The Tight Junction-specific Protein Occludin Is a Functional Target of the E3 Ubiquitin-protein Ligase Itch*

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Tight junctions create a highly selective diffusion barrier between epithelial and endothelial cells by preventing the free passage of molecules and ions across the paracellular pathway. Although the regulation of this barrier is still enigmatic, there is evidence that junctional transmembrane proteins are critically involved. Recent evidence confirms the notion that occludin, a four-pass integral plasma-membrane protein, is a functional component of the paracellular barrier. The overall hydrophilicity of occludin predicts two extracellular loops bounded by NH₂- and COOH-terminal cytoplasmic domains. To date, the binding of the COOH terminus of occludin to intracellular proteins is well documented, but information concerning the function of the cytoplasmic NH₂ terminus is still lacking. Using yeast two-hybrid screening we have identified a novel interaction between occludin and the E3 ubiquitin-protein ligase Itch, a member of the HECT domain-containing ubiquitin-protein ligases. We have found that the NH₂-terminal portion of occludin binds specifically to a multi-domain of Itch, consisting of four WW motifs. This interaction has been confirmed by our results from in vivo and in vitro co-immunoprecipitation experiments. In addition, we provide evidence that Itch is specifically involved in the ubiquitination of occludin in vivo, and that the degradation of occludin is sensitive to proteasome inhibition.

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† The abbreviations used are: TJ, tight junction; MDCK, Madin-Darby canine kidney cells; MEM, minimal essential medium; aa, amino acid(s).

EXPERIMENTAL PROCEDURES

Cell Culture, DNA Transfection, Immunofluorescence—LLC-PK1 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. HEK 293 cells were kept in MEM with 10% horse serum. MDCK cells were cultured in MEM enriched with 10%
fetal calf serum. For transfection experiments cells were grown to 70–80% confluence and transfected with Effectene™ transfection reagent (Qiagen) according to the manufacturer’s protocol.

For immunofluorescence, cells were grown on coverslips, washed in PBS−, and fixed with ethanol/acetic acid (95:5) for 20 min at −20°C. Immunolabeling was done according to standard procedures. Fluorescent images were taken using a Zeiss Axioplan™ microscope and the Zeiss AxioVision Imaging System™.

Constructs—pGBK7-N-Occl was generated by subcloning the coding sequence of the entire NH2 terminus of mouse occludin (aa 1–67; GenBank™ accession number U49185) in-frame with the GAL4 DNA-binding domain into the NdeI/SalI sites of the yeast two-hybrid vector pGBK7 (CLONTECH). PCR primers were: 5′-ctgcatatgtccgtgaggccttt-3′ and 5′-agggatcctgatgccatgggtc-3′, flanked by NdeI and SalI sites.

For cloning of pCR™2.1-TOPO™- Itch a full-length cDNA encoding mouse Itch (GenBank™ accession number NM_008395) was amplified from a cDNA template derived from mouse liver RNA. Primers for the PCR reaction were: 5′-ctgcatatgtccgtgaggccttt-3′ and 5′-agggatcctgatgccatgggtc-3′, flanked by NdeI and SalI sites. The amplified product was cloned into the pCR™2.1-TOPO™ vector as recommended by the manufacturer (Invitrogen).

Myc-tagged Wt-Occl (aa 1–521), Occl-ΔN (aa 64–521), Occl-ΔN1/2 (aa 28–521), Occl-mut (aa 1–521, NH2-terminal mutated), and Claudin-1 were generated using the pDNA3.1-(−)/Myc-His/LacZ expression vector (Invitrogen). The putative WW recognition motif Pro9-Pro10-Tyr11-Pro12 was mutated to Ala9-Ala9-Tyr11-Pro12 by PCR primer modification. For Itch-EGFP the full-length cDNA coding for mouse Itch was subcloned into the XhoI-BamHI sites of pEGFP-N2 (CLONTECH).

DNA fragments encoding the different WW domains of Itch were amplified by PCR from pCR 2.1-TOPO™-itch and were subcloned into the NdeI/BamHI sites of the AD-vector pGADT7. pGADT7-WW1 encodes as 1–311, pGADT7-WW1/2 aa 1–366, pGADT7-WW3 aa 377–422, and pGADT7-WW3/4 aa 377–460. A poly-HA-ubiquitin construct was kindly provided by M. Treier (22), Itch-pEP neo was cloned as described previously (19). Full-length human ZO-1 and canine ZO-2 were kindly provided by E. Witten.

