (46). In the presence of orthovanadate, the interaction between ZO-1 or occludin and DEP-1 D/A was inhibited (Fig. 1*D*), indicating that both proteins bind the active site of the catalytic domain of DEP-1. These results suggest that DEP-1 specifically interacts with a small subset of junctional proteins that includes ZO-1 and occludin.

DEP-1 Dephosphorylates Occludin and ZO-1—Having shown that occludin and ZO-1 can bind to DEP-1, we tested whether they are in fact substrates of the phosphatase. First, MCF10A cells were treated with pervanadate to induce phosphorylation of occludin and ZO-1, and then lysates were incubated with GST, GST-DEP-1 WT, and either the substrate-trapping mutant GST-DEP-1 D/A or the catalytically inactive mutant GST-DEP-1 C/S. ZO-1 or occludin were immunoprecipitated from the lysates and phosphotyrosine levels were measured by blotting with an anti-phosphotyrosine antibody. As shown in Fig. 2, *A* and *B*, wild type DEP-1 dephosphorylated both occludin and ZO-1, whereas GST and DEP-1 D/A or GST and DEP-1 C/S did not. Note that the extensive band shift of immunoprecipitated occludin incubated with DEP-1 WT is consistent with dephosphorylation of occludin by the PTP. These results confirm that occludin and ZO-1 are in fact substrates of DEP-1 *in vitro*. Next, we asked whether DEP-1 can dephosphorylate occludin when both are expressed together in cells. Full-length DEP-1 mammalian expression constructs (pMT2.DEP-1 WT, pMT2.DEP-1 D/A, or pMT2.DEP-1 C/S) or empty vector were co-expressed with occludin in HEK 293 cells, occludin was immunoprecipitated and phosphotyrosine levels were analyzed (Fig. 2*C*). In cells overexpressing DEP-1 WT phosphorylation of occludin was dramatically reduced compared with cells expressing empty vector, DEP-1 D/A, or DEP-1 C/S (Fig. 2*C*).

We also tested the ability of DEP-1 to dephosphorylate ZO-1 *in vivo*. However, we had difficulty immunoprecipitating ZO-1 with the same methods used for occludin. Therefore, a different experimental system was used. EGF stimulation has been shown to phosphorylate ZO-1 in A431 cells (47). We treated

A431 cells with EGF for varying time points and saw an increase in tyrosine phosphorylation of endogenous ZO-1, which peaked at 2–5 min (Fig. 2*D*). We transfected A431 cells with empty vector, pMT2.DEP-1 WT, or pMT2.DEP-1 C/S and left cells either untreated or treated with EGF for 5 min (Fig. 2*E*). The expression of DEP-1 WT in A431 cells induced dephosphorylation of ZO-1 following EGF treatment compared with the expression of empty vector or DEP-1 C/S (Fig. 2*E*). These combined experiments suggest that both ZO-1 and occludin are substrates of DEP-1 and that DEP-1 catalytic activity is required for the dephosphorylation of ZO-1 and occludin *in vivo*.

