The Fate of Desmosomal Proteins in Apoptotic Cells*

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Activation of caspases results in the disruption of structural and signaling networks in apoptotic cells. Recent biochemical and cell biological studies have shown that components of the cadherin-catenin adhesion complex in epithelial adherens junctions are targeted by caspases during apoptosis. In epithelial cells, desmosomes represent a second type of anchoring junctions mediating strong cell-cell contacts. Using antibodies directed against a set of desmosomal proteins, we show that desmosomes are proteolytically targeted during apoptosis. Desmogleins and desmocollins, representing desmosome-specific members of the cadherin superfamily of cell adhesion molecules, are specifically cleaved after onset of apoptosis. Similar to E-cadherin, the desmoglein-3 cytoplasmic tail is cleaved by caspases. In addition the extracellular domains of desmoglein-3 and desmocollin-3 are released from the cell surface by a metalloproteinase activity. In the presence of caspase and/or metalloproteinase inhibitors, both cleavage reactions are almost completely inhibited. As reported previously, the desmosomal plaque protein plakoglobin is cleaved by caspase-3 during apoptosis. Our studies now show that plakophilin-1 and two other major plaque proteins, desmoplakin-1 and -2, are also cleaved by caspases. Immunofluorescence analysis confirmed that this cleavage results in the disruption of the desmosome structure and thus contributes to cell rounding and disintegration of the intermediate filament system.

Apoptosis is a highly conserved process important for the destruction of excess or damaged cells during the development and in the homeostasis of multicellular organisms (1). Impaired regulation of programmed cell death is involved in the pathogenesis of cancer and immune and neuronal diseases (2, 3). Once the cell death program is started, dramatic changes in cell morphology can be observed such as nuclear/cytoplasmic condensation, formation of membrane protrusions, DNA fragmentation, and disruption of the structural integrity followed by fragmentation into “apoptotic bodies” that are removed by subsequent engulfment by neighboring cells or macrophages. Many of these morphological changes can be attributed to the cleavage of structural and regulatory proteins by members of the caspase family of cysteine proteases. Caspases specifically cleave substrate proteins C-terminal to aspartate residues (for review see Refs. 4–6). Caspase-3-mediated cleavage of inhibitor of caspase-activated DNase releases caspase-activated DNase, which is responsible for the generation of the nucleosomal ladder. Cleavage of cytoskeletal proteins and regulators such as fodrin (7), gelsolin (8), Gas2 (9), and focal adhesion kinase (10–12) and adhesion molecules such as cadherins (13–16) results in disruption of the cytoarchitecture.

Desmosomes are punctate intercellular junctions located on the basolateral side primarily of epithelial cells. They provide mechanical strength to epithelial tissues by forming stable cell-cell contacts that are anchored to the keratin intermediate filaments, thereby connecting the intermediate filament system of neighboring cells within a tissue. Two types of transmembrane glycoproteins of the cadherin superfamily, the desmogleins (Dsg1–3)1 and the desmocollins (Dsc1–3), mediate cell-cell adhesion in desmosomes. At present conflicting results about the mechanism of desmosomal cell-cell adhesion exist (for review see Ref. 17). From studies in a nonadhesive fibroblastic host cell environment it appears that transfection with plakoglobin together with desmoglein and desmocollin results in substantial adhesion, suggesting that both types of cadherins are important for cell adhesiveness (18, 19). For the anchorage of the intermediate filament cytoskeleton, a number of proteins have to be associated with the cytoplasmic tails of the desmosomal cadherins to form the desmosomal plaque. Two families of proteins appear to play a central role in the functional assembly and stabilization of desmosomes: members of the Armadillo protein family, namely plakoglobin and plakophilin1–3 (20), and the plakin protein family (21–24). A number of studies performed to analyze the molecular structure of the desmosomal plaque suggest a highly complex network of interactions between these proteins. Within this network plakoglobin and plakophilin-1 are able to interact directly with the desmosomal cadherins and are involved in the recruitment of desmplakin1 to the membrane. Moreover, it also appears that desmplakins can bind to desmosomal cadherins (25). Association with the intermediate filament system is mediated by plakoglobin and desmplakins (25–27). From these interaction studies a model for the structural assembly of the

