The specific fates of tight junction proteins in apoptotic epithelial cells

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Summary

The polarized morphology of epithelial cells depends on the establishment and maintenance of characteristic intercellular junctions. The dramatic morphological changes observed in apoptotic epithelial cells were ascribed at least in part to the specific fragmentation of components of adherens junctions and desmosomes. Little, however, is known about tight junctions during apoptosis. We have found that after induction of apoptosis in epithelial cells, tight junction proteins undergo proteolytic cleavage in a distinctive manner correlated with a disruption of tight junctions. The transmembrane protein occludin and, likewise, the cytoplasmic adaptor proteins ZO-1 and ZO-2 are fragmented by caspase cleavage. In addition, occludin is cleaved at an extracellular site by a metalloproteinase. The caspase cleavage site in occludin was mapped C-terminally to Asp^{320} within the C-terminal cytoplasmic domain. Mutagenesis of this site efficiently blocked fragmentation. In the presence of caspase and/or metalloproteinase inhibitors, fragmentation of occludin, ZO-1 and ZO-2 was blocked and cellular morphology was almost fully preserved. Interestingly, two members of the claudin family of transmembrane tight junction proteins exhibited a different behavior. While the amount of claudin-2 protein was reduced similarly to occludin, ZO-1 and ZO-2, claudin-1 was either fully preserved or was even increased in apoptotic cells.

Supplemental data available online

Key words: Apoptosis, Tight junctions, Occludin, Caspase, Metalloproteinase

Introduction

Tight junctions represent the most apical cell-cell contacts in epithelial and many endothelial cell sheets and are important for their barrier function (for reviews, see Tsukita and Furuse, 2002; Tsukita et al., 2001). In addition, tight junctions act as a kind of fence maintaining the specific lipid and protein composition of apical and basolateral membrane domains in polarized epithelia. In freeze-fracture electron micrographs tight junctions appear as branched beaded strands of particles that fuse the outer leaflets of the plasma membranes of opposing cells. Occludin (Furuse et al., 1993) and claudins (Furuse et al., 1998a) were identified as the major integral membrane proteins forming the continuous tight junction strands. The junctional adhesion molecule (JAM), a member of the Ig superfamily of single transmembrane domain proteins, was found associated with tight junction strands (Martin-Padura et al., 1998). In contrast to occludin and claudins, JAM is not able to reconstitute tight junction strands in L-cell transfectants (Tsukita et al., 2001). Both, occludin and claudins have four transmembrane domains and their N- and C-terminal ends are located in the cytoplasm. This topology generates two extracellular loops that are supposed to provide the intercellular interaction sites (Furuse et al., 1993; van Itallie and Anderson, 1997; Wong and Gumbiner, 1997). The C-terminal domain of occludin and claudins serves as a binding site for a complex set of proteins including a number of PDZ-domain proteins (ZO-1, ZO-2, ZO-3), kinases and phosphatases (D'Atri and Citi, 2002). This association with different signaling molecules indicates that tight junctions are more than simple barriers separating compositionally distinct environments. In contrast, they appear to be involved in the reception and conversion of signals from and to the cell interior (Matter and Balda, 2003).

The number of tight junction strands and the branches within these strands appear to be involved in the specification of the barrier properties of tight junctions. There is increasing evidence now that the molecular composition of tight junction strands plays a central role in this respect. Claudin-1 was shown to be crucial for the epidermal barrier function, since claudin-1-deficient mice exhibited severe dehydration caused by excessive transepidermal water loss across the skin (Furuse et al., 2002). Recently, claudin-5 was shown to determine the blood-brain barrier in mice (Nitta et al., 2003). Moreover, there is evidence now that the tightness of the tight junction strands is determined by their claudin composition and that the different members of the claudin family specifically interact with other distinct family members (Sasaki et al., 2003; Turksen and Troy, 2002). Overexpression of claudin-2 in

MDCK I cells results in a conversion from tight to leaky strands by formation of cation-selective pores (Amasheh et al., 2002; Furuse et al., 2001). Claudin-8 expression down-regulates endogenous claudin-2 expression and acts primarily as a nonspecific cation barrier (Yu et al., 2003). From mutagenesis and domain swapping experiments it was concluded that the first extracellular domain determines both paracellular charge selectivity and transepithelial resistance (Colegio et al., 2003; Colegio et al., 2002). Together with the specific expression patterns of different claudins these data suggest, that claudins are directly involved in the paracellular transport of molecules. The identification of hereditary diseases with affected claudin genes further supports the important function of these molecules for tight junction structure and function. A mutation in the claudin-14 gene was found to be responsible for human hereditary deafness (Wilcox et al., 2001). Patients with hereditary hypomagnesaemia were shown to have a mutated claudin-16/paracelllin-1 gene (Simon et al., 1999; Weber et al., 2001). In inflammatory bowel disease tight junction dysfunction also appears to contribute to the clinical phenotype (Gitter et al., 2001; Schmitz et al., 1999b).

Apoptotic cell death in epithelial tissues involves typical morphological alterations including membrane blebbing, condensation of cytoplasmic structures and fragmentation of the nucleus, and also results in the loss of cell-cell contacts. The death program can be activated by either an extrinsic or an intrinsic pathway induced by the stimulation of death receptors in the plasma membrane or by mitochondrial membrane permeabilization in response to perturbation of intracellular homeostasis (Ferri and Kroemer, 2001). Members of the caspase family of aspartate-specific cysteine proteinases are specifically activated and represent the central executioners responsible for most of the morphological changes observed in apoptotic cells (Cryns and Yuan, 1998; Hengartner, 2000). During recent years a number of caspase substrates have been identified. Among these, cytoskeletal proteins and regulators (Brancolini et al., 1995; Jaenicke et al., 1998) and components of cell-cell adhesion complexes such as cadherins (Herren et al., 1998; Schmeiser and Grand, 1999; Steinhusen et al., 2001) and catenins (Brancolini et al., 1998; Schmeiser et al., 1998; Steinhusen et al., 2000) contribute to the disruption of the cytoarchitecture. Furthermore, we recently showed that desmosomal proteins are targeted by caspases (Weiske et al., 2001). Induction of apoptosis in epithelial cells is followed by a dramatic decrease in transepithelial resistance (Bojarski et al., 2001). Currently, however, nothing is known about the fate of tight junction proteins in cells after induction of apoptosis.

With this in mind, we performed a detailed analysis of the fate of the tight junction proteins in apoptotic epithelial cells. After induction of apoptosis by staurosporine, occludin is cleaved by caspase and metalloproteinase generating a 31 kDa and 55 kDa fragment, respectively. While apoptosis induces a loss of claudin-2, as it does for occludin, claudin-1 was not affected, indicating a specific response of the different tight junction proteins to apoptosis. In addition to the integral membrane proteins of the tight junctions, the cytoplasmic adaptor proteins ZO-1 and ZO-2 were fragmented in a caspase-dependent reaction. Inhibitor studies confirmed these observations. Consistent with the biochemical data, confocal immunofluorescence microscopy revealed a complete disruption of tight junction structure after induction of apoptosis. Comparable effects were observed in different cell lines and when apoptosis was induced by TNF-α or anti-Fas/CD95 antibody.

Materials and Methods

Cell lines

Experiments were performed with the human breast epithelial cell line H184A1, the colon-carcinoma cell line HT-29/B6, Madine-Darby canine kidney (MDCK) cells and mouse fibroblast Ltk$^-$ cells grown at 37°C in a 5% CO_2 atmosphere. H184A1 cells, obtained from Dr Ulrike Steinhusen (Max-Delbrück-Center for Molecular Medicine, Berlin-Buch, Germany) were routinely cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (1:1) (Biochrom, Berlin, Germany) supplemented with 5% (v/v) fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml transferrin (Sigma), 10 μg/ml insulin and 1.8 μg/ml hydrocortisone (Biochrom). HT-29/B6 cells originally subcloned from HT-29 cells (Kreusel et al., 1991) were cultivated in RPMI 1640 medium (Biochrom) containing 2% stabilized L-glutamine and supplemented with 10% (v/v) FCS. MDCK and Ltk$^-$ cells were grown in DMEM supplemented with 10% (v/v) FCS and 100 U/ml penicillin, 100 μg/ml streptomycin.