Occludin and ZO-1 Interaction Is Specific to DEP-1—We wanted to determine whether the interaction of DEP-1 with occludin and ZO-1 was specific or if these proteins were substrates for additional PTPs. Lysates of untreated or pervanadate-treated MCF10A cells were incubated with GST fusion proteins of substrate-trapping mutants of DEP-1 (GST-DEP-1 D/A), PTP-PEST (GST-PTP-PEST D/A), and other junctional PTPs including Shp2 (GST-Shp2 C/S) and PTPμ (GST-PTPμ D/A). DEP-1 D/A was the only PTP of those tested that was able to interact with ZO-1 and occludin, and it did so in a tyrosine phosphorylation-dependent manner (Fig. 3, *A* and *B*). To confirm that the substrate-trapping mutants were functional, pull downs were blotted with an anti-phosphotyrosine antibody, and all were found to bind tyrosine-phosphorylated proteins (data not shown). In addition, membranes were stained with Coomassie Blue to verify the use of equal amounts of PTP fusion proteins (Fig. 3, *A* and *B*). We also confirmed that other PTPs were comparatively inefficient at dephosphorylating occludin and ZO-1. MCF10A cells were treated with pervanadate to induce protein phosphorylation and incubated with wild type GST fusion proteins of DEP-1, PTP-PEST, PTPα, and PTPμ. Following incubation, fusion proteins were removed by centrifugation, and then occludin and ZO-1 were immunoprecipitated and the phosphotyrosine levels analyzed. As shown in Fig. 3, *C* and *D*, GST-WT DEP-1 was able to completely dephosphorylate occludin and ZO-1, whereas the other PTPs had little effect. Again, notice the decrease in molecular weight of the occludin that accompanied dephosphorylation by DEP-1 WT (Fig. 3*C*). These results suggest that occludin and ZO-1 are specific substrates of DEP-1 and not other PTPs tested.

DEP-1 and Occludin Co-localize at Areas of Cell-Cell Contact—Previous studies have shown that DEP-1 localizes at points of cell-cell contact as well as along the apical plasma membrane (27, 42). We examined the extent of DEP-1 and occludin co-localization at tight junctions. MDCK II cells were transfected with GFP or DEP-1 WT-GFP and were stained with anti-occludin antibodies to visualize endogenous occludin at tight junctions. As shown in Fig. 4, there was significant colocalization of DEP-1 and occludin at sites of cell-cell contact.

DEP-1 Expression Enhances Barrier Function as Junctions Reassemble—We next determined whether our previously observed dephosphorylation of occludin and ZO-1 had functional consequences with respect to tight junction physiology. MDCK II cells were infected with GFP, DEP-1 WT, or DEP-1 D/A adenovirus, plated onto transwell filters, and TER of the stable junctions was measured 4 days post plating. In epithelial