1 The abbreviations used are: Dsg, desmoglein; Dsc, desmocollin; MMPI-1, matrix metalloproteinase inhibitor 1, 2-amino-benzoyl-Gly-Pro-p-D-Leu-p-D-Ala-NH-Oh; STS, staurosporine; TAPI, N-[(1,1,2(3[S-Hydroxyaminocarboxylnethyl-(methyl)]-4-methylpentanoyl)-3-(2-naphthyl)allyl)-alanin-1-amine, 2-aminoethyl amide; Z-DEV-D-FMK, Z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-Glu(OMe)-CH₂-F; MDCK, Madin-Darby canine kidney; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; GST, glutathione S-transferase.
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Desmosomal plaque has emerged whereby plakoglobin links desmoplakin to the desmosomal cadherin tails, and plakophilin-1-desmoplakin interactions extend the plaque laterally (17). This model is consistent with data obtained by high resolution immunoelectron microscopy (28).

Cell matrix and cell-cell adhesion mechanisms regulate cell growth, differentiation, and survival of epithelial and endothelial cells. When cultured cells lose their contacts to the extracellular matrix and are grown in suspension, they rapidly undergo apoptosis (29). However, cells are able to survive and to proliferate when they are permitted to form E-cadherin-mediated multicellular aggregates (30, 31). This in consequence implicates that cells irreversibly destined for cell death must have highly efficient mechanisms to inactivate cell-cell contacts. Cell junctions representing sites of cytoskeletal anchorage therefore appear to be predestined sites for cleavage by effector caspases. Adhesion complexes of epithelial and endothelial adherens junctions have previously been shown to be targeted by caspases during apoptosis (13, 14, 16).

However, at present our knowledge about the fate of desmosomes during apoptosis is limited. It is known that plakoglobin, a protein localized in the desmosomal plaque and in adherens junctions, is cleaved by caspases after induction of apoptosis (32). Here we report an extensive study analyzing the fate of desmosomes after induction of apoptosis. Similar to the classical cadherin family member E-cadherin, desmoglein-3 (Dsg3) is cleaved by caspase(s) and a metalloproteinase(s) during programmed cell death. Inhibitor studies, however, indicated that the metalloproteinase responsible for shedding of the desmoglein extracellular domain is not identical with the enzyme releasing the E-cadherin extracellular domain into the cell culture supernatant. Desmocollin-3 (Dsc3) cleavage was inhibited by the same metalloproteinase inhibitor. Moreover, the desmosomal plaque proteins desmoplakin-1 and -2 and plakophilin-1 were fragmented by caspase activity. This results in the disruption of desmosomes and concomitant breakdown of the keratin cytoskeleton as shown by immunofluorescence microscopy.

EXPERIMENTAL PROCEDURES

Cell Culture—The cell lines HaCat and MDCK were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.).

Reagents and Antibodies—The monoclonal antibody directed against the desmoglein cytoplasmic domain (clone 62) was purchased from BD Transduction Laboratories (Heidelberg, Germany); antibodies directed against desmocollin-3 (Dsc3-U114), desmoplakin-1 and -2 (DP1&2–2.15) (33), and cytokeratin pan were obtained from Progen (Heidelberg, Germany). The desmoglein-3 antibody (5H10) against the extracellular domain is described by Prohy et al. (34). The rabbit polyclonal antibodies against plakophilin-1 head (anti-667) and repeat domains (anti-670) are described elsewhere (27).

Horse radish peroxidase-labeled anti-mouse and anti-rabbit antibodies were purchased from Dianova (Hamburg, Germany). Alexa FluorTM488 goat anti-mouse IgG (Alexa FluorTM594 goat anti-rabbit IgG antibodies were obtained from Molecular Probes (Schwalbach, Germany). Caspase-3 inhibitor Z-DEVD-FMK and matrix metalloproteinase inhibitor-1 were purchased from Calbiochem (Schwalbach, Germany), and TAPI was kindly provided by Dr. R. Black (Immunex, Seattle, WA). The active recombinant human caspase-3 (CPP32) was purchased from BD Pharmingen (Heidelberg, Germany). Immunoprecipitations—The cell culture supernatants were collected at different time points after induction of apoptosis. After centrifugation (10 min, 20,800 × g), 1 ml of the supernatant was preincubated for 30 min with 50 μM Z-DEVD-FMK and/or 100 μM MMP inhibitorI before addition of staurosporine. For solubilization of desmoplakins, the cells were incubated in lysis buffer, and after the addition of an equal volume of 8 M urea they were incubated for 24 h at 4 °C under constant agitation.