Yeast Two-hybrid Screening and β-Galactosidase Assay—The MATCHMAKER GALA Two-Hybrid System 3 (CLONTECH) was used to identify new interaction partners for the NH2 terminus of occludin. First, the Saccharomyces cerevisiae strain AH109 was transformed with pGBK7-N-Occl using a small scale lithium acetate/single-stranded carrier DNA/polyethylene glycol (LiAc/ss-DNA/PEG) transformation protocol (20). Selected transformants were transformed with a mouse liver MATCHMAKER cDNA library (in AD-vector pGADT7; CLONTECH) using a large scale LiAc/ss-DNA/PEG transformation protocol. Positive transformants were selected on SIB–Ade−/His−/−Leu−/−Trp+ 5 mM 3-aminotriazole selection media. All yeast clones were restreaked onto SIB−/−Trp−/−Leu media and assayed for activity of β-galactosidase by a β-galactosidase plate assay (21). DNA was isolated from all lacZ positive clones and the AD-library plasmids were rescued by S. cerevisiae strain Y190 (2) but no interaction with the DNA-BD expressed by pGBK7 was found (3). Furthermore, WW Itch and WW Itch-Hect did not interact with human lamin C, an unrelated protein (4). C, additional yeast two-hybrid analysis for WW Itch. A frameshift mutation of WW Itch (position indicated by arrow) did not interact with N-Occl (upper row), but β-galactosidase activity was observed when N-Occl was shifted from the DNA-BD vector to the AD vector and vice versa for WW Itch (lower row). D, domain mapping for occludin-Itch interaction. Schematic diagrams of the various constructs used in the yeast two-hybrid assay are shown. β-Galactosidase activity was only detected for transformants expressing the NH2-terminal domain of occludin and the 2nd and 4th WW domain of Itch. The 1st and 3rd WW domain were not capable of interacting with N-Occl. E, mutation of the putative WW recognition motif from Pro9-Pro10-Tyr11-Pro12 to Ala9-Ala9-Tyr11-Pro12 within the NH2 terminus of occludin (N-Occl-mut; upper row) leads to a disruption of the interaction with WW Itch. Furthermore, co-transformation of WW Itch with a NH2-terminal deleted occludin (AN-Occludin) abolishes β-galactosidase activity (lower row).
pGBK7-N-Occl, empty pGBK7, and pGBK7-LAM (CLONTECH; encodes human lamin C and provides a control for a fortuitous interaction). Transformation reactions were plated onto appropriate minimal synthetic dropout media, followed by a $\beta$-galactosidase plate assay.

Furthermore, the library insert was transferred from the pGAD10-AD vector into the BamHI site of the pGBK7-BD vector, and N-Occl (bait) was subcloned from the pGBK7-BD vector into the NdeI/XhoI sites of the pGADT7-AD vector, followed by a two-hybrid assay in yeast strain Y190.

Additionally, a frameshift mutation just upstream of the cDNA insert in the activation domain vector pGAD10 was created by digesting the plasmid with MluI, filling in the overhangs, and then religating the library plasmid. The frameshifted prey was then co-transformed with pGBK7-N-Occl into yeast strain Y190 and transformants were assayed for $\beta$-galactosidase activity as described above.

For domain mapping, the appropriate expression constructs (see constructs) were co-transformed into the yeast strain Y190 using a small scale LiAc/ss-DNA/PEG transformation protocol. Transformants were plated onto appropriate minimal synthetic dropout media and tested for $\beta$-galactosidase activity.

**Antibodies**—A mouse monoclonal anti-c-Myc antibody was purchased from Santa Cruz Biotechnology Inc. Rabbit anti-occludin and anti-claudin-1 antibody was from Zymed Laboratories Inc. Alexa Fluor™ 568 goat anti-rabbit IgG was used from Molecular Probes. A rabbit anti-Itch polyclonal antibody was generated as previously described (19). Monoclonal anti-HA was kindly provided by M. Gimona (Institute of Molecular Biology, Salzburg, Austria).