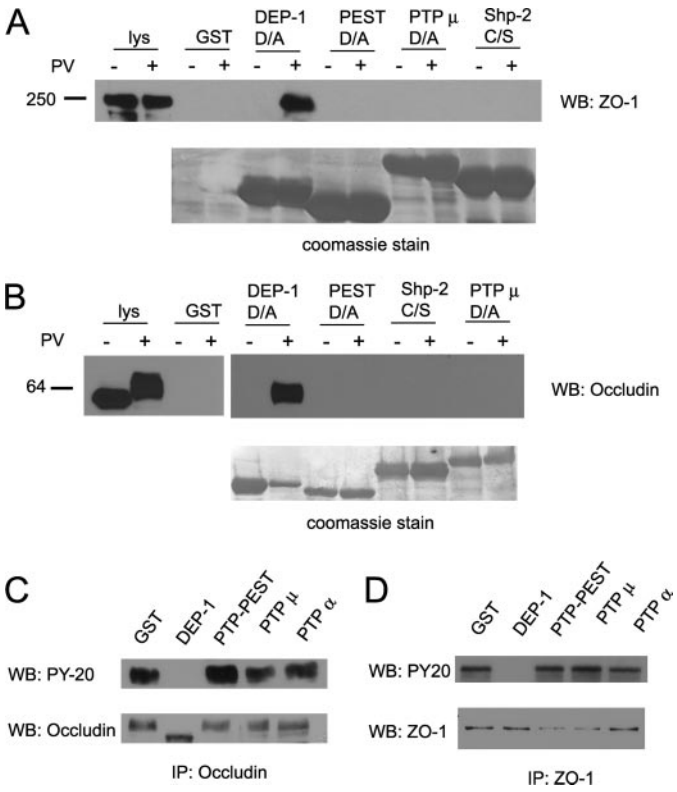

FIGURE 3. **Occludin and ZO-1 are specific substrates of DEP-1.** *A* and *B*, substrate-trapping mutants of different PTPs. MCF10A cells were either left untreated (−) or were treated (+) with pervanadate prior to lysis. Lysates were incubated with the GST fusion proteins of the substrate-trapping mutants of the indicated cytoplasmic and receptor PTPs. Protein complexes were analyzed by SDS-PAGE and immunoblotted for: *A*, ZO-1; and *B*, occludin. Membranes were stained with Coomassie Blue to confirm that equal amount of PTP fusion proteins were used. *C* and *D*, *in vitro* dephosphorylation assay. MCF10A cells were treated with pervanadate prior to lysis. Lysates were incubated with GST or wild type GST-PTP fusion proteins bound to glutathione-Sepharose, the fusion proteins were removed by centrifugation and occludin (*C*) or ZO-1 (*D*) were immunoprecipitated from the lysates. Immunocomplexes were analyzed by SDS-PAGE and immunoblotted with PY-20, occludin, or ZO-1 antibodies. *WB*, Western blot.

cells overexpressing DEP-1 we saw a small but significant increase in TER compared with the GFP control (100% *versus* 109 ± 2%) ($p < 0.05$) (data not shown), supporting the hypothesis that expression of DEP-1 can decrease the permeability of steady state tight junctions albeit very slightly.

To better address the ability of DEP-1 to regulate tight junction function, we measured the permeability of epithelial monolayers during junctional reassembly following a calcium switch (48, 49). First we looked at FITC-dextran flux across the monolayers. MDCK II cells were infected with GFP, DEP-1 WT, or DEP-1 D/A adenovirus and plated on transwell filters. After cells had adhered, growth media was replaced with low calcium media overnight (5 μM). Normal calcium-containing media (1.8 mM) was then added to the transwells and the ability of FITC-dextran to pass across the monolayer was assessed at 0, 6, 12, 24, and 48 h. The amount of FITC-dextran that crossed the monolayers was significantly reduced as junctions reassembled (Fig. 5*A*). At early stages of junction reassembly (6 h), the presence of additional DEP-1 WT decreased permeability indicated by the further decrease of FITC-dextran flux compared with GFP and DEP-1 D/A control cells (Fig. 5*A*). After 12 and

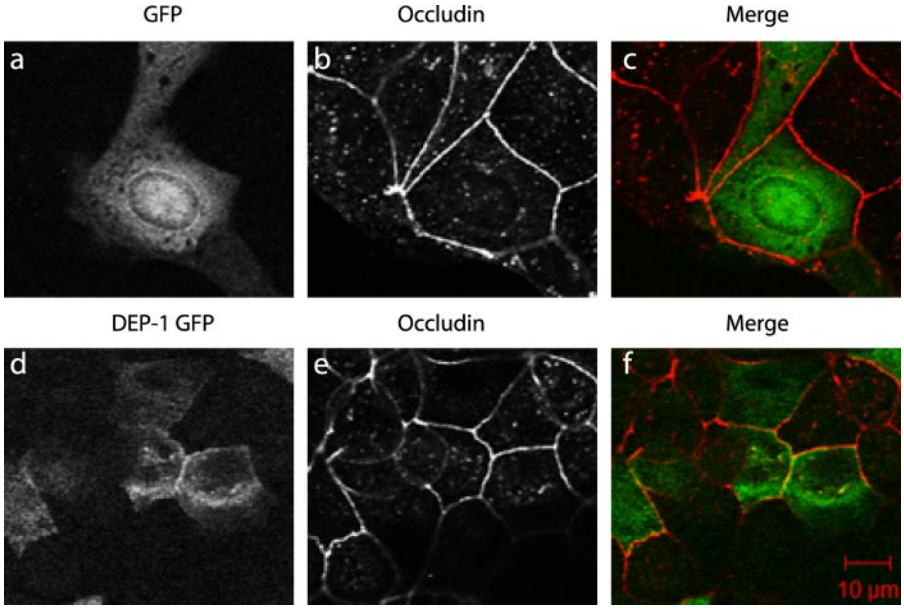

FIGURE 4. **DEP-1 localizes to areas of cell-cell contact.** MDCK II cells were transfected with pEGFP or pEGFP-DEP-1 (*a* and *d*) and stained for occludin (*b* and *e*). Merged images show colocalization in *yellow* (*c* and *f*). *Scale bar = 10 μm.*