Western Blot Analysis—50 μg of total protein in 4× SDS loading buffer was separated by SDS-polyacrylamide gel electrophoresis and transferred onto PolyScreen polyvinylidene fluoride transfer membranes (PallBiorad Life Sciences). Membranes were blocked with TBST buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20) for 1 h at room temperature and incubated with the first antibody (1 μg/ml for anti-desmoglein (Clone 62) and anti-desmoplakin (DP1&2–2.15); anti-plakophilin-1 antibody (anti-670) was diluted 1:5,000; anti-desmocollin-3 and anti-desmoglein-3 (5H10) antibodies were diluted 1:2,000 and 1:200, respectively) in TST for 1 h. After three washes, the membranes were incubated with horseradish peroxidase-conjugated second antibody diluted 1:10,000 in TST for 30 min. Chemiluminescence detection was performed by exposure of Lumiglo Western blotting substrate (Roche Molecular Biochemicals) treated membranes to Biomax MR films (Eastman Kodak Co.). Molecular masses of fragments were determined using BenchMarkTM Protein Ladder (Life Technologies, Inc.).

Immunoprecipitations—The cell culture supernatants were collected at different time points after induction of apoptosis. After centrifugation (10 min, 20,800 × g), 1 ml of the supernatant was preincubated for 30 min with 50 μM Z-DEVD-FMK and/or 100 μM MMP inhibitorI before addition of staurosporine. For solubilization of desmoplakins, the cells were incubated in lysis buffer, and after the addition of an equal volume of 8 M urea they were incubated for 24 h at 4 °C under constant agitation.

Immunofluorescence—The cells were grown for 18 h on gelatine-coated glass coverslips. 3 or 6 h after induction of apoptosis cells were washed with PBS and fixed immediately in ice-cold methanol for 10 min. For staining with anti-Dsg3 (clone 62) antibody and anti-plakophilin-1 (anti-670) cells were washed with PBS, permeabilized with 0.5% (v/v) Triton X-100, and fixed in 3.7% (w/v) paraformaldehyde for another 20 min. Subsequently the cells were washed in PBS after blocking with 0.1% (v/v) goat serum in PBS for 30 min at room temperature, the cells were incubated with first antibodies for 30 min at room temperature (anti-plakophilin-1 antibody (anti-670) was diluted 1:250). After three washes in PBS, the cells were incubated with Alexa FluorTM488 goat anti-mouse IgG or Alexa FluorTM594 goat anti-rabbit IgG antibodies for another 30 min and washed again before mounting with ProTags Mount Fluor (Biocyt, Luckenwalde, Germany). For double staining the cells were incubated with Alexa FluorTM488 goat anti-mouse IgG antibodies (30 μg/ml for anti-plakophilin-1, and 32 μg/ml for anti-desmoglein-3 clone 5H10; 1:100 for anti-cytokeratin pan) under otherwise identical conditions. Analysis and photography were performed on a Zeiss LSM510 confocal microscope with 63× magnification at excitation wavelengths 543 and 488 nm. Details on the microscopy setup are available on request.

In Vitro Caspase Cleavage—A recombinant glutathione S-transferase (GST)-tagged cytoplasmic domain of human desmoglein-3 was expressed in E. coli and adsorbed onto GST-agarose beads. Recombinant protein (5 μg) was digested with 60 ng of recombinant caspase-3 in 20 mM PIPES, pH 7.2, 0.1% (w/v) CHAPS, 10 mM dithiothreitol, 100 mM NaCl, 1 mM EDTA, 10% sucrose, 1 mM phenylmethylsulfonyl fluoride, 50 μM leupeptin, and 200 μg/ml aprotinin at 37 °C for 2 to 4 h. After separation by SDS-polyacrylamide gel electrophoresis, the cleavage products were transferred to polyvinylidene fluoride membrane. Coomassie-stained protein bands were excised and sequenced by automated Edman degradation on a Applied Biosystems protein sequencer (model 473A).
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RESULTS