**In Vitro Binding Assay**—WW Itch was subcloned from pGAD10 into the BamHI site of the AD-vector pGADT7. Using the TNT™ T7 Coupled Reticulocyte Lysate System (Promega), WW Itch and the unmodified and modified versions of occludin (wt-Occl, Occl-N, Occl-N1/2, and Occl-mut) were in vitro transcribed/translated from the T7 promoter, which lies downstream of the GAL4 coding sequences. Equal amounts of the $^{35}$S-labeled occludins and WW Itch were pooled and incubated at 30 °C for 1½ h. Then, 500 μl of co-immunoprecipitation buffer (0.5% Triton X-100, 20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM CaCl$_2$, 5 mM Na$_2$VO$_4$, 10 mM NaF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol) and 2 μg of mouse monoclonal anti-c-Myc (Santa Cruz Biotechnology Inc.) were added. The samples were rotated for 2 h at 4 °C followed by the addition of 20 μl of 1:1 slurry of Protein G-Sepharose™ 4 Fast Flow (Amersham Bioscience Inc.). Collection of the immunocomplexes was

![Fig. 2. WW Itch binds a putative WW recognition motif in the N terminus of occludin.](image)

**Fig. 3.** Endogenous Itch interacts with occludin, but not with claudin-1. Homogenized (E13) mouse embryo tissue was subjected to immunoprecipitation using a polyclonal anti-occludin (lanes 1 and 2) or anti-claudin-1 (lanes 5 and 6) antibody. As negative controls anti-c-Myc antibody (lanes 3 and 7) or no antibody (lanes 4 and 8) was added to the sample. The immunoprecipitates were analyzed by immunoblotting using polyclonal anti-occludin (lane 1), anti-claudin-1 (lane 5), or polyclonal anti-Itch (lanes 2–4 and 6–8). Endogenous Itch (~120 kDa) co-precipitated with occludin (lane 2) but not with claudin-1 (lane 6). Minimal unspecific binding of Itch to the beads (lanes 3, 4, 7, and 8) was observed. HC, heavy chain; LC, light chain.
carried out at 4 °C for 3 h. The samples were centrifuged at 1600 × g for 30 s and washed 4 times with 1 ml of co-immunoprecipitation buffer. The samples were then resuspended in 15 μl of SDS sample buffer, boiled for 5 min, and analyzed on 10% SDS-polyacrylamide gels. After electrophoresis, the gel was fixed for 30 min in 30% methanol, 10% acetic acid, soaked for 30 min in Amplify™ (Amersham Biosciences Inc.), and dried under vacuum at 80 °C overnight. The samples were then resuspended in 15 μl of 1:1 slurry of Protein G-Sepharose™ 4 Fast Flow were added to each sample and immunoprecipitations were carried out at 4 °C overnight. The beads were recovered by centrifugation at 1,600 × g for 30 s and washed four times with 1 ml of lysis buffer. Bound proteins were eluted by boiling in SDS sample buffer and analyzed on 4–12% SDS-polyacrylamide gels.

**Fig. 4. In vitro ubiquitination of occludin.** Occludin (lane 1), claudin-1 (lane 2), lamin C (TJ-unrelated protein; lane 8), ZO-1 (lane 11), and ZO-2 (lane 14) were in vitro transcribed/translated in the presence of [35S]methionine. Comparable amounts of protein were incubated with His6×-tagged ubiquitin in the presence of ATP-regenerating system. Subsequently His6×-ubiquitin-conjugates were purified by metal chelation chromatography (Novagen) and bound proteins were analyzed by SDS-PAGE and fluorography. Only occludin was efficiently ubiquitinated (lane 2), whereas no Ub-conjugates for claudin-1 (lane 5), lamin C (lane 9), ZO-1 (lane 12), or ZO-2 (lane 15) could be detected. As a negative control the assay was performed in the absence of His6×-ubiquitin (lanes 3, 6, 10, 13, and 16).

**Fig. 5. In vivo ubiquitination of occludin.** A, HEK 293E cells expressing HA-tagged ubiquitin were left untreated or were treated with MG-132 (50 μM, 1 h). Endogenous occludin was immunoprecipitated from equal amounts of total protein and collected immunocomplexes were subjected to an immunoblot analysis using monoclonal anti-HA antibody. As a negative control the assay was performed with untransfected cells. HA-ubiquitin conjugates of occludin are highlighted by a bracket. B, Triton X-100-soluble (sol) and -insoluble (insol) fractions of HEK 293E cells expressing HA-tagged ubiquitin were immunoprecipitated using anti-occludin antibody after treatment with MG-132 (50 μM, 1 h). Bound proteins were analyzed as described above. Polyubiquitinated occludin could be detected in the Triton X-100-soluble and -insoluble fraction.