Reagents and antibodies

Two antibodies directed against the cytoplasmic tail of occludin were used in our analyses. The monoclonal anti-occludin (clone 19) antibody directed against amino acid 396-507 of mouse occludin and the polyclonal rabbit anti-occludin antibody directed against the C-terminal 150 amino acids of human occludin were purchased from BD Transduction Laboratories (Heidelberg, Germany) and Zymed (Zytomed, Berlin, Germany). The rabbit polyclonal antibodies against ZO-1, ZO-2, claudin-1 and claudin-2 were obtained from Zymed (Zytomed, Berlin, Germany). Monoclonal anti-CD95/Fas (clone CH11) and anti-CD95/Fas (clone ZB4) blocking antibody were from Beckman Coulter (Unterschleissheim, Germany). Anti-poly-(ADP-ribose)-polymerase (PARP) polyclonal antibody was purchased from Roche Applied Science (Mannheim, Germany). Horseradish peroxidase-labeled anti-mouse and anti-rabbit antibodies were purchased from Dianova (Hamburg, Germany). Alexa Fluor™488 goat anti-rabbit IgG and Alexa Fluor™594 goat anti-rabbit IgG antibodies were obtained from Molecular Probes (MoBiTec, Göttingen, Germany). Recombinant TNF-α and active recombinant caspase-3 were purchased from Biomol (Hamburg, Germany) and BD PharMingen (Heidelberg, Germany), respectively. INF-γ was from PeproTech (London, UK). The caspase inhibitor Z-DEVD-FMK (Z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH_2F) and the metalloproteinase inhibitors TAPI-2 (N–{Δ,L[2(hydroxy amino ncarbonyl)methyl]-4-methyl pentanoyl}L-3-(2′naphthyl)-alanyl-L-alanine, 2-aminoethyl amide) and MMPI-1 (matrix metalloproteinase inhibitor 1, 2-aminobenzoyl-Gly-Pro-D-Leu-D-Ala-NH-OH) were obtained from Calbiochem (Schwalbach, Germany). The caspase inhibitor Z-VAD-FMK was from ICN Biomedicals Inc. (Aurora, Ohio, USA). M30 CytoDeath mouse monoclonal antibody (clone M30) was from Roche Applied Science (Mannheim, Germany). Staurosporine was from Sigma (Deisenhofen, Germany) and the Complete™-EDTA protease inhibitor mix and Lumi-Light western blotting substrate were from Roche Applied Science (Mannheim, Germany). Benzonase was obtained from Merck (Darmstadt, Germany).

Induction of apoptosis and preparation of cell lysates

Cells were plated at a density of 1×10^6 per well in 6-well plates and apoptosis was induced by treatment with 1 μM staurosporine. Subsequently floating cells were harvested from the culture medium, pooled with adherent cells and lysed in ice-cold lysis buffer (10 mM imidazole pH 6.8, 0.1 M KCl, 0.3 M sucrose, 2 mM $MgCl_2$, 10 mM

EGTA, 1 mM NaF, 1 mM $Na_2MoO_4^{2-}$, 1 mM $NaVO_3$, 0.2% (v/v) Triton X-100 and Complete™-EDTA protease inhibitor mixture) for 10 minutes at 4°C (Weiske et al., 2001). Otherwise apoptosis was induced, after preincubation with 1 U/ml IFN-γ for 6 hours (Abreu-Martin et al., 1995) and washing cells twice with PBS/Ca^{2+}/Mg^{2+}, by addition of 200 ng/ml of the monoclonal anti-CD95/Fas (clone CH11) antibody crosslinking the Fas-receptors on cells. As a control, cells were pre-incubated with 500 ng/ml of the anti-CD95/Fas (clone ZB4) inhibitory antibody for 2 hours before addition of anti-CD95/Fas (clone CH11) antibody. For induction of apoptosis with TNF-α, 100 ng/ml of the recombinant polypeptide were added. At different time points after induction of apoptosis with anti-CD95/Fas (clone CH11) antibody, detached apoptotic cells in the cell culture supernatant were collected by gentle centrifugation at 310 ***g*** for 10 minutes. Pelleted cells were subsequently lysed in ice-cold lysis buffer for 10 minutes at 4°C. Inhibitors (Z-DEVD-FMK and TAPI-2 at 50 μM and MMPI-1 at 100 μM) were added 1 hour prior to induction of apoptosis. For analysis of ZO-1 and ZO-2, cells were lysed in 50 mM Tris-HCl pH 6.8 + 2% (w/v) SDS + 100 U/ml benzonase.

The rate of apoptosis of adherent cells was determined by DAPI staining as the percentage of fully condensed nuclei, indicating late stages of apoptosis, per high power field. Four fields in six samples per condition were counted according to the protocol described previously (Ren et al., 2002).

Western blot analysis

For western blot analyses cell lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (PolyScreen, Perkin Elmer Life Sciences) by semidry transfer. Membranes were blocked in TST buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20) for 1 hour at room temperature (RT). Incubation with the first antibody at a concentration of 1 μg/ml in TST was performed at RT for 1 hour. After three washes with TST, membranes were incubated for 30 minutes with horseradish peroxidase-conjugated second antibody diluted 1:10,000 in TST and subsequently washed. Chemiluminescence detection was performed by exposure of Biomax MR films (Kodak, Rochester, NY) to Lumi-Light western blotting substrate-treated membranes. The molecular masses of the protein fragments were determined using the BenchMark™ Prestained Protein Ladder (Invitrogen Life Technologies, Karlsruhe, Germany).

Immunofluorescence studies

Cells were grown on 18×18 mm glass coverslips in 6-well plates. At different time points after induction of apoptosis, cells were washed twice with PBS + 0.9 mM $CaCl_2$ + 0.5 mM $MgCl_2$ (PBS/Ca^{2+}/Mg^{2+}) at 37°C, fixed in –20°C methanol for 10 minutes and washed again in PBS/Ca^{2+}/Mg^{2+}. After a 10-minute blocking step with 0.1% (v/v) goat serum in PBS/Ca^{2+}/Mg^{2+} and two washes with PBS/Ca^{2+}/Mg^{2+}, cells were incubated with polyclonal anti-occludin antibody (2.5 μg/ml) or polyclonal anti-ZO-1 antibody (2.5 μg/ml) for 30 minutes at RT. After two further washing steps cells were incubated with Alexa Fluor™594 goat anti-rabbit IgG or Alexa Fluor™488 goat anti-rabbit IgG for 30 minutes at RT. Second antibodies was diluted 1:500 in DAKO antibody diluent (DAKO Diagnostika, Hamburg, Germany). Before mounting in ProTaqs MountFluor (Biocyc, Luckenwalde, Germany) cells were washed again. Analysis and photography were performed on a confocal laser scanning microscope (LSM 510 META, Zeiss, Jena, Germany) with 63× magnification at excitation wavelengths of 543 nm and 488 nm. Details on the microscopy setup can be obtained upon request.