Cleavage of Desmoglein-3 and Desmocollin-3 during Apoptosis—Our recent analysis of E-cadherin fragmentation during apoptosis revealed that the cytoplasmic tail of E-cadherin is cleaved by caspase-3 near the transmembrane domain, and, simultaneously, the extracellular domain is released from the cell surface by a metalloproteinase (16). To examine whether desmosomal cadherins are cleaved in a similar way at intracellular and/or extracellular sites, apoptosis in HaCat cells was induced by staurosporine treatment, and the fate of desmoglein-3 (Dsg3) and desmocollin-3 (Dsc3) was analyzed in detergent extracts of cells by Western blotting. The intracellular domain of desmoglein was detected with the monoclonal anti-desmoglein antibody (clone 62). The specificity of this antibody for Dsg1 or Dsg3 was tested against GST-Dsg1 and GST-Dsg3 cytoplasmic domain fusion proteins. The antibody interacted strongly with Dsg3 and exhibited weak cross-reactivity with Dsg1 (not shown). The extracellular domain of Dsg3 was analyzed with the monoclonal anti-Dsg3 antibody (clone 6H10) (34). Dsc3 was detected with the monoclonal anti-desmocollin-3 (clone Dsc3-U114) antibody (35). HaCat cells responded to the apoptotic stimulus by changes in cell shape, fragmentation of the nucleus, and detachment from the substrate. Stimulation of apoptosis was confirmed biochemically in analyses for poly (ADP-ribose) polymerase cleavage products (not shown).

Dsg3 was almost completely cleaved within 24 h after staurosporine-induced apoptosis, and two Dsg3 fragments with apparent molecular masses of about 55 kDa (fragment 1) and 100 kDa (fragment 2) both reacting with the monoclonal antibody directed against the cytoplasmic domain were detectable in detergent extracts after 3–12 h (Fig. 1A). Quantification of the signal intensities for fragment 2 was highest around 9 h after induction of apoptosis and then declines during the next 12–15 h, suggesting that fragment 2 is further fragmented (not shown). A similar fragmentation pattern was observed in both staurosporine- or camptothecin-treated MDCK and HaCat cells (Fig. 1B), confirming that fragmentation of Dsg3 is not dependent on a specific cell line or apoptosis inducing agent. Analysis of the fragmentation pattern with the anti-Dsg3 (5H10) antibody revealed cleavage products with apparent molecular masses of 80 and 100 kDa (Fig. 1C). The 80-kDa cleavage product was assigned as fragment 3. The 100-kDa fragment appears to be identical to fragment 2 because both fragments perfectly aligned when analyzed on the same gel (not shown). The molecular masses of fragments 1–3 suggested that Dsg3 is fragmented during apoptosis by three distinct intracellular and extracellular cleavages. In this context, it was expected that the extracellular cleavage reaction should release an extracellular domain fragment of Dsg3 into the cell culture supernatant. In immunoprecipitation experiments with anti-Dsg3 (5H10) antibody, a 75-kDa fragment (fragment 4) was precipitated from the supernatants of apoptotic cells that increased in a time-dependent manner after induction of apoptosis (Fig. 1D). To further confirm these data, inhibitor studies were performed. In the presence of the caspase inhibitor Z-DEVD-FMK, formation of fragment 2 was strongly inhibited when analyzed with anti-Dsg3 (clone 62) antibody, whereas generation of fragment 1 was less reduced (Fig. 2A). The formation of fragments 2 and 3 was blocked when analyzed with anti-Dsg3 (5H10) antibody (Fig. 2B). In our previous studies (16) we have shown that the metalloproteinase inhibitor TAPI inhibits shedding of the E-cadherin extracellular domain. Using this inhibitor, Dsg3 shedding could not be blocked (not shown). However, in the presence of the matrix metalloproteinase inhibitor-1 (MMPI-1) inhibiting matrix metalloproteinases 1, 8, 9, and 3 (36), formation of fragment 1 was blocked, whereas fragment 2 was not affected when analyzed with anti-Dsg3 (clone 62) antibody (Fig. 2A). Fragments 2 and 3 where not affected when analyzed with anti-Dsg3 (5H10) antibody (Fig. 2B). Consistent with these data, shedding of the soluble fragment 4 was not affected in the presence of Z-DEVD-FMK (Fig. 2C). In the presence of MMPI-1, however, the amount of fragment 4 in the cell culture supernatant was reduced (Fig. 2C). All figures are representatives of at least three independent experiments. From these data we propose the following model (Fig. 2D). Fragments 2 and 3 result from two distinct caspase cleavages in the cytoplasmic tail of Dsg3 and encompass the entire extracellular domain, the transmembrane domain, and different parts of the intracellular domain dependent on the caspase cleavage site. Fragment 1 represents part of the extracellular domain C-terminal to the metalloproteinase.
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Fig. 2. Inhibition of cytoplasmic domain cleavage and extracellular domain shedding. A, formation of fragments 2 and 1 is inhibited in the presence of the caspase inhibitor Z-DEVD-FMK (DEVD) and the matrix metalloproteinase inhibitor-1 (MMPi), respectively, as analyzed with anti-desmoglein (clone 62) antibody. B, in the presence of DEVD, fragments 2 and 3 were not detectable when analyzed with anti-Dsg3 (SH10) antibody. The addition of MMPI-1 did not affect the formation of fragments 2 and 3. C, in the presence of DEVD shedding of fragment 4 was not affected, whereas addition of MMPI-1 inhibited formation of fragment 4 as shown by immunoprecipitation with anti-Dsg3 (SH10) antibody and subsequent Western blotting. For all experiments cells or cell culture supernatants were harvested 6 h after induction of apoptosis. D, schematic model of the apoptotic cleavage fragments of Dsg3 deduced from the detected fragmentation pattern. The epitopes detected by the anti-desmoglein antibody (clone 62) and the anti-Dsg3 (SH10) antibodies are indicated as deduced from our data and from Ref. 34, respectively. Co, control.