**Interaction of Occludin with Itch**

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**Co-immunoprecipitation and Immunoblotting—Co-immunoprecipitation of endogenous Itch with occludin or claudin-1 was examined using E13 whole mouse embryo homogenate. In short, embryonic tissue was homogenized in 2 ml of lysate buffer (0.5% Triton X-100, 0.5% Nonidet P-40, 20 mM Tris-Cl (pH 7.5), 250 mM NaCl, 0.2 mM EDTA, 5 mM Na3VO4, 25 mM NaF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride) using a tight fitting glass/Teflon homogenizer. The homogenate was kept on ice for 30 min, centrifuged at 16,000 × g for 10 min and the supernatant was split into 4 tubes. 2.5 μg of anti-occludin or anti-claudin-1 antibody, 2 μg of anti-c-Myc (an unrelated antibody as a control), and no antibody (negative control) were added to the samples, and incubated for 2 h at 4 °C. 30 μl of 1:1 slurry of Protein G-Sepharose™ 4 Fast Flow were added to each sample and immunoprecipitations were carried out at 4 °C overnight. The beads were recovered by centrifugation at 1,600 × g for 30 s and washed four times with 1 ml of
lysulfonyl fluoride), and kept on ice for 30 min. The lysate was centrifuged at 16,000 × g for 10 min at 4 °C, the supernatant was collected, and the protein content was determined using a BCA™-200 Protein Assay Kit (Pierce). 2.25 μg of anti-occludin or anti-claudin-1 antibody was added to equal amounts of total protein (500 μg) and immunoprecipitations were carried out as described above. Bound proteins were eluted by boiling in SDS sample buffer and were analyzed by SDS-PAGE and fluorography. Densitometric measurements were performed using E.A.S.Y. Win32 software (Herolab).

Ubiquitination of Occludin in Vitro and in Vivo—Radiolabeled Myc-occludin, claudin-1-Myc, Myc-lamin-C, ZO-1, and ZO-2 were synthesized using the TNT™ T7 Coupled Reticulocyte Lysate System (Promega) as indicated by the manufacturer. Conjugation reaction mixtures contained 16 μl of in vitro translated protein, 14 μl of untreated RRL, 12.5 μg of His6-Ub (Sigma), 50 mM Tris-Cl (pH 7.5), 1 mM ATP, 1 mM dithiothreitol, 1 mM phosphocreatine (Sigma), and 0.2 mg/ml creatine phosphokinase (Sigma) in a total volume of 50 μl. After an incubation period of 1 h at 37 °C 500 μl of binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-Cl (pH 7.9), 0.1% Triton X-100) and 30 μl of 1:1 slurry of charged His-Bind Resin (Novagen) were added. Samples were rotated for 2 h at room temperature and beads were recovered by centrifugation at 1,600 × g for 1 min. Beads were then washed 4 times with 1 ml of 60 mM imidazole, 500 mM NaCl, 20 mM Tris-Cl (pH 7.9), 0.1% Triton X-100 and 2 times with 1 ml of 125 mM imidazole, 500 mM NaCl, 20 mM Tris-Cl (pH 7.9), 0.1% Triton X-100. Radiolabeled ubiquitin conjugates were analyzed by electrophoresis and fluorography as described above.

In vivo ubiquitination assays were performed as previously described (19). In short, HEK 293 cells were transiently co-transfected with full-length Myc-occludin cloned into pcDNA3.1 (Invitrogen), HA-ubiquitin (kindly provided by M. Treier) (22), and Itch cDNA cloned into pEFneo. MG-132 (Calbiochem) was applied at a final concentration of 50 μM for 30 min. Cell lysates were immunoprecipitated with anti-Myc and blotted with anti-HA. Polyubiquitinated occludin is indicated by a bracket (top panel). The blot was reprobed with anti-Myc (middle panel). Aliquots of cell lysates were blotted with anti-Itch antibody (bottom panel).

FIG. 6. Overexpression of Itch promotes formation of polyubiquitinated occludin. 293 T cells transiently transfected with plasmids containing HA-Ub and Myc-occludin (Occl) without or with Itch were left untreated or treated with MG-132 (50 μM, 30 min). The cell lysates were immunoprecipitated with anti-Myc and blotted with anti-HA. Polyubiquitinated occludin is indicated by a bracket (top panel). The blot was reprobed with anti-Myc (middle panel). Aliquots of cell lysates were blotted with anti-Itch antibody (bottom panel).