In vitro caspase cleavage

The cDNA encoding the C-terminal cytoplasmic domain of occludin (amino acids 264-522) was amplified by PCR from the full-length cDNA with the oligonucleotide primer pair 5′-CGCGGATCC-GCCGCCATGGCTGTGAAAACTCGAAGAAAG-3′ and 5′-CGC-GGATCCCTATGTTTTCTGTCTATCATA-3′. The *Bam*HI-digested PCR product was cloned into the *Bam*HI sites of pGEX4T1. The sequence of the construct was confirmed by cycle sequencing and subsequent analysis on an ABI Prism 310 Genetic Analyzer. Expression and purification of GST-occludin$_{264\text{-}522}$ was performed according to the procedures described previously (Huber et al., 1996). Recombinant protein (5 μg) was digested with 150 ng of recombinant caspase-3 as described previously (Weiske et al., 2001). Cleavage products were separated by SDS-PAGE and transferred to a PVDF membrane. After Coomassie Blue staining, protein bands were excised and sequenced by automated Edman sequencing on an Applied Biosystems 473A protein sequencer.

Site-directed mutagenesis and transient transfection

Full-length occludin cDNA was amplified by PCR with the oligonucleotide primers 5′-CATGTCATCCAGGCCTCTTG-3′ and 5′-CTGTTTTCTGTCTATCATAGTC-3′. The PCR product was ligated into the pCS2+myc$_6$ vector that was cleaved by *Bam*HI and subsequently treated with Klenow DNA polymerase to generate blunt ends. Site-directed mutagenesis of the caspase cleavage site was performed using the QuickChange™ Site-directed Mutagenesis Kit (Stratagene, La Jolla, USA). The primer pair 5′-GACTA-TGTGGAAAGAGTTGCTAGCCCCATGGCATACTCTT-CC-3′ and 5′-GGAAGAGTATGCCATGGGGCTAGCAACTCTTTCCACATA-GTC-3′ introducing a new *Nhe*I restriction site was used to change Asp^{320} into Ala. The sequence of the constructs was confirmed by cycle sequencing and subsequent analysis on a ABIPrism 310 genetic analyzer. Full-length claudin-2 cDNA was amplified by PCR with the oligonucleotide primers 5′-GCGGAATTCGCCACCATGGCCTCT-CTTGGCCTCCAAC-3′ and 5′-GCGGAATTCACATACCCTGTCA-GGCTG-3′. The PCR product was ligated into the *Eco*RI site of p3XFLAG-CMV-14 vector (Sigma, Deisenhofen, Germany).

Ltk$^-$ cells were transiently transfected with the Lipofectamine Plus reagent (Invitrogen Life Technologies, Karlsruhe, Germany) according to the manufacturer's instructions. After 48 hours, apoptosis was induced with staurosporine and 3 hours or 6 hours later cells were lysed. Further analyses were performed as described above.

Measurement of transepithelial resistance

The transepithelial resistance (R^t; Ω cm^2) of the HT-29/B6 monolayers was determined in Ussing chambers specifically designed for insertion of Millicell filters (Kreusel et al., 1991). Briefly, electrical measurements were performed by two fixed pairs of electrodes (STX-2, World Precision Instruments, USA) connected with an impedance meter (D. Sorgenfrei, Department of Clinical Physiology). R^t was calculated from the voltage deflections caused by an external 10 μA, 21 Hz rectangular current. Depth of immersion and position of the filters was standardized mechanically. The temperature was maintained at 37°C during the measurements by a temperature-controlled warming plate. Resistance values were corrected for the resistance of the empty filter and the bathing solution. HT-29/B6 cells were seeded on Millicell PCF filters (effective membrane area 0.6 cm^2). Three filters were placed together into one conventional culture dish (o.d. 60 mm) and filled with 10 ml of culture medium. Confluence of the monolayers was reached after 7 days and the experiment was performed on day 8. Z-VAD-FMK (50 μM) was added to the basolateral and apical compartment 2 hours before the addition of 10^4 U/ml TNF-α to the basolateral compartment.