Fig. 3. Desmocollin-3 cleavage during apoptosis. A, kinetics of the Dsc3 cleavage analyzed by Western blotting with anti-Dsc3-U114 antibody. The antibody reacts with the 109- and 100-kDa desmocollin-3 splice isoforms. B, Dsc-3 cleavage was inhibited in the presence of MMPi-1 (MMPi) but not by Z-DEVD-FMK (DEVD). The cells were analyzed 6 h after induction of apoptosis by STS. Co, control; MW, molecular mass.

To confirm this model we tried to identify the caspase cleavage site(s) in the Dsg3 cytoplasmic domain. However, the epitope detected by the anti-desmoglein antibody (clone 62) maps to the N-terminal half of the Dsg3 cytoplasmic domain and thus it was not possible to isolate the apoptotic fragment(s) generated from the Dsg3 cytoplasmic domain in vivo. Therefore, we tried to map the caspase cleavage site(s) by in vitro cleavage using a GST-Dsg3 cytoplasmic domain fusion protein as substrate for recombinant caspase-3. Two cleavage fragments were detectable (not shown), and subsequent N-terminal sequencing of the cleavage fragment(s) by Edman degradation allowed us to map a caspase-3 cleavage site C-terminal to amino acid Asp791. Unfortunately, no sequence data could be obtained for the second fragment.

Analysis of the fate of desmocollin-3 after induction of apoptosis indicated that apart from desmogleins desmocollins are also proteolytically targeted in apoptotic cells. Desmocollins are subjected to alternative splicing resulting in two isoforms that differ in the length of their cytoplasmic domains (37, 38). After induction of apoptosis both the 109-kDa and the 100-kDa splice variants of Dsc3 were cleaved within 24 h (Fig. 3A). As compared with the 109-kDa form, the 100-kDa splice variant appeared to be cleaved more efficiently. No specific cytoplasmic or membrane-bound cleavage products were detectable in cellular detergent extracts. This can be explained by the specificity of the anti-Dsc3 (clone Dsc3-U114) antibody that is directed against an epitope located in the Dsc3 extracellular domain. Moreover, analysis of the cytoplasmic domain amino acid sequence revealed only one obvious caspase cleavage site C-terminal to Asp734 in human Dsc3. If this site is used by endogenous caspases, an 18-amino acid fragment would be derived from full-length Dsc3, generating a molecular mass shift that is not specifically detectable in our assays. To examine whether the decrease in the Dsc3 signal is the result of a matrix metalloproteinase activity, inhibitor studies were performed as described above. The addition of Z-DEVD-FMK did not effect the decrease of both Dsc3 variants, whereas MMPI-1 inhibited cleavage of both full-length Dsc3 forms, indicating that both splice variants are proteolytically fragmented in their extracellular domains (Fig. 3B). We could not show the accumulation of the Dsc3 extracellular domain fragments in the cell culture supernatant because the only antibody available to us (anti-Dsc3-U114) did not react with soluble or detergent extracted Dsc-3 in immunoprecipitation experiments.