24 h in calcium-containing media, the permeability between control and DEP-1 expressing cells was not significantly different (Fig. 5*A*). In identical experiments, barrier function was measured by TER following the calcium switch. Monolayers overexpressing DEP-1 WT have higher TER than GFP and DEP-1 D/A controls at 6, 12, and 24 h of junction reassembly (Fig. 5*B*). The differences between DEP-1 D/A and DEP-1 WT at 12 and 24 h are statistically significant. Together the TER and FITC-dextran data reveal that expression of DEP-1 WT enhances the barrier function of epithelial junctions during reassembly. Thus, the presence of DEP-1 is particularly important at the dynamic stages of junctional assembly.

Reduction of DEP-1 Protein Expression Increases Epithelial Permeability—With an increase in DEP-1 levels resulting in strengthened barrier function, conversely we tested whether loss of DEP-1 expression reduced junctional integrity. We transfected MCF10A cells with siRNA oligos targeting DEP-1 or with non-targeting (NT) control oligos and saw reduced expression of DEP-1 after 72 h (Fig. 6*A*). MCF10A cells were plated onto transwell filters 24 h post-transfection and cultured for an additional 48 h before TER measurements were taken. The loss of DEP-1 expression in MCF10A cells reduced the electrical resistance of the monolayer by 13.1% (±1%) (Fig. 6*B*).

DISCUSSION

Cell-cell junctions can be regulated through reversible tyrosine phosphorylation. Tyrosine phosphorylation of junction proteins has been shown either to promote the dissociation of protein complexes at the junction (22–24, 50) or to promote the internalization and degradation of junctional proteins (51). Whereas most work in this area has focused on the tyrosine kinases that act on junctional proteins, it is equally important to identify the PTPs and their targets within cell-cell junctions. Here we show for the first time that the prominent tight junction proteins, occludin and ZO-1, interact with and are dephosphorylated by the PTP DEP-1. Experimentally increasing the levels of DEP-1 reduced the permeability of epithelial monolayers during the early stages of junction assembly. Conversely, reducing DEP-1 protein levels increased permeability of monolayers.

Similar to the adherens junctions, tyrosine phosphorylation has been correlated with the dissociation of tight junction components, promoting the detachment of ZO-1 from occludin (12, 19, 52–54). This increased tyrosine phosphorylation of tight junction components is paralleled by increased epithelial permeability. Because DEP-1 has previously been localized to regions of cell-cell contact (27, 42), we examined whether the phosphorylation of tight junction proteins was regulated by this PTP. We found that the catalytically dead trapping mutant of DEP-1 binds to phosphorylated forms of occludin and ZO-1 and that catalytically active DEP-1 dephosphorylates these proteins. Previous work has shown that DEP-1 interacts with and acts on the adherens junction proteins p120 catenin and β-catenin (27, 40), and we have confirmed these interactions (Fig. 1*B* and data not shown). However, we found that DEP-1 does not bind all tyrosine-phosphorylated junctional proteins such as AF-6 (Fig. 1*B*). It is striking that several PTPs are concentrated within epithelial junctions (55) and it is likely that there is some redundancy with respect to their targets. We were surprised to find that two junctional PTPs, PTPμ and SHP-2, did not significantly interact with or dephosphorylate occludin and ZO-1 (Fig. 3). This result suggests that the different PTPs within cell-cell junctions have specific targets and that they may regulate distinct signaling pathways. These pathways may either be initiated within junctions in response to cell-cell interactions or be triggered by a stimulus such as growth factors or oxidative stress to regulate junction stability, strength, and permeability.