Results

Cleavage of occludin in apoptotic epithelial cells

Cell lysates generated at different time points after induction of

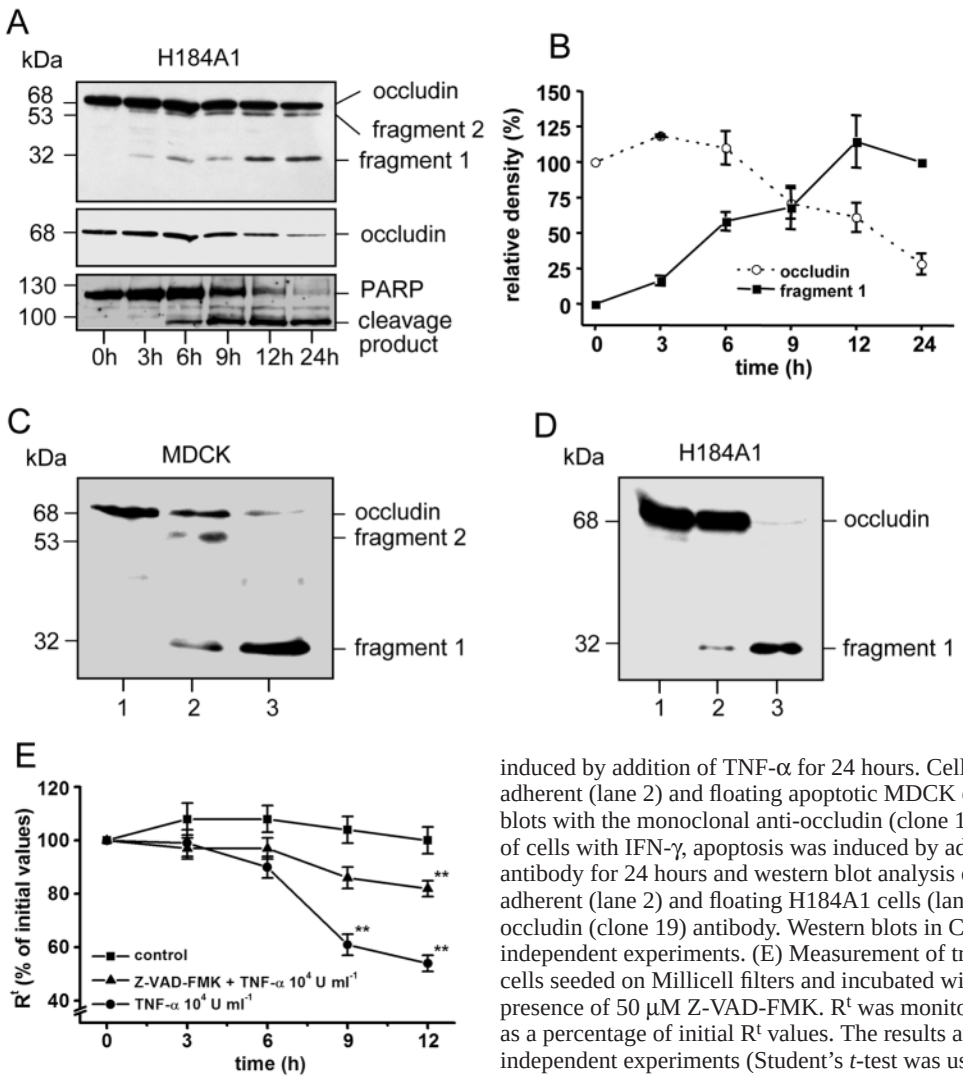


Fig. 1. Occludin is cleaved during apoptosis of epithelial cells. (A) H184A1 cell lysates (35 μg) were analyzed by western blotting with the monoclonal anti-occludin (clone 19) antibody at different times after induction of apoptosis with staurosporine. Two specific occludin fragments (fragment 1 and 2) were generated with time. The middle panel shows a shorter exposure of the above western blot to show the decrease in full-length occludin more clearly. The lower panel shows the degradation of PARP in the same cell lysates that were used to analyze occludin cleavage. (B) The generation of fragment 1 and decrease of full-length occludin were quantified by chemiluminescence imaging on a FujiFilm LAS-1000 system. The signal for occludin at 0 hours and the strongest signal for fragment 1 at 12 hours were set to 100%. Data represent mean values of five independent experiments with A as a representative blot. (C) Apoptosis was induced by addition of TNF-α for 24 hours. Cell lysates (15 μg) of control (lane 1), adherent (lane 2) and floating apoptotic MDCK cells (lane 3) were analyzed using western blots with the monoclonal anti-occludin (clone 19) antibody. (D) After 6 hours pretreatment of cells with IFN-γ, apoptosis was induced by addition of anti-CD95/Fas (clone CH11) antibody for 24 hours and western blot analysis of lysates (20 μg) of control (lane 1), adherent (lane 2) and floating H184A1 cells (lane 3) was performed with monoclonal anti-occludin (clone 19) antibody. Western blots in C and D are representatives of at least four independent experiments. (E) Measurement of transepithelial resistance R^t in HT-29/B6 cells seeded on Millicell filters and incubated with 10^4 U/ml TNF-α alone and TNF-α in the presence of 50 μM Z-VAD-FMK. R^t was monitored over a period of 12 hours and specified as a percentage of initial R^t values. The results are representative of at least three independent experiments (Student's *t*-test was used to compare results, $^{**}P<0.01$).

cell death with staurosporine were characterized by western blot analyses with a monoclonal antibody directed against the C-terminal cytoplasmic tail of occludin. In H184A1 epithelial breast cells specific cleavage fragments with apparent molecular masses of 31 kDa (fragment 1) and 55 kDa (fragment 2) were detected 3 hours after induction of apoptosis. Concomitant with the generation of these cleavage fragments the amount of full-length occludin (68 kDa) decreased (Fig. 1A). The induction of occludin cleavage was compared with cleavage of poly (ADP-ribose) polymerase, which is a characteristic marker for apoptotic cells. Similar to occludin fragmentation, the characteristic 89 kDa cleavage fragment of PARP became detectable 6 hours after induction of apoptosis concomitant with a decrease in full-length PARP (115 kDa). When cleavage of cytokeratin 18 was analyzed with the M30 cyto death monoclonal antibody using immunofluorescence microscopy, cytokeratin 18 fragmentation was weakly detectable 3 hours after induction of apoptosis (see Fig. S1, http://jcs.biologists.org/supplemental/). To quantify the decrease in full-length occludin and generation of the 31 kDa fragment, chemiluminescence signals on western blots were analyzed with a FujiFilm LAS-1000 system. The amount of full-length occludin was reduced to about 50% within 10-12 hours and to 25% after 24 hours. The amount of the 31 kDa fragment reached a maximum after 12 hours and then started to decrease suggesting it is further degraded (Fig. 1B). Quantification of fragment 2 was not unequivocally possible because of the relatively low signal intensity. To show that fragmentation of occludin is not restricted to H184A1 cells, we also tested MDCK (canine renal tubular epithelial) and HT-29/B6 (human colon carcinoma) cells (not shown). Occludin fragments of identical molecular mass and similar fragmentation kinetics could be observed.

In the next set of experiments we wanted to know whether occludin fragmentation is also induced by other apoptotic stimuli such as TNF-α and CD95/Fas. Since our H184A1 cells did not show an apoptotic response to TNF-α we used MDCK cells. TNF-α treatment of MDCK cells resulted in a much higher number of floating cells compared to staurosporine treatment. Lysates of adherent and floating MDCK cells were analyzed on western blots 24 hours after addition of TNF-α (Fig. 1C). In floating apoptotic MDCK cells full-length occludin was almost completely fragmented, resulting in an accumulation of fragment 1 while fragment 2 was not detectable. In adherent cells the amount of full-length occludin was reduced to a lesser extent and the 31 kDa and 55 kDa fragments were both detectable. In another approach, apoptosis

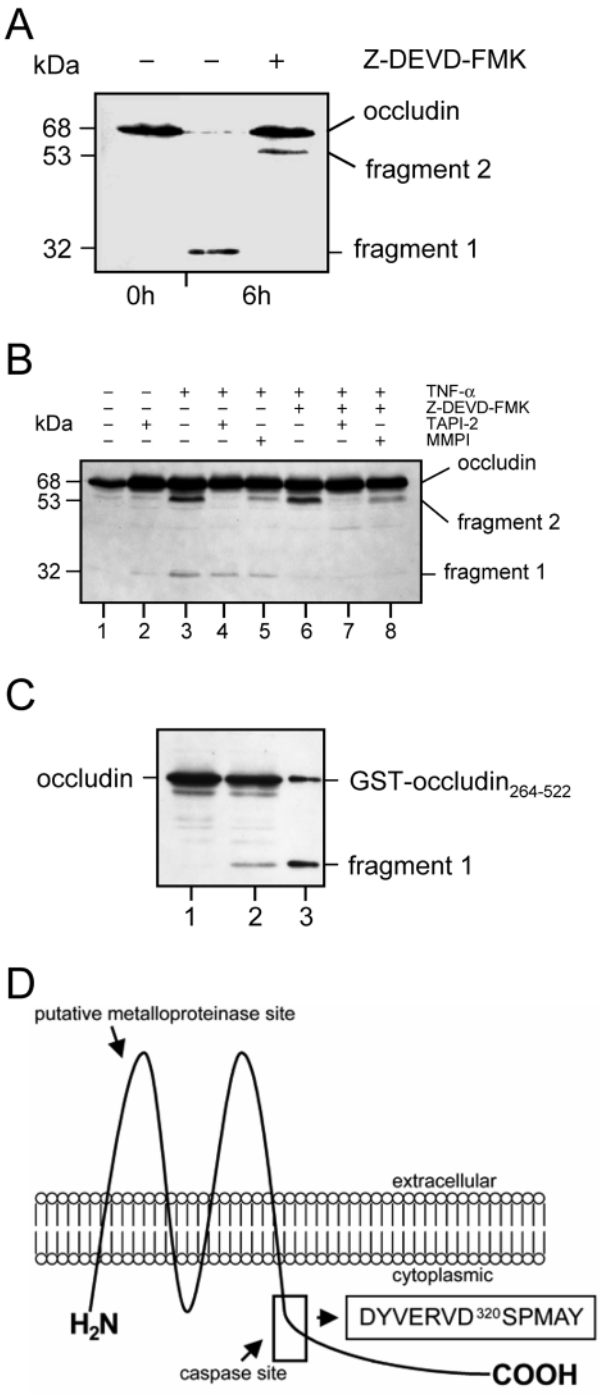


Fig. 2. Fragment 1 and 2 are generated by caspase and metalloproteinase cleavage, respectively. Western blots in A, B and C are representative of at least four independent experiments. (A) MDCK cell lysates (10 μg) were analyzed 6 hours after induction of apoptosis by staurosporine in the presence or absence of Z-DEVD-FMK with the monoclonal anti-occludin (clone 19) antibody. (B) Apoptosis was induced by 24 hours TNF-α treatment and equal amounts of protein from lysates (15 μg) of MDCK cells were analyzed in the presence of caspase and/or metalloproteinases inhibitors (TAPI-2, 50 μM; MMPI-1, 100 μM) using the monoclonal anti-occludin (clone 19) antibody. (C) In vitro cleavage of GST-occludin$_{264\text{-}522}$ by recombinant caspase-3 generates a cleavage fragment co-migrating with fragment 1 from H184A1 cell lysates. In vivo cleavage of occludin in H184A1 cells at 0 hours (lane 1) and 12 hours (lane 2) after induction of apoptosis. In vitro cleaved GST-occludin$_{264\text{-}522}$ (lane 3). Western blots were analyzed with the polyclonal anti-occludin antibody. (D) Schematic model of occludin and the apoptotic cleavage sites.

was induced in H184A1 cells by addition of the cross-linking anti-CD95/Fas (clone CH11) antibody (Abreu-Martin et al., 1995) after preincubation with 1 U/ml of IFN-γ for 6 hours. This treatment has previously been shown to induce apoptosis in epithelial cells (Abreu-Martin et al., 1995). Again occludin was cleaved to form fragment 1 in floating and adherent cells (Fig. 1D). By contrast, in both floating and adhering cells fragment 2 was not detectable with monoclonal anti-occludin (clone 19) antibody. In control experiments when cells were preincubated with the blocking anti-CD95/Fas (clone ZB4) antibody, subsequent addition of cross-linking anti-CD95/Fas (clone CH11) antibody no longer induced apoptosis. In consequence, cells did not detach from the substrate and the occludin cleavage products were not generated (not shown).

