Perturbation of the tight junction permeability barrier by occludin loop peptides activates β -catenin/TCF/LEF-mediated transcription

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Here we show that interference with the integrity of the trans-epithelial permeability barrier of mouse mammary epithelial cells by treatment with synthetic peptides, homologous to the second extracellular domain of occludin, decreased the amount of occludin protein present at tight junctions and led to the formation of multilayered, unpolarized cell clusters. In addition, transcription of the adherens junction protein β -catenin was induced. Following accumulation of soluble β -catenin protein, transcription by β -catenin/TCF/LEF was increased, as revealed by transcriptional assays following transient transfection of the reporter construct. Furthermore, treatment with occludin-II peptides up-regulated RNA levels of the known β -catenin/TCF/LEF downstream target gene *c-myc*. The data presented imply a functional cross-talk between tight and adherens junctions that possibly contributes to the stepwise transformation during oncogenesis.

INTRODUCTION

Tight junctions (TJs) are the most apical intercellular junctions of epithelial and endothelial cells. Besides their function as a regulatable semipermeable diffusion barrier between individual cells, there is increasing evidence suggesting their involvement in cellular signaling events (Balda and Matter, 2000; Li and Mrsny, 2000; Reichert *et al.*, 2000). So far, mainly adherens junctions (AJs) have been implicated in signaling to the nucleus. The AJ protein β -catenin plays a dual role as a structural component of those junctions and as a signaling molecule in the Wnt signaling pathway. Stabilized β -catenin can translocate into the nucleus where, in association with members of the TCF/LEF transcription factor family, it regulates gene expression of Wnt target genes

such as *c-myc* (He *et al.*, 1998). Oncogenic transformation of cells is closely linked to the signaling function of β -catenin in intestinal epithelial cells. These cells, when carrying mutations in adenomatous polyposis coli (APC) that activate β -catenin/TCF/LEF signaling, develop into adenomas and adenocarcinomas (Korinek *et al.*, 1997). Several recent observations suggest that TJ proteins, in addition to their structural function, might also play a role in the regulation of cell growth and differentiation.

Occludin is an integral membrane protein of TJ strands (Furuse *et al.*, 1993). It spans the membrane four times, has three cytoplasmic domains and two extracellular loops. Occludin is not only critical for the regulation of paracellular permeability (Balda *et al.*, 1996, 2000), but also controls phenotypic changes associated with epithelial oncogenesis. Occludin may act as a pivotal signaling molecule in oncogenic Raf-1-activated disruption of TJs in salivary gland epithelial cells (Li and Mrsny, 2000) and thereby regulate phenotypic changes associated with epithelial cell transformation.

Mammalian ZO-1 associates with the cytoplasmic domain of occludin. The protein is related to the *Drosophila* discs-large tumor suppressor (Dlg-A), a component of septate junctions in *Drosophila* implicated in signaling during mitosis. Dlg proteins with mutations in their PDZ and SH3 domains cause neoplastic outgrowth of larval imaginal disc epithelial cells (Woods *et al.*, 1996). Recently, Reichert *et al.* (2000) showed that expression of mutant ZO-1 proteins that encode only the PDZ domains in MDCK cells leads to a fibroblastic, transformed phenotype of these cells *in vitro* and an increased tumorigenicity *in vivo*, paralleled by the activation of β -catenin/TCF/LEF transcriptional activity. Interestingly, loss of ZO-1 expression at the plasma

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Fig. 1. Treatment with the synthetic occludin loop II peptide caused a statistically significant decrease in the TER ($p < 0.05$). EpH4 cells were treated for 60 h with the indicated peptides (10 μ M final concentration) or left untreated (control). Control (DMSO-treated) cells (6713 Ω cm²) are depicted as 100%. TER values for all other samples were compared with this control as mean $\pm$ SD ($n = 3$). Statistical significance was calculated with one-way ANOVA and Dunnett's post-test.

membrane was recently correlated with tumor formation in breast cancers (Hoover *et al.*, 1998). It has been shown that interference with extracellular loops of occludin by treatment with the synthetic peptides corresponding to the second extracellular domain (Occ-II) disrupted the TJ permeability barrier in *Xenopus* kidney epithelial cell lines (Wong and Gumbiner, 1997; Lacaz-Vieira *et al.*, 1999). Intriguingly, Occ-II peptides depleted occludin from TJs without affecting other known TJ proteins such as ZO-1. Therefore, Occ-II peptides resemble a very specific and completely reversible means of selective perturbation of the TJ paracellular barrier.

Here, we used polarized EpH4 mammary epithelial cells (Fialka *et al.*, 1996) to assay the ability of Occ-II peptides to interfere with the TJ barrier function and its possible effect on signaling.