Having found that DEP-1 dephosphorylates occludin and ZO-1, we explored the effect of DEP-1 on tight junction function in epithelial cells. Mature junctions are thought to exist in a tyrosine-dephosphorylated state, with increased tyrosine phosphorylation leading to increased junction disassembly, and increased paracellular permeability (16, 17). Thus, dephosphorylation of occludin, as well as other junctional proteins, is believed to increase the barrier function of the junction. Our overexpression of DEP-1 in epithelial cells increased TER (data not shown), a measure of increased barrier properties of the junctions, consistent with phosphorylation regulating the permeability of tight junctions. Although the difference was subtle (5–10% increase), it was reproducible and statistically significant. We suspect the small effect on TER is due to the fact that the cells had been confluent for several days. At this point, the junctions have reduced dynamics and the level of tyrosine

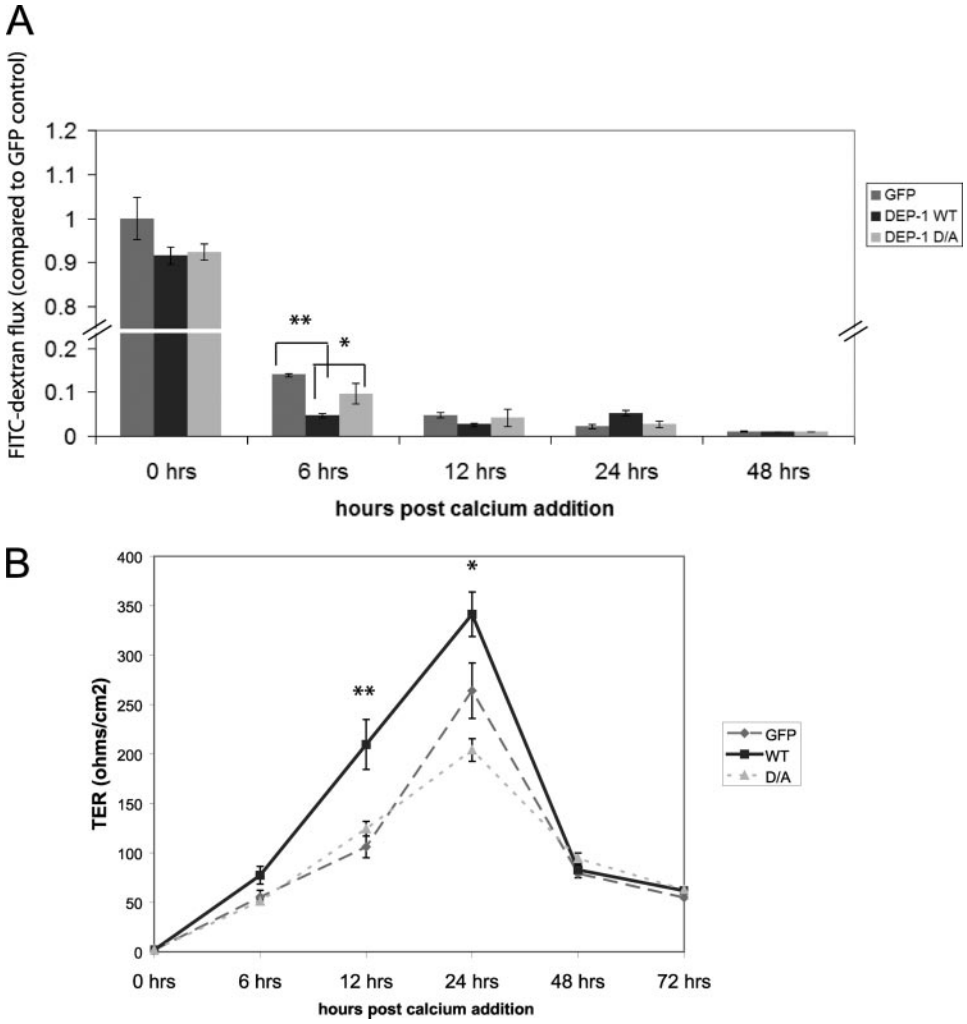

FIGURE 5. **Expression of DEP-1 enhances barrier function as junctions reassemble.** MDCK II cells were infected with GFP, DEP-1 WT, or DEP-1 D/A adenovirus and grown on filters as confluent monolayers. Cells were incubated in low calcium media for ~16 h to completely disrupt junctions (as measured by TER), and then transwell filters were switched to normal calcium media. *A*, at varying time points after the addition of calcium media, 10-kDa FITC-dextran was added to the top chamber of the transwell filter and allowed to cross the cell monolayer for 1 h. Values across all times were normalized to the amount of FITC-dextran flux that crossed the GFP control cell monolayer at 0 h. Data shown are the ratio ± S.E. for samples in triplicate. *Double asterisk* indicates significance values at $p < 0.01$, and *single asterisk* indicates significance values at $p < 0.05$. The graph shows a representative experiment. *B*, TER was measured over 72 h after the calcium switch. Data are expressed as mean ± S.E. of filters in triplicate. This graph shows a representative experiment. *Double asterisk* indicates that the value is significantly ($p < 0.01$) different from the corresponding value for DEP-1 D/A. *Single asterisk* indicates that the value is significantly ($p < 0.05$) different from the corresponding value for DEP-1 D/A.