Transepithelial resistance (R^t) was measured to determine changes in tight junction barrier function after induction of apoptosis. HT-29/B6 cells, which form a highly polarized, tight epithelial cell layer, were treated with TNF-α. This resulted in a time-dependent decrease in R^t. Apoptosis in most cases was associated with the activation of caspases. Inhibition of caspase activity with Z-VAD-FMK at least in part prevented this breakdown of R^t (Fig. 1E). However, it should be noted that impaired barrier function may not be an effect induced solely by occludin cleavage. Other tight junction components are also targeted during apoptosis (see below). Moreover, it is not possible to specifically differentiate between direct effects induced by impaired tight junction proteins or secondary effects, e.g. by disruption of adherens junction components that may indirectly contribute to the decrease in R^t.

Caspase- and metalloproteinase-mediated cleavage of occludin

To analyze the mechanisms of occludin fragmentation in more detail, inhibitor studies were performed. If fragments 1 and 2 are the result of caspase-mediated cleavage reactions, their generation should be blocked by the addition of a caspase inhibitor. In the presence of the membrane-permeable caspase inhibitor Z-DEVD-FMK formation of fragment 1 was strongly reduced whereas the amount of fragment 2 was increased (Fig. 2A). This indicates that two distinct cleavage reactions participate in the fragmentation of occludin during apoptosis. Since the anti-occludin antibody is directed against the C-terminus of occludin the apparent molecular mass of fragment 2 suggested that it is generated by cleavage within the first extracellular loop of occludin. Therefore, we assumed that, similar to the apoptotic cleavage of cadherin extracellular domains (Herren et al., 1998; Steinhusen et al., 2001; Weiske et al., 2001), occludin is cleaved by metalloproteinase(s) in the extracellular loop. To address this question cells were preincubated with metalloproteinase inhibitors before induction of apoptosis. Indeed, formation of fragment 2 was blocked in the presence of TAPI-2 (Fig. 2B) whereas the broad-

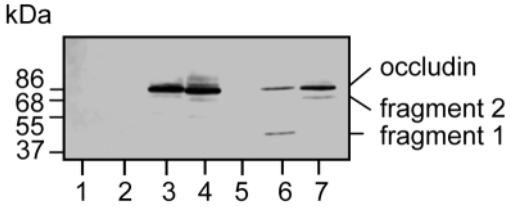


Fig. 3. Mutagenesis of the caspase cleavage site prevents cleavage of the occludin cytoplasmic domain. Ltk- cells were transiently transfected with either full-length occludin-myc$_6$ or with occludin (D^{320}A)-myc$_6$ with the mutated caspase cleavage site. Apoptosis was induced with 1 μM staurosporine for 3 hours. Western blots of cell lysates (25 μg) were analyzed with monoclonal anti-occludin (clone 19) antibody. Lanes 1-4 control cells, lanes 5-7, cells 3 hours after induction of apoptosis. Lane 1, untransfected Ltk$^-$ cells; lanes 2 and 5, Ltk$^-$ cells transfected with empty vector; lanes 3 and 6, Ltk$^-$ cells transfected with wild-type occludin; lane 4 and 7, Ltk$^-$ cells transfected with mutant occludin (D^{320}A). Analysis with an anti-myc antibody gives comparable results (not shown). This western blot is a representative of at least five independent experiments.

range matrix metalloproteinase inhibitor MMPI-1 inhibited fragment 2 formation to only a minor extent. In the presence of both TAPI-2 and Z-DEVD-FMK, generation of both fragments 1 and 2 was inhibited. Taken together, these data show that during apoptosis occludin is fragmented by TAPI-2 inhibited metalloproteinase(s) and by caspase(s) in the extracellular loop and the C-terminal cytosolic domain, respectively.

The molecular mass of fragment 1 suggested that it may represent the total or at least a large part of the C-terminal cytoplasmic tail of occludin. Since we were not able to identify the cleavage sites from the fragments generated in vivo, we expressed the occludin C-terminus-encoding amino acids, 264-522, as a glutathione S-transferase (GST) fusion protein (molecular mass of 68 kDa) and used it as substrate for in vitro cleavage by recombinant caspase-3. The specific in vitro cleavage product co-migrated perfectly on SDS-polyacrylamide gels with the 31 kDa fragment generated in vivo (Fig. 2C). Subsequent sequencing by Edman degradation defined the caspase cleavage site C-terminal to amino acid Asp^{320} in a Asp-Tyr-Val-Glu-Arg-Val-Asp^{320}-Ser-Pro-Met-Al-Tyr sequence motif (Fig. 2D). From this assay, however, we cannot exclude the possibility that in vivo other caspases might also be involved in the generation of the 31 kDa fragment.

To prove that this cleavage site is also used in vivo, amino acid Asp^{320} was mutated to Ala. Myc-tagged wild-type and mutant full-length occludin were transfected into Ltk$^-$ cells. These cells do not express endogenous occludin and claudin molecules and, therefore, signals from endogenous protein do not hamper interpretation of the results. When transfected with occludin, a small number of short and straight tight junction strand-like structures are formed (Furuse et al., 1998b). Consistent with these reports, expression of occludin protein was detectable in lysates of transiently transfected cells, whereas wild-type or mock-transfected cells showed no expression. When cells were analyzed after induction of apoptosis with staurosporine, the amount of full-length occludin was reduced and fragment 1 was generated in Ltk$^-$ cells transfected with wild-type occludin. In contrast, in Ltk$^-$ cells expressing occludin with the mutated caspase cleavage site no fragment 1 was generated whereas fragment 2 became detectable (Fig. 3). These observations indicate that the cleavage site we identified in vitro corresponds to the in vivo site.

Localization of occludin in apoptotic cells

Confocal immunofluorescence microscopy was performed to investigate the distribution of occludin in apoptotic MDCK cells. At time point 0 hours the polyclonal anti-occludin antibody revealed strong staining of the membrane at sites of cell-cell contacts (Fig. 4A). Three hours after induction of apoptosis by staurosporine, occludin staining at the cell membrane was slightly reduced (Fig. 4B). Cells were more irregular in shape and appeared more rounded in differential interference contrast images (not shown). After 6 hours, numerous cells were detached from the substratum and the remaining adherent cells showed only weak and irregular occludin staining (Fig. 4C). In the presence of Z-DEVD-FMK occludin staining was less reduced and the cell morphology was more preserved (Fig. 4D). Preincubation with TAPI-2 also resulted in fewer changes in cell shape (Fig. 4E). Treatment with both caspase and metalloproteinase inhibitors even more efficiently preserved occludin staining at cell-cell contact sites (Fig. 4F) and cell morphology was close to untreated cells. Rates of cells in apoptosis are given in Table 1.