Fig. 2. Treatment with synthetic Occ-II peptides caused subcellular redistribution of occludin, E-cadherin and β -catenin. Cells grown on filter inserts formed polarized monolayers as documented by occludin (A), ZO-1 (C), E-cadherin (E) and β -catenin (G). Upon treatment with Occ-II peptides, they formed multilayers (B, D, F, H and x-z sections in the corresponding bottom panels), and occludin, β -catenin and E-cadherin redistributed all around the cells and partially into the cytoplasm (B, F, H). White arrowheads point towards layers where sections were taken. Size bar, 20 μ m. DNA staining with DAPI is in red; staining of proteins with the respective antibodies is in green.

RESULTS AND DISCUSSION

On permeable filter supports, the EpH4 cell line forms monolayers that have high trans-epithelial electrical resistance (TER; 6000–7000 Ω cm²; Figure 1). Occludin localization at the lateral cell boundaries (Figure 2A) correlated with the formation of TJs as measured by TER. Filter-grown polarized control epithelium

Fig. 3. Synthetic Occ-II peptides changed the morphology of three-dimensional epithelial cell structures and localization of TJ proteins. Control EpH4 cells formed tubular three-dimensional structures with a hollow lumen composed of a cell monolayer in collagen gels (left top image) visible in a cross-section. Treatment with Occ-II peptides caused the formation of stunted structures with diminished branching, multilayering of cells, closure of the lumina, and redistribution of ZO-1 (green) and occludin (red).

also showed the characteristic outlining of the lateral membrane for ZO-1 (Figure 2C), E-cadherin (Figure 2E) and β -catenin (Figure 2G). Treatment with synthetic peptides corresponding to the entire second extracellular loop of occludin (Occ-II) gave rise to a statistically significant decrease in TER (for Occ-II $p < 0.05$; Figure 1).

Occ-II treatment did not affect all cells in culture evenly, but the responding cells typically grew in islands. This was also reflected in the subsequent biochemical analysis of the samples. Responding cells formed multilayered cultures with occludin randomly redistributed within affected cells (Figure 2B). ZO-1 (Figure 2D) and E-cadherin (Figure 2F) were not affected; however, β -catenin protein staining was disorganized, especially in cell clusters overgrowing the primary monolayer (Figure 2H). These phenotypes were only observed with Occ-II

peptides [e.g. $22.6 \pm 7.3\%$ ($n = 530$) and $24.9 \pm 4.9\%$ ($n = 725$) of cells displayed disorganized immunofluorescence (IF) staining in randomly chosen fields for occludin and β -catenin, respectively], whereas peptides corresponding to the first extracellular domain of occludin had no effect. In addition, specificity was documented by treatment with Occ-II peptides with scrambled amino acid sequence, as described previously (Wong and Gumbiner, 1997), which did not cause detectable morphological changes (as documented by TER values in Figure 1).

Here, EpH4 mammary cells were used that are able to reconstitute a physiological mammary gland morphology when grown embedded in reconstituted collagen I matrices (Fialka *et al.*, 1996). To test whether the presence of occludin peptides would cause a similar perturbation of TJs as in two-dimensional cultures, Occ-II peptides were incorporated in the collagen

Fig. 4. Treatment with Occ-II peptides caused an increase in β -catenin mRNA and protein levels and the subcellular redistribution of the β -catenin protein. Cells were treated for 96 h either with Occ-II peptides or left untreated (control). Total RNA (10 μ g) samples were analyzed by northern blotting. Treatment with synthetic Occ-II peptides induced β -catenin gene expression (A) (quantification in B); values are expressed as mean $\pm$ SD ($n = 4$). Total protein extracts and soluble proteins only were separated by SDS-PAGE and β -catenin was analyzed by western blotting (C). Equal loading of protein lysates is documented by amido black staining shown in (E). In an independent experiment, cells were mechanically homogenized and β -catenin protein was analyzed by western blotting in nuclear and cytoplasmic fractions separately (D).

matrix. Untreated controls showed the typical hollow ducts with monocellular cross-sections (Figure 3, left panels). In IF of semi-thin cross-sections, ZO-1 and β -catenin labeling typically appears in a ring-form shaped, reflecting the angle of cutting the structure. Incorporation of Occ-II peptides in the collagen matrix (Figure 3, right panels) led to a stunted morphology with tightly packed cell clusters. In contrast to similar treatments in two-dimensional cultures of *Xenopus* epithelia (Wong and Gumbiner, 1997), in three-dimensional cultures Occ-II peptides caused the formation of solid cords, complete loss of TJs, and cytoplasmic distribution of both ZO-1 and occludin, respectively. Specificity of the Occ-II peptide was shown by treatment with scrambled peptides as described in Methods, which did not cause any detectable morphological changes (data not shown).