FIG. 7. Turnover of occludin and claudin-1 and the effect of MG-132, a proteasome inhibitor, on the stability of the cellular pool of occludin. A, LLC-PK1 cells were pulse-labeled with [35S]Met and Cys for 1 h and were chased with nonradioactive medium for 0–3 h. At appropriate time intervals cell lysates were prepared. Occludin and claudin-1 were immunoprecipitated from equal amounts of total protein and extensively washed immunoprecipitates were analyzed by SDS-PAGE and fluorography. B, LLC-PK1 cells were labeled in the presence of 80 μM MG-132 (top panel, right figure) or DMSO as a vehicle control (top panel, left figure); MG-132 and DMSO were present during the starving, the pulse, and the chase period. Occludin immunoprecipitation was performed as described above and densitometric measurements were carried out using E.A.S.Y. Win32 software (Herolab). In the presence of MG-132 ~30% of initially labeled occludin could be detected after a 3-h chase period, whereas only ~1% was left in the control experiment.
The isolated cDNAs showed 100% identity to the coding sequence of E. coli in GenBank™ and EMBL using a BLAST2 homology search. Following plasmid purification from E. coli, inserts of both clones were sequenced and compared with the sequence entries in GenBank™ and EMBL using a BLAST2 homology search. The isolated cDNAs showed 100% identity to the coding sequence of Itch, a mouse E3 ubiquitin-protein ligase (GenBank™ accession number AF037454) (18).

Itch is a member of the HECT domain-containing subfamily of E3 ubiquitin-protein ligases (24). Fig. 1A shows the functional domains of mouse Itch. The HECT (homologous to the E6-associated protein carboxyl terminus) domain has been suggested to be responsible for the ubiquitinating activity of the E3 ligases, whereas the binding specificity for target proteins is mediated by sequences within the NH2 terminus. Disruption of Itch was found to induce a spectrum of immunological diseases in mutant mice, including inflammations of the large intestine with infiltrates consisting of mainly neutrophils (18). Whether occludin is affected in this mutant phenotype is unknown.

The cDNA inserts of the clones WW Itch and WW Itch Hect comprise 851 and 1636 bp of mouse Itch cDNA, extending from nucleotides 851–1636 and 494–2114, respectively (Fig. 1B). Interestingly, both clones encode an overlapping region containing all four WW domains of Itch.

The predicted protein sequences were submitted to a two-hybrid assay using a MATCHMAKER™ library. Two clones comprising 851 and 1636 bp of mouse Itch cDNA were sequenced. Both clones encode an overlapping region containing all four WW domains of Itch.

We have further analyzed which of the WW domains present in WW Itch and WW Itch-Hect are responsible for the binding of Itch to the NH2-terminal region of occludin. As a result, the presence of WW2 and WW4 was found to be critical for the association of Itch with occludin. Neither WW1 nor WW3 alone were able to bind to the occludin bait. The partial HECT domain which is included in WW Itch-Hect does not seem to be of importance for the interaction, since occludin-Itch interaction has also been found with WW Itch, lacking the HECT domain.

Additional evidence for the association of Itch and occludin was provided by in vitro pull-down assays. Labeled modified and unmodified versions of Myc-occludin and WW Itch protein were synthesized using a rabbit reticulocyte lysate system. WW Itch was found to co-precipitate with wild-type occludin but not with NH2-terminal truncated occludins (occl-ΔN, occl-ΔN1/2) (Fig. 2B). This was also confirmed by our

FIG. 8. Proteasome inhibition influences TER in MDCK cells. A, MDCK cells were grown to confluence on filter supports in normal medium and were subsequently changed to low calcium medium for 12 h. After low calcium incubation, medium was switched to normal MEM and TER was monitored in the presence or absence of MG-132. The time point of medium exchange was considered as t = 0. Addition of 50 μM MG-132 markedly accelerated TER increase within the first hour. Incubation times >3 h appeared to be toxic to the cells. B, confluent MDCK cells, exhibiting maximum TER values, were switched to low calcium medium with or without 25 μM MG-132 and TER was measured at various time intervals. TER decrease was inhibited by 31% at the time point of 150 min. Again, the time point of medium exchange was considered as t = 0. Filters were used in triplicate. Three independent experiments were performed and means of one representative experiment are shown in each panel. Variations were below statistical significance (error bars not shown).
results from a two-hybrid assay, showing that the NH2 terminus of occludin is indispensable for the interaction of WW Itch with occludin (Fig. 1E).