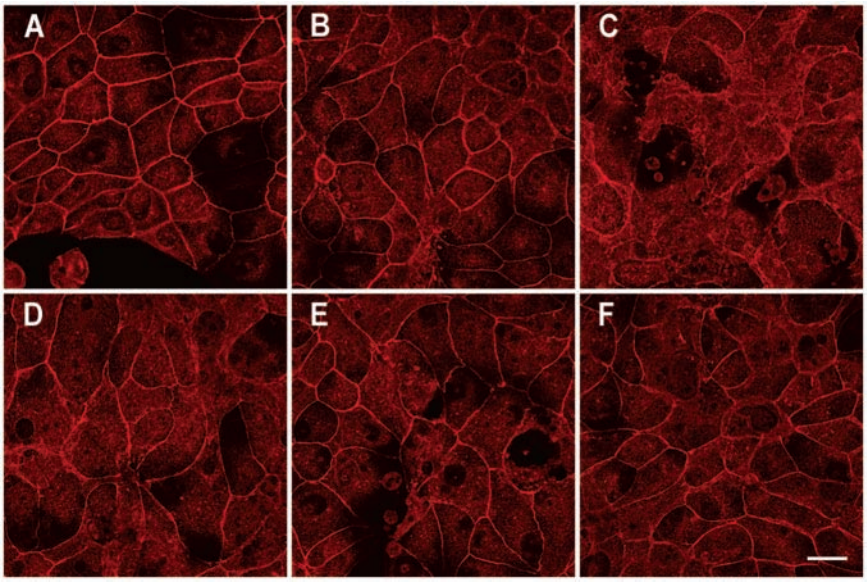


Fig. 4. Localization of occludin in apoptotic cells. MDCK cells were stained with polyclonal anti-occludin antibody. (A) 0 hours; (B) 3 hours STS (staurosporine); (C) 6 hours STS; (D) 6 hours STS + Z-DEVD-FMK; (E) 6 hours STS + TAPI-2; (F) 6 hours STS + Z-DEVD-FMK + TAPI-2. STS, staurosporine. Scale bar: 20 μm.

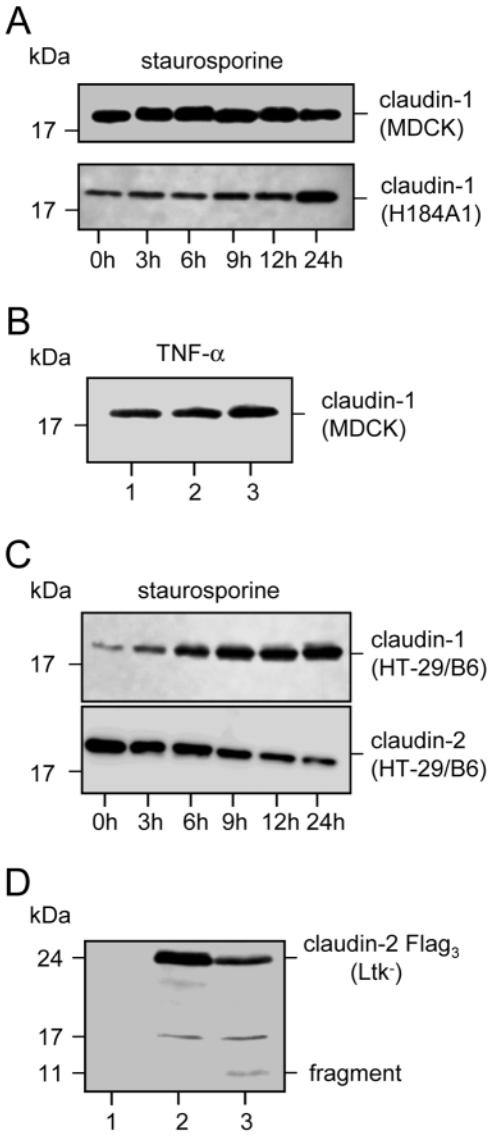
Table 1. Evaluation of the rate of apoptosis per high power field in MDCK cells after induction of apoptosis

	Control	STS 3h	STS 6h	TAPI-2/STS 6h	Z-DEVD-FMK/STS 6h	TAPI-2/ Z-DEVD-FMK/STS 6h
Rate of apoptosis (%±s.e.m.)	1.7±0.2	5.5±0.9***	14.6±2.2***	9.9±1.2***	12.3±1.1***	7.8±0.6***

Apoptosis was induced with staurosporine (STS) for 3 and 6 hours, and after preincubation with 50 μM TAPI-2, 20 μM Z-DEVD-FMK or both inhibitors. DAPI staining was performed and the number of adherent cells with condensed nuclei were counted per high power field. Results were representative of at least six independent experiments (Student's *t*-test was used to compare results, ***P<0.001).

Claudin-1 and claudin-2 in apoptotic epithelial cells

The finding of a specific fragmentation of occludin in apoptotic cells prompted us to also focus on two members of the second type of structural tight junction transmembrane proteins, the claudins. Claudin-1 expression did not significantly change after induction of apoptosis with staurosporine in H184A1 and MDCK cells (Fig. 5A) or after induction of apoptosis with TNF-α (Fig. 5B) or anti-CD95/Fas (clone CH11) antibody (data not shown) in MDCK cells. Surprisingly, in HT-29/B6 cells the amount of claudin-1 protein even strongly increased after 24 hours of staurosporine treatment. In contrast, claudin-2 protein dramatically decreased when the same amount of protein from the same HT29/B6 cell lysate was analyzed (Fig. 5C). We could not detect claudin-2 fragments with the polyclonal anti-claudin-2 antibody which is directed against the claudin-2 C terminus. Therefore, we generated a claudin-2 with a C-terminal 3x-FLAG tag. When Ltk⁻ cells transiently transfected with p3xFLAG-claudin-2 were analyzed by western blotting 6 hours after induction of apoptosis with staurosporine, a cleavage fragment with an apparent molecular mass of 11 kDa was detectable concomitant with a decrease of the full-length protein. The apparent molecular mass of this cleavage fragment suggested that it may be generated by cleavage in the second extracellular loop. Only low amounts of this fragment were detectable, suggesting that it is quickly degraded further. This is similar to the fate of the E-cadherin fragment 1 during apoptosis (Steinhusen et al., 2001) but in contrast, further degradation of the claudin-2 fragment was not inhibited in the presence of the proteasome inhibitor ALLN (not shown).



Analysis of ZO-1 and ZO-2 in apoptotic cells

The C-terminal tail of occludin and claudins binds to PDZ domains of the adapter proteins ZO-1, ZO-2 and ZO-3. Caspase-mediated cleavage within the cytoplasmic tail of occludin thus should release the interaction site of these proteins. Moreover, it could not be excluded that the zonula occludens (ZO) adapter proteins themselves are the targets of caspases. To analyze this, lysates of H184A1 cells generated either 12 hours after induction of apoptosis by staurosporine or

Fig. 5. Fate of claudin-1 and claudin-2 during apoptosis of epithelial cells. (A) MDCK and H184A1 cell lysates (5 μg) were analyzed at different time points after induction of apoptosis with staurosporine by western blotting with polyclonal rabbit anti-claudin-1 antibody. (B) Apoptosis was induced in MDCK cells by treatment with TNF-α for 24 hours (lane 1, 0 hours; lane 2, adherent cells 24 hours; lane 3, floating cells 24 hours). (C) HT-29/B6 cell lysates (5 μg) were analyzed at different time points after induction of apoptosis with staurosporine by western blotting with polyclonal anti-claudin-1 or anti-claudin-2 antibodies, respectively. On both western blots identical amounts of protein from the same cell lysate were analyzed. Western blots show representative data from four independent experiments. (D) Ltk⁻ cells were transiently transfected with full-length claudin-2-FLAG$_3$. Apoptosis was induced with 1 μM staurosporine for 6 hours. Western blots of cell lysates (40 μg) were analyzed with monoclonal anti-FLAG M2 antibody. Lane 1, Ltk⁻ cells transfected with empty vector; lane 2, control Ltk⁻ cells transfected with claudin-2-FLAG$_3$; lane 3, Ltk⁻ cells transfected with claudin-2-FLAG$_3$ 6 hours after induction of apoptosis with staurosporine. Western blots are representatives of at least three independent experiments.