To examine the potential effects of Occ-II peptides at the molecular level, mRNA expression of β -catenin was determined.

Only treatment with Occ-II peptides resulted in the transcriptional increase of β -catenin (Figure 4A). To investigate this response further biochemically, we performed western blot analysis of the β -catenin pool in total cell lysates as well as of the detergent-extractable (soluble) protein fraction after Occ-II treatment of filter-grown cultures (Hsu *et al.*, 1998). The total amounts of β -catenin changed only slightly, whereas the soluble pool was clearly increased (Figure 4C). Occ-II treatment also substantially increased the nuclear pool of β -catenin (Figure 4D).

Redistribution and up-regulation of β -catenin prompted us to analyze its transcriptional activities in response to TJ disruption by occludin peptides. Both control and peptide-treated filter cultures were transiently transfected with pTOPFLASH-luciferase reporter plasmid responsive to β -catenin/TCF/LEF activation (van de Wetering *et al.*, 1997). Occ-II peptides caused

Fig. 5. Treatment with Occ-II peptides increased transcriptional activity of the β -catenin/Tcf/Lef complex. EpH4 cells were co-transfected with the pTOPFLASH reporter plasmid (or negative control pFOPFLASH), pGK-Geo construct (for normalization of the transfection efficiency) and hTcf-4 expression plasmids. One day after transfection, cells were treated with the indicated Occ peptides or left untreated. Treatment with Occ-II peptides for 48 h caused a significant decrease in the TER ($p < 0.05$; **A**) expressed as mean $\pm$ SD ($n = 3$). In the same cultures, occludin loop II peptide treatment induced pTOPFLASH transcriptional activity in a statistically significant manner ($p < 0.01$; **B**).

a significant reduction of TER (Figure 5A) and increased luciferase activity $\sim$ 5-fold (Figure 5B).

Finally, mRNA expression of *c-Myc*, a well characterized downstream target of β -catenin/TCF/LEF signaling, was evaluated after 96 h peptide treatment of EpH4 cells. Treatment with Occ-II peptides resulted in an increase of *c-myc* RNA levels (Figure 6). Expression of the protooncogene *c-myc*, which regulates cell growth and differentiation, was previously shown by others to be repressed by wild-type APC and activated by β -catenin and TCF (He *et al.*, 1998).

Fig. 6. Treatment with Occ-II peptides increased *c-myc* mRNA levels. Ninety-six hours following the treatment of EpH4 cells with Occ-II peptides, total RNA was isolated and analyzed by northern blotting. Treatment-induced *c-myc* gene expression is shown in the top part of (**A**), which was normalized on loading due to the GAPDH signal shown in the bottom part. The relative change in the *c-myc* RNA levels was calculated as a ratio between the intensity of *c-myc* and *GAPDH* bands after phosphorimager analysis (**B**). Values are expressed as mean $\pm$ SD ($n = 4$).

Transformed epithelial cells usually display an unpolarized phenotype, forming clusters and multiple layers with disrupted TJ strands. Here we show that depletion of occludin from TJs of polarized mammary epithelial cells by synthetic peptide treatment caused similar morphological changes, i.e. loss of TJ and AJ structure, as observed in transformed cells. Treatment with Occ-II peptides also caused up-regulation and translocation of β -catenin. We could further show that the ultimate consequence of this response was induction of the β -catenin/TCF/LEF signal transduction pathway, which determines epithelial cell fate during neoplastic growth. Interestingly, expression of mutants of the TJ protein ZO-1, encoding only the N-terminus including the PDZ domains, induced a dramatic loss of the polarized epithelial

phenotype in MDCK-I cells (Reichert *et al.*, 2000). These cells underwent epithelial to mesenchymal transition (EMT), and in analogy to our system β -catenin/TCF/LEF signaling was activated. Another recent example of the involvement of TJ proteins in epithelial transformation is occludin *per se*. Li and Mrsny (2000) demonstrated an essential role for occludin in the loss of structure and function of epithelial TJs in Ras–Raf-driven oncogenic transformation.

It has been shown recently in *Drosophila* that the Armadillo degradation machinery, consisting of E-APC and Shaggy (the *Drosophila* GSK-3 β homolog), is associated with the apicolateral adhesive zones of epithelial cells (Yu *et al.*, 1999). These authors suggested that the Armadillo destabilizing complex needs anchorage to adhesive zones to actively promote the degradation of Armadillo. Interestingly, these authors have also observed a substantial loss of junctional Armadillo and accumulation of extra free Armadillo in zonula adherens mutants. They proposed that the destabilizing complex translocate into the cytoplasm and is inactive. This model could be consistent with our observations that disruption of junctional architecture by treatment with Occ peptides leads to translocation and activation of β -catenin.