phosphorylation is very low (essentially undetectable). Because tyrosine phosphorylation is elevated during disassembly/assembly of junctions, this led us to explore the effect DEP-1 had on junctional permeability as the junctions were assembling. Using the established calcium switch technique (49, 56) to modulate cell-cell adhesion, we measured the permeability of epithelial junctions with both TER and FITC-dextran flux over a time course of junction reassembly. As shown in Fig. 5*A*, as junctions reassemble after calcium addition, the permeability decreases over time. Importantly, when comparing the permeability of GFP-or DEP-1 D/A-expressing *versus* DEP-1 WT-expressing cells at 6 h following calcium restoration, the presence of DEP-1 WT significantly reduces the permeability of FITC-dextran flux, suggesting that these junctions reassembled more rapidly (Fig. 5*A*). By 12 and 24 h the difference in permeability between control cells and DEP-1-expressing cells was not statistically significant (Fig. 5*A*). The TER data mimics the FITC-dextran flux experiment (Fig. 5*B*) in that the cells overexpressing DEP-1 WT have increased TER compared with GFP and DEP-1 D/A expressing cells at earlier time points during reassembly. However, the TER remains higher through the 24-h time point, leveling off at 48 h, although the FITC-dextran flux levels were similar by 12 h of reassembly. This difference may reflect the difference between measuring the permeability of ions *versus* macromolecules in epithelial cells. Knockdown of DEP-1 protein levels in epithelial cells compromised barrier function when compared with control cells. The reduction in TER was small but statistically significant. This phenotype is mirrored in the DEP-1 knock-out mice, which are fertile, viable, and show no gross anatomical alterations (57). This may be due to other PTPs that can compensate at some level in diverse signaling pathways. In future studies it would be interesting to examine whether protein levels of other PTPs increase in DEP-1 knockdown cells. In addition, it is also possible that examining the effect of loss of DEP-1 in cells with extremely tight junctions, such as brain endothelial cells, would produce a more dramatic effect in permeability. Overall, our results suggest a model in which DEP-1 is a receptor PTP that localizes to cell-cell junctions, and is able to regulate phosphorylation levels of junctional proteins. In this way, it aids in the reformation of the tight junction and enhances barrier function of epithelial junctions during calcium-induced reassembly.