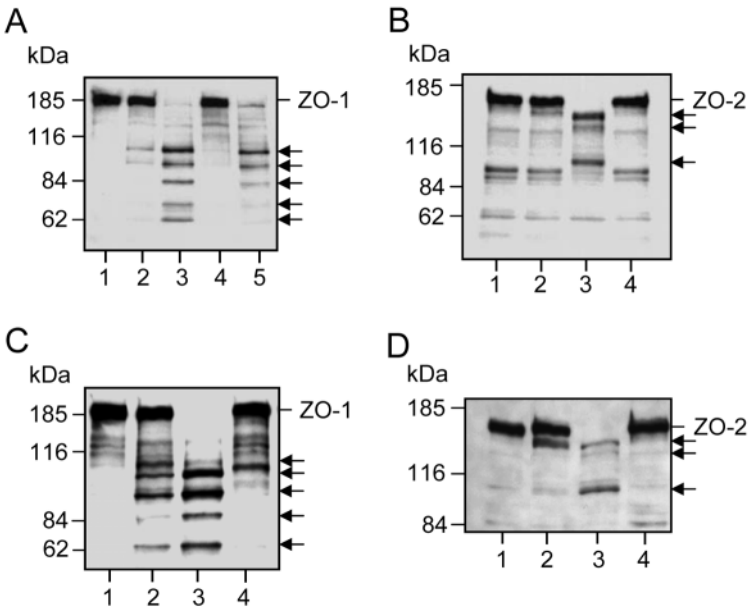


Fig. 6. Cleavage of ZO-1 and ZO-2 in apoptotic H184A1 cells. (A) Apoptosis was induced either by staurosporine (lanes 2 and 3) for 12 hours or anti-CD95/Fas (clone CH11) antibody (lanes 4 and 5) for 24 hours. Lysates (10 μg) of adherent (lanes 2 and 4) and floating (lanes 3 and 5) cells were analyzed by western blotting with a polyclonal anti-ZO-1 antibody. Lane 1, control at 0 hours. (B) Western blot analysis of ZO-2 24 hours after induction of apoptosis with the anti-CD95/Fas (clone CH11) antibody. Lane 1, control at 0 hours; lane 2, adherent cells; lane 3, floating cells; lane 4, lysate of pooled adherent cells and remaining floating cells in the presence of inhibiting anti-CD95/Fas (clone ZB4) antibody. Both western blots are representative of at least four independent experiments. (C,D) Apoptosis was induced by staurosporine and 10 μg of lysates of adherent (lane 2) and floating (lane 3) cells were analyzed by western blotting with anti-ZO-1 (C) or anti-ZO-2 (D) antibody. In the presence of Z-DEVD-FMK cells remain attached and fragmentation of ZO-1 and ZO-2 is inhibited (lane 4). Lane 1, control at 0 hours. Arrows in A-D indicate ZO-1 and ZO-2 cleavage products.

24 hours after activation by anti-CD95/Fas (clone CH11) antibody were analyzed in western blots with polyclonal anti-ZO-1 (Fig. 6A) or anti-ZO-2 (Fig. 6B) antibodies. In apoptotic cells full-length ZO-1 and ZO-2 protein was fragmented and, independent of the type of the apoptotic stimulus, at least five ZO-1 cleavage products and three major ZO-2 fragments could be detected. In the presence of inhibiting anti-CD95/Fas (clone ZB4) antibody or of Z-DEVD-FMK, respectively, a marked reduction in the number of floating cells was observed. Moreover, Z-DEVD-FMK (Fig. 6C,D) and inhibiting anti-CD95/FAS antibody (Fig. 6A, lane 5 and B, lane 4) inhibited fragmentation of ZO-1 and ZO-2. The amount of full-length ZO-1 and ZO-2 protein in adherent cells was similar to control cells.

To further confirm this observation, immunofluorescence staining for ZO-1 was performed. At time point 0 h characteristic ZO-1 staining was present at sites of cell contact of MDCK cells (Fig. 7A). After induction of apoptosis continuous ZO-1 staining at the cell membranes disappeared rapidly (Fig. 7B,C). In the presence of the caspase inhibitor Z-DEVD-FMK (Fig. 7D) ZO-1 degradation appeared to be reduced. However, ZO-1 staining did not reveal the typical predominant membrane localization at sites of cell-cell contact. Membrane association similarly was not completely restored in the presence of TAPI-2 (Fig. 7E). In the presence of both inhibitors ZO-1 staining was detected predominantly at sites of cell-cell contacts (Fig. 7F). Similar results were obtained in H184A1 cells (not shown).

Discussion

In multicellular organisms epithelia and endothelia represent the borders between different spatial compartments. This requires the establishment of stable cell-cell contacts between adjacent cells to withstand mechanical stress and prevent the diffusion of solutes by paracellular flux. Adherens junctions and desmosomes provide the major mechanical linkage between neighboring cells whereas tight junctions form the intercellular seals that are of central importance for the maintenance of the polarized phenotype of epithelial and endothelial cells. During apoptosis of epithelial cells, among other things, dramatic morphological changes and loss of polarization can be observed. This is due, in part, to the disruption of cadherin- and desmosome-mediated cell-cell contacts. For E-, P- and VE-cadherin it was shown that their cytoplasmic tail is targeted by caspases and the extracellular domain is shed from the cell surface after induction of the programmed cell death (Herren et al., 1998; Schmeiser and Grand, 1999; Steinhusen et al., 2001). In addition, both β- and γ-catenin (plakoglobin) associated with the cytoplasmic tail are fragmented by caspases, whereas α-catenin is not affected (Brancolini et al., 1997; Brancolini et al., 1998; Steinhusen et al., 2000). Moreover, as we were able to show recently, the desmosomal cadherins desmoglein-3 and desmocollin-3 are shed from the cell surface and the desmoglein-3 cytoplasmic domain is cleaved twice by caspases (Weiske et al., 2001). In addition to plakoglobin, the desmosomal plaque proteins plakophilin-1 and desmoplakin were found to be fragmented by caspases.

Tight junctions have turned out to be platforms for trafficking and signaling protein complexes (Zahraoui et al., 2000) but a detailed analysis of the fate of tight junction proteins during apoptosis is missing. Here we show that the C-terminal cytoplasmic tail of occludin, a central transmembrane protein of tight junction strands, is cleaved by caspases C-terminal to Asp^{320}, generating a specific 31 kDa fragment. Since the antibodies used in the presented experiments were directed against the cytoplasmic domain, and other suitable antibodies were not available, it cannot be definitely excluded that caspases might also cleave within the cytoplasmic loop between transmembrane domain 2 and 3 of occludin. However, analysis of the amino acid sequence did not reveal a caspase consensus sequence in the cytoplasmic loop. Moreover, it is an open question whether the remaining part of occludin stays associated with the apical tight junction structures or becomes laterally distributed. Overexpression of an occludin fragment