Although epithelial cancers appear to be lacking both proper AJ and TJ complexes (Quinonez and Simon, 1988), a role for functional signaling through TJs similar to that observed through AJ components has not been described. It is possible that anti-neoplastic signaling through TJs is similar to that observed for AJs and that structural disturbance of the TJ permeability barrier function uncouples these processes. These data, together with the observations described here, raise the possibility of signaling cross-talk (communication) between TJs and AJs. Signaling from cell–cell junctions is an important and understudied area, and will surely receive more attention in the future.

METHODS

Cell cultures. Cells were cultured on filter culture inserts (Costar, Transwell) and the functional integrity of TJs was assayed by measuring the TER of cell layers as described (Fialka *et al.*, 1996). EpH4 cells were grown in collagen gels as described previously (Fialka *et al.*, 1996). Where indicated, cells were treated with synthetic occludin peptides (Occ-I or Occ-II, 10 μ M, respectively).

Antibodies. Monoclonal antibodies against β -catenin, E-cadherin and occludin were from Transduction Laboratories. Rat monoclonal anti-ZO-1 antibody was purchased from Chemicon. Secondary antibodies were either Cy-3- or Cy-5-labeled anti-primary antibodies from Jackson ImmunoResearch.

Peptides. Occ-I peptide corresponded to amino acids 81–125 and Occ-II peptides to amino acids 184–227 of chick occludin, as described previously (Wong and Gumbiner, 1997). As an additional control, a scrambled peptide composed of the same residues as Occ-II was used in some experiments (Wong and Gumbiner, 1997).

Protein analysis. Cytoplasmic cell extracts were prepared from cells by adding lysis buffer (100 mM NaCl, 50 mM Tris–HCl pH 7.5, 0.5% NP-40, protease inhibitors) and pre-clearing the lysates by centrifugation. For the preparation of whole-cell extracts, cells were lysed in 2 $\times$ sample buffer (120 mM Tris–HCl

pH 6.8, 4% SDS, 20% glycerol, 200 mM dithiothreitol, 0.01% bromophenol blue), boiled for 5 min and clarified by centrifugation. Western blotting was performed as described previously (Fialka *et al.*, 1996). For the analysis of β -catenin subcellular distribution, cells were mechanically homogenized and nuclei separated from cytoplasm and membrane fractions by centrifugation as described in Fialka *et al.* (1997).

RNA analysis. RNA was extracted by the guanidinium thiocyanate procedure (Chomczynski and Sacchi, 1987). Transfer and hybridization with 32 P-labeled probes were carried out according to standard protocols. Northern blots were measured with a phosphorimager and quantified by ImageQuant 1.1 (Molecular Dynamics). Experiments were repeated several times, all samples were loaded more than once, and specific RNA signals (β -catenin and *c-myc*) were normalized to *GAPDH* expression on the same blot. A 1.3 kb fragment of human *c-myc* exon 3 and a 2.4 kb fragment of β -catenin were used as probes.

Transient transfections and luciferase assay. Cells were grown in 6-well insert plates and transfected with 0.5 μ g of luciferase reporter, 0.5 μ g of the pGKGeo β -galactosidase and 0.5 μ g of hTCF-4 expression plasmids using the Lipofectamine Plus Reagent (Life Technologies). As reporters we used pTOPFLASH containing three copies of the optimal TCF motif and pFOPFLASH containing mutant motif, upstream of a minimal c-Fos promoter driving luciferase expression (van de Wetering *et al.*, 1997). Cells were harvested 48 h post-transfection in 0.25 M Tris pH 7.5, 1% Triton X-100 buffer and assayed for both luciferase and β -galactosidase activities (Galacto-light chemiluminescence reporter assay; Tropix) with the 96-well plate luminometer Lucy 2 (Berthold). Luciferase values were corrected for transfection efficiency by dividing them by the β -galactosidase values. Transfections were performed in triplicate and all experiments repeated several times (values are given as mean $\pm$ SD, $n = 3$). Statistical significance was calculated with one-way ANOVA and Dunnett's post-test.

Confocal microscopy. Fixation of cells, antibodies and fluorescent labeling was performed as described (Oliferenko *et al.*, 1999). For cryo-sectioning, cells were grown in collagen I gels, fixed in 4% paraformaldehyde (PFA) in 250 mM HEPES for 10 min, 8% PFA for 60 min and 4% PFA overnight. Cells were washed in TBS, blocks cut and placed in PVP solution (1.61 M sucrose, 0.066 M Na₂CO₃ and 30% w/v PVP) overnight and semi-thin sectioned. Stainings were analyzed in horizontal confocal microscopy sections (50–100 sections of 0.12 μ m) recorded by a Leica TCS NT and images deconvoluted (Huygens software). Signals were overlaid and sections projected into one image are presented as an extended focus view.

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