To further substantiate the interaction found, endogenous Itch was co-precipitated with endogenous occludin from homogenized embryonic mouse tissue (Fig. 3). As controls, immunoprecipitations were carried out either in the absence of an antibody or in the presence of an unrelated antibody (mouse monoclonal anti-c-Myc). Itch specifically co-precipitated with antibody or in the presence of an unrelated antibody (mouse monoclonal anti-c-Myc). Itch specifically co-precipitated with occludin and reaction products were purified by metal chelation chromatography. In parallel, in vitro translated radiolabeled claudin-1, ZO-1, ZO-2, and lamin C (control) were tested for ubiquitination. Strong ubiquitination of occludin was observed as evidenced by a high molecular weight smear (>60,000), representing mono- and polyubiquitinated forms of occludin (Fig. 4 lane 2). In contrast to occludin, claudin-1, ZO-1, and ZO-2 were not ubiquitinated in vitro, nor was the junction-unrelated protein lamin C (Fig. 4, lanes 5, 9, 12, and 15).

We have also demonstrated that ubiquitination of occludin occurs in vivo. For these experiments cell lysates from HEK 293 cells overexpressing HA-ubiquitin were immunoprecipitated with an anti-occludin antibody and Western blots were probed with anti-HA antibody. We found that short-term treatment with the proteasome inhibitor MG-132 significantly led to an accumulation of ubiquitin-containing conjugates in these cells (Fig. 5A).

To further confirm that Itch is critically involved in the ubiquitination of occludin, HEK 293 cells were co-transfected with Myc-occludin and HA-ubiquitin plasmids in the absence or presence of an Itch expression construct. Following immunoprecipitation of cell lysates with anti-Myc and immunoblotting with anti-HA antibody, ubiquitination of occludin was found to be specifically induced in cells overexpressing Itch (Fig. 6). Cells containing only endogenous Itch (detectable upon overexposure of immunoblots) exhibited substantially lower levels of ubiquitinated occludin. Again, MG-132 increased the levels of ubiquitin conjugates in HA-ubiquitin overexpressing cells (Fig. 6).

To address the important question which pool of occludin is ubiquitinated, ubiquitin-ubiquitin conjugates were determined in Triton X-100-extracted fractions of HEK 293 cells overexpressing HA-ubiquitin. The major portion of ubiquitinated occludin was observed in the soluble fraction (Fig. 5B, lane 1). However, substantial amounts of occludin-ubiquitin conjugates were also detectable in the insoluble fraction (Fig. 5B, lane 2), suggesting that Itch is capable of associating with junctional and lateral occludin as well.

Since ubiquitination of proteins is usually associated with their rapid turnover, we initially investigated the turnover rate of occludin using pulse-chase experiments. Fig. 7A shows SDS-PAGE patterns of occludin immunoprecipitates from pulse-labeled LLC-PK1 cells. Our results indicate that occludin is a short-lived protein with a t1/2 of about 1.5 h. After a 3-h chase, only 1.2% of 35S-labeled occludin was still detectable in cell lysates of LLC-PK1 cells, while the amount of 35S-labeled claudin-1 was unchanged. When the proteasome inhibitor MG-132 was added during a 1-h pulse and the subsequent chase period, degradation of occludin was reduced by 30% (Fig. 7B). This is in line with previous reports showing that proteasome inhibition led to the stabilization of junctional proteins such that experimentally induced scattering of epithelial cell monolayers could be inhibited (27). Similarly, the half-life of β-catenin was increased by about 3-fold upon proteasome inhibition (28), and reducing connexin degradation with inhibitors of the proteasome was found to induce assembly and function of gap junctions (29).

Although the rapid turnover of occludin is reminiscent of degradation rates reported for connexins (29, 30), it appears rather unusual compared with the relatively long half-lives shown for E-cadherin (27) and desmosomal proteins (31). A first attempt to study the degradation of occludin in cultured cells was made by Wong and Gumbiner (32), showing that a synthetic peptide, corresponding to the second extracellular loop of occludin, enhances the degradation of occludin in steady-state 35S-labeled Xenopus A6 epithelial cells (32). In contrast to our findings, occludin appeared remarkably stable in this experiment, showing a first significant decrease only after 12 h of peptide treatment. It has to be mentioned that the properties of various types of epithelia and endothelia differ substantially as reflected by TER values ranging from 5 Ωcm² to 8,000 Ωcm². Thus, the slow turnover of occludin reported by Wong and Gumbiner (32) may be explained by the fact that exceptionally tight A6 monolayers exhibiting TER values of up to 8,000 Ωcm² were used for their study. In our experiments, maximum TER values of LLC-PK1 and MDCK cells leveled at 150–300 Ωcm² which is consistent with most reports found in the literature.