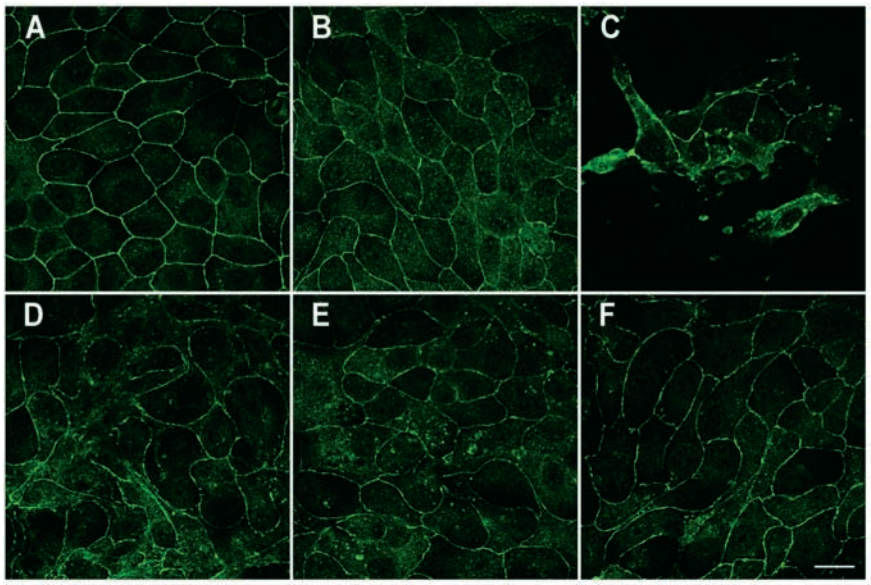


Fig. 7. Localization of ZO-1 in apoptotic cells. MDCK cells were stained with polyclonal anti-ZO-1 antibody. (A) 0 hours; (B) 3 hours; (C) 6 hours STS; (D) 6 hours STS + Z-DEVD-FMK; (E) 6 hours STS + TAPI-2; (F) 6 hours STS + Z-DEVD-FMK + TAPI-2. STS, staurosporine. Scale bar: 20 μm.

with a deleted C-terminal cytoplasmic domain does not interfere with tight junction strand formation and appearance of tight junctions in thin sections but results in increased paracellular permeability and loss of the intramembrane diffusion barrier (Balda et al., 1996). Thus, release of the occludin C-terminus during apoptosis might generate an occludin fragment that could act similarly in a dominant-negative way on tight junctions thus providing a mechanism that enhances the apoptotic disruption of tight junctions.

Inhibitor studies revealed that a second cleavage site in occludin is present presumably in the first extracellular loop of occludin and that this cleavage is mediated by a metalloproteinase. Interestingly, this cleavage reaction is efficiently blocked by the metalloproteinase inhibitor TAPI-2, that was originally described as a TACE (TNF-α converting enzyme) inhibitor, but is only inhibited to a minor extent by MMPI-1. Similarly, E-cadherin cleavage was blocked by TAPI-2 but not by MMPI-1, consistent with the strong reciprocal interaction between adherens and tight junctions. Inhibition of protein tyrosine phosphatases in epithelial and endothelial cells by phenylarsine oxide (PAO) treatment was previously reported to induce metalloproteinase-dependent cleavage of occludin generating a 50 kDa fragment (Wachtel et al., 1999). It will be interesting to analyze whether the generation of this 50 kDa fragment can be inhibited by TAPI-2. Moreover, PAO treatment elevated permeability and led to the disappearance of occludin from sites of cell contact and its diffuse accumulation in cells. A similar redistribution of occludin was detectable after induction of apoptosis by staurosporine. The extracellular loops of occludin were reported to also be targeted by other proteinases. Cleavage of occludin by cysteine and serine proteinases from house dust mite fecal pellets (HDMFP) results in disruption of tight junction barrier function (Wan et al., 2001; Wan et al., 1999). Similarly, treatment of MDCK cell layers with rat mucosal mast cell chymase (RMCP-II) altered the permeability and distribution of the tight junction proteins ZO-1 and occludin (Scudamore et al., 1998), which may also depend on an extracellular cleavage of occludin. Moreover, the bacterial cytotoxin hemagglutinin/ protease (HA/P) was shown to perturb barrier function by fragmentation of occludin (Wu et al., 2000). The electrophysiological data presented above are in line with these observations although other components of tight junctions and cell-cell contacts may contribute to the observed effects.

Similar to occludin, claudins show a 4-transmembrane domain structure. They represent a heterogenous family of at least 20 tight junctional transmembrane proteins that were shown to be involved in the determination of the different paracellular permeability properties reported for different epithelia. When we analyzed the fate of claudin-1 and -2 we found a clearly different behavior of the two proteins in response to induction of apoptosis. The amount of claudin-2 protein was reduced, whereas claudin-1 levels did not show strong changes or were even increased in HT-29/B6 cells. This suggests that, depending on their claudin patterns, epithelial cells may specifically respond to apoptotic stimuli. Moreover, the reduction of the pore-inducing claudin-2 and the up-regulation of the tightening claudin-1 may represent a physiological compensatory mechanism when single apoptotic cells are removed from an epithelial cell layer to maintain at least a limited barrier function until the neighboring cells have reestablished new cell-cell contacts (Abreu et al., 2000).

Removal of the C-terminal cytoplasmic domain generates an occludin variant that is no longer able to associate with the cytoplasmic adapter proteins ZO-1, -2 and -3 and, as a consequence, with the actin cytoskeleton. The observed fragmentation of ZO-1 and ZO-2 further contributes to impairment of tight junction structure and function. Especially since claudins are also associated with ZO-1, ZO-2 and ZO-3, the tight junction strand-forming proteins thus completely lose their linkage to the actin cytoskeleton and other ZO-1-, ZO-2-, ZO-3-interacting proteins. Currently it is not known which secondary effects might be induced by the release of the occludin C-terminus from the plasma membrane and the fragmentation of ZO-1 and ZO-2 proteins. Overexpression of ZO-1 mutants with deleted C-terminal domains was reported to lead to EMT (epithelium to mesenchyme transition)-like changes in cell morphology (Ryeom et al., 2000). Thus ZO-1 fragments could contribute to the dramatic changes in cell morphology seen in apoptotic cells. Moreover, ZO-1 was shown to interact with the transcription factor ZONAB (Balda and Matter, 2000). In consequence, apoptotic ZO-1 fragments might interact with ZONAB and affect transcriptional processes.

The regulated disruption of tight junctions, adherens junctions and desmosomes appears to represent a crucial step

during extrusion of apoptotic cells from epithelial and endothelial cell layers. Especially when apoptotic cells lose their contacts to neighboring cells and detach, it is critical that the remaining cells establish new contacts to maintain barrier function (Abreu et al., 2000) to avoid infiltration of noxious agents or allergens. Impairment of epithelial barrier function is a typical phenomenon observed in the intestinal mucosa of inflammatory bowel disease (IBD) patients. An accumulation of polymorphic mononuclear cells at sites of inflammation, changes in cytokine levels (Andus et al., 1991; McCormack et al., 2001; Nikolaus et al., 1998) and increased rates of epithelial apoptosis (Anderson, 2000; Iwamoto et al., 1996; Levine, 2000; Sträter et al., 1997) were found to be associated with an increased permeability of the intestinal barrier. In human HT-29/B6 cells, increased cytokine levels resulted in a decrease in the transepithelial resistance and altered tight junction strand formation (Schmitz et al., 1999a). This, at least in part, appears to be regulated by the repression of the occludin promoter in response to TNF-α and IFN-γ (Mankertz et al., 2000). In this respect it was recently shown that spontaneous and TNF-α-induced single-cell apoptosis results in leaks in the epithelial barrier (Gitter et al., 2000). Moreover, it was demonstrated that metalloproteinase levels are elevated in inflammatory bowel diseases (Baugh et al., 1999; von Lampe et al., 2000). Down-regulation of occludin thus appears to be at least one of the molecular events participating in the increase of paracellular permeability (Kucharzik et al., 2001). Current investigations are attempting to elucidate the molecular mechanisms involved in the down-regulation of occludin. In this context our results now indicate that, together with TNF-α- and IFN-γ-induced transcriptional repression of the occludin promoter (Mankertz et al., 2000), caspase- and metalloproteinase-mediated proteolytic fragmentation of occludin represents a relevant pathomechanism that may contribute to IBD.

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