The identification of DEP-1 substrates at the tight junction in this paper strengthens the hypothesis that DEP-1 localizes to and dephosphorylates junction proteins, contributing to the regulation of junctional permeability. A growing body of literature suggests that tyrosine phosphorylation of TJ proteins may play a key role in regulating epithelial tight junctions; however, the role of tyrosine phosphatases in this regulation has not been investigated. Increases in tyrosine phosphorylation of occludin and ZO-1 disrupts their association with each other and reduces their localization at junctions (12, 19), as well as decreases transepithelial electrical resistance of monolayers (16, 17). Recently, two C-terminal tyrosine residues of occludin,

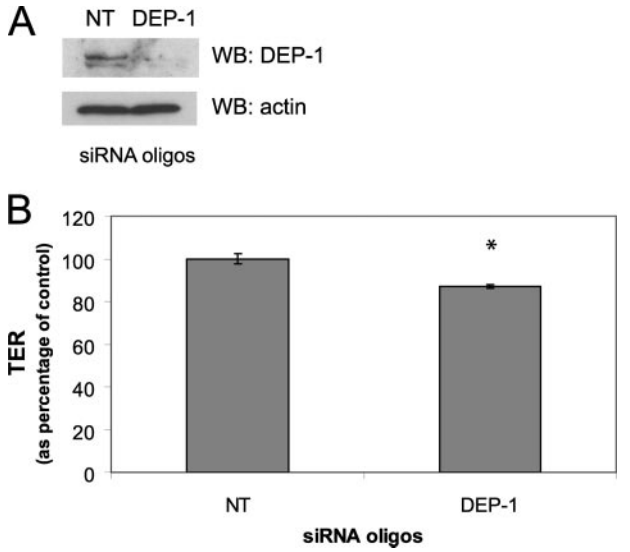

FIGURE 6. **Reduced expression of DEP-1 increases permeability.** *A*, MCF10A cells were transfected with non-targeted (*NT*) control or DEP-1 siRNA oligos for 72 h. Cells were lysed and blotted for DEP-1 or actin (loading control). *B*, MCF10A cells were transfected with non-targeted or DEP-1 siRNA oligos. 24 h post-transfection, cells were trypsinized and seeded on transwell filters (0.4 μm pore). Transepithelial electrical resistance measurements were taken 48 h after plating on filters. *Asterisk* indicates the value is significantly ($p < 0.01$) different from corresponding values for control group (*NT*) as determined by Student's *t* test. *WB*, Western blot.

Tyr-398 and Tyr-402, have been identified as Src-induced phosphorylation sites (21). This study found that phosphorylation of these residues prevented ZO-1 binding to occludin *in vivo*, building on the *in vitro* study performed previously (12). The crystal structure of occludin demonstrates that the coiled-coiled region located C-terminal to these residues is also important for ZO-1 interaction (58). Tyrosines in this region may also be sites for potential phosphoregulation of ZO-1-occludin interactions. With the identification of specific tyrosine residues that are regulated by phosphorylation, it would be interesting to test whether DEP-1 is able to dephosphorylate Tyr-398 and Tyr-402, enhancing the interaction of occludin and ZO-1. Development of phosphospecific antibodies to these newly identified residues would aid in determining how phosphorylation at these sites may be involved in signaling pathways downstream of certain stimuli such as growth factors and oxidative stress.

Previous studies have implicated DEP-1 as a factor in the deregulation of growth and differentiation in numerous types of cancers (59–61), and led to the proposal that DEP-1 is a tumor suppressor. The action of DEP-1 on both adherens and tight junctions suggests another means by which DEP-1 may protect cells from oncogenic transformation and metastasis; controlling the strength and stability of cell-cell interactions. Diminished interaction of a cell with its neighbors is a hallmark of cancer and particularly associated with tumor invasion and metastasis. In future work, we hope to examine whether the relationship of DEP-1 with occludin and ZO-1 is changed in specific epithelial tumors. The continued identification of junctional substrates of DEP-1 will aid in furthering our understanding of the role of this PTP in diseases such as cancer.

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