As a next step we examined whether a reduction in the rate of occludin degradation influences functional properties of TJJs. To this end, MDCK cells were grown on filter supports and TER measurements were performed in the presence or absence of the proteasome inhibitor MG-132. In a first experiment, confluent MDCK cells were grown in low calcium medium (LC) for 12 h to disrupt pre-existing junctional complexes. Subsequently, the culture medium was switched to normal calcium (NC) levels with or without MG-132 and TER was measured at various time intervals. We found that MG-132 significantly accelerated the TER increase compared with control cells (Fig. 8A). On the other hand, MG-132 diminished TER decrease in MDCK monolayers switched to LC medium by about 30% (Fig. 8B). Although it is reasonable to assume that MG-132 leads to the accumulation/stabilization of any junctional protein normally degraded by the proteasome, the exceptionally rapid increase in TER indicates that preferably short-lived junctional proteins may have been affected.

The molecular mechanism underlying the “stabilizing” effect
of proteasome inhibition on TJs is still unclear. However, taking into account that deubiquitinating enzymes are active at junction sites (33, 34), it is tempting to speculate that ubiquitinated junctional proteins, which accumulate upon proteasome inhibition, may well serve as a pool for rapid reintegration into TJs following deubiquitination. In this way, spontaneous alterations of TJ properties could be explained.

Taken together, our results provide evidence to suggest that occludin directly interacts with the E3 ubiquitin-protein ligase Itch, a member of the HECT domain-containing ubiquitin-protein ligases. Our molecular studies have shown that occludin is ubiquitinated in vivo and in vitro and its turnover is slowed down by the proteasome inhibitor MG-132. Overexpression of Itch substantially increased the amount of occludin ubiquitin conjugates predominantly present in the soluble fraction in cultured epithelial cells. Interestingly, overexpression of Itch did not alter the total amount of occludin in HEK 293 cells (not shown) nor did it perturb the localization of occludin at plasma membranes (Fig. 9).

Since ubiquitination of proteins is considered to be sufficient to induce their internalization, E3 ubiquitin-protein ligases exert a crucial function in the endocytosis of plasma-membrane proteins. So far, several receptors and one ion channel have been shown to undergo ubiquitination at the plasma membrane (Fig. 9). Notably, claudin-1 did not interact with Itch nor was it ubiquitinated in HEK 293 cells. Together with our results showing that turnover rates of occludin and claudin differed markedly in pulse-chase experiments, this finding supports the notion that occludin and claudins exert physiologically distinct functions at TJs and thus are subjected to different degradation pathways. Previous reports have already suggested that TJ function can be up-regulated at the level of occludin turnover (32). We now provide further molecular evidence to understand the regulatory steps underlying this dynamic process of TJ modulation.

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REFERENCES
1. Stevenson, B. R., and Keon, B. H. (1998) Annu. Rev. Cell Dev. Biol. 14, 89–109
2. Citi, S., and Cordenonsi, M. (1998) Biochim. Biophys. Acta 1448, 1–11
3. Mitic, L. L., and Anderson, J. M. (1998) Annu. Rev. Physiol. 60, 121–142
4. Balda, M. S., and Matter, K. (1998) J. Cell Sci. 111, 541–547
5. Denker, B. M., and Nigam, S. K. (1998) J. Cell Biol. 141, 1777–1788
6. Fanning, A. S., Jameson, B. J., Jesaitis, L. A., and Anderson, J. M. (1998) J. Biol. Chem. 273, 29745–29753
7. Wittchen, K. S., Haskins, J., and Stevenson, B. R. (1999) J. Biol. Chem. 274, 17975–17985
8. Cordenonsi, M., D’Atri, F., Hammari, E., and Parry, D. A. (1999) J. Cell Biol. 147, 1569–1582
9. Furuse, M., Hirase, T., Itoh, M., Nagafuchi, A., Yonemura, S., and Tsukita, S. (1998) J. Cell Biol. 142, 1777–1788
10. Furuse, M., Itoh, M., Hirase, T., Nagafuchi, A., Yonemura, S., Tsukita, S., and Tsukita, S. (1994) J. Cell Biol. 121, 1617–1626
11. Taya, S., Skre, I. B., Staniszewski, M. C., Furuse, M., Tsukita, S., Rogers, R. A., Lynch, R. D., and Schneeberger, E. E. (1996) J. Cell Sci 109, 2287–2298
12. Van Itallie, C. M., and Anderson, J. M. (1997) J. Cell Sci. 110, 1113–1121
13. Balda, M. S., Flores-Maldonado, C., Cereijido, M., and Matter, K. (2000) J. Cell. Biochem. 8, 85–96
14. Saitou, M., Furuse, M., Sasaki, H., Schulze, J. D., Fromm, M., Takano, H., Noda, T., and Tsukita, S. (2000) Mol. Cell. Biol. 20, 4131–4142
15. Saitou, M., Fujimoto, K., Rei, Y., Itoh, M., Fujimoto, T., Furuse, M., Takano, H., and Noda, T. (1998) J. Cell Biol. 141, 397–408
16. Tsukita, S., and Furuse, M. (1999) Trends Cell Biol. 9, 268–273
17. Huber, D., Balda, M. S., and Matter K. (2000) J. Cell Biol. 127, 5773–5778
18. Perry, W. L., Hustad, C. M., Swing, D. A., O’Sullivan, T. N., Jenkin, N. A., and Copeland, N. G. (1998) Nat. Genet. 18, 143–146
19. Qiu, L., Joazeiro, C., Fang, N., Wang, H. Y., Elly, C., Altmann, Y., Fang, D., Wang, Y., Hunter, T., and Li, Z. (1999) Cell 98, 3574–3577
20. Giertz, R. D., Triggs-Raine, B., Robbins, A., Graham, R. C., and Woods, R. A. (1997) Mol. Cell. Biol. 17, 67–79
21. Duttweiler, H. M. (1996) Trends Genet. 12, 340–341
22. Treier, M., Staniszewski, L. M., and Bohman, D. (1994) Cell 78, 787–798
23. Bazanini, G., Martinez-Estrada, O. M., Orsenigo, F., Cordenonsi, M., Citi, S., and Dejana, E. (2000) J. Biol. Chem. 275, 20520–20526
24. Huibregtse, J. M., Scheffner, M., and Howley, P. M. (1993) Mol. Cell. Biol. 13, 775–784
25. Zarrinpar, A., and Lim, W. A. (2000) Nat. Struct. Biol. 7, 611–613
26. Kay, B. K., Williamson, M., and Sudol, M. (2000) FASEB J. 14, 231–241
27. Tsochatzidis, F., and Nigam, S. K. (1999) J. Biol. Chem. 274, 24579–24584
28. Aberle, H., Bauer, A., Stuppert, J. Kirsch, A., and Kemler, R. (1997) EMBO J. 16, 3797–3804
29. Mesulam, L. S., Le, A.-Ch. N., VanSlyke, J. K., and Roberts, L. M. (2000) J. Biol. Chem. 275, 25207–25212
30. Lai, J. G., and Beyer, E. C. (1995) J. Biol. Chem. 270, 26399–26403
31. Penn, E. J., Burdett, I. D., Holson, C., Magee, A. I., and Rees, D. A. (1987) J. Cell Biol. 105, 2327–2334
32. Wang, Y., and Gumbiner, B. M. (1997) J. Cell Biol. 136, 399–409
33. Taya, S., Yamamoto, T., Kato, K., Kawano, Y., Iwamatsu, A., Tsujiya, T., Tanaka, K., Kanai-Azuma, M., Wood, S. A., Matick, J. S., and K adulthood, K. (1988) J. Biol. Chem. 142, 1053–1062
34. Taya, S., Yamamoto, T., Kanai-Azuma, M., Wood, S. A., and Kaibuchi, K. (1999) Genes Cells 4, 757–767
35. Hicke, L. (1999) Trends Cell Biol. 9, 107–112
36. Dioudou, A., Harvey, K. F., Konwatana, P., Young, J. A., Kumar, S., and Cook, D. I. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7169–7173
37. Abriel, H., Kamny, E., Horsiberger, J.-D., and Staub, O. (2000) FEBS Lett. 466, 377–380
38. Dunn, R., and Hicke, L. (2001) Mol. Biol. Cell 12, 421–435