Localization of Desmoglein-3 in Apoptotic Cells—To examine the fate of Dsg3 in cells during apoptosis, confocal immunofluorescence microscopy was performed. At time 0, anti-Dsg3 (SH10) antibody directed against the extracellular domain of Dsg3 revealed strong staining at sites of cell-cell contact (Fig. 4A). Anti-Dsg3 (clone 62) antibody staining showed mainly punctate immunofluorescence at sites of cell contact (Fig. 4D). After induction of apoptosis, staining at cell contacts was strongly reduced (Fig. 4C and E). Moreover, cell morphology changes from a flat to a more thickened phenotype as seen in differential interference contrast images (not shown). Co-stain-
Fig. 4. Localization of Dsg3 in apoptotic HaCat cells. The cells were analyzed by indirect immunofluorescence double-staining with anti-desmoglein (5H10) antibody (5H10; green) and anti-pan-cytokeratin antibody (pan-ck; red) in comparison with anti-Dsg3 (clone 62) antibody staining (Dsg3; green). Panels a, e, i, m, and q, anti-desmoglein (5H10) antibody staining directed against the Dsg extracellular domain; panels b, f, j, n, and r, pan-cytokeratin staining; panels c, g, h, o, and s, merged images of Dsg extracellular domain and pan-cytokeratin stainings; panels d, h, l, p, and t, anti-Dsg3 (clone 62) antibody staining of the Dsg3 cytoplasmic domain. Panels a–d, 0 h; panels e–h, 6 h STS; panels i–l, 6 h STS + Z-DEVD-FMK; panels m–p, 6 h STS + MMPI-1; panels q–t, 6 h STS + Z-DEVD-FMK + MMPI-1. The cells stained with anti-desmoglein (5H10), and anti-pan-cytokeratin antibodies were fixed in methanol. For the staining with anti-Dsg3 (clone 62) antibody cells were permeabilized with Triton X-100 and fixed in paraformaldehyde.

Fig. 5. Apoptotic fragmentation of plakophilin-1 and desmoplakin-1 and -2. A, Western blot analysis of HaCat cell lysates with anti-plakophilin 670 antibody directed against the Arm repeat domain of plakophilin-1 at different time points after the addition of STS. B, formation of the 62 kDa cleavage product is inhibited in the presence of Z-DEVD-FMK. C, time-dependent desmoplakin-1 and -2 cleavage in apoptotic HaCat cells. D, addition of Z-DEVD-FMK inhibited the generation of the 92 kDa fragment in HaCat and MDCK cells. Co, control; MW, molecular mass.

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Analysis of Desmosomal Plaque Proteins Plakophilin-1 and Desmoplakin-1 and -2 During Apoptosis—In previous studies it was shown that plakoglobin, an Armadillo protein family member associated with the cytoplasmic domains of classical and desmosomal cadherins, is cleaved during apoptosis (32). Plakophilin-1, a second member of the Armadillo multigene family, is localized in desmosomal plaques and in the cell nucleus, suggesting both structural and signaling functions. Recently, plakophilin-1 was shown to support desmosome assembly and to be involved in actin filament organization (27). After induction of apoptosis, plakophilin-1 is rapidly cleaved in HaCat cells within 12 h as shown by Western blot analysis with the anti-plakophilin 670 antibody (27) directed against the plakophilin-1 Arm repeat domain. Concomitant with the decrease of full-length plakophilin-1, a 62-kDa cleavage product became detectable reacting with this antibody (Fig. 5A). In the presence of Z-DEVD-FMK, formation of the 62-kDa cleavage fragment was inhibited in both HaCat and MDCK cells (Fig. 5B).

Analysis of desmoplakin with the monoclonal antibody DP1&2–2.15 showed a rapid cleavage of desmoplakin and generation of a 92-kDa fragment (Fig. 5C) that is blocked by inhibition of caspase activity (Fig. 5D). Again for both proteins comparable results were obtained in HaCat and MDCK cells.
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Immunodetection of Plakophilin and Desmoplakin during Apoptosis—Probing of HaCat cells with the anti-plakophilin 670 antibody at 0 h revealed cell contact and strong nuclear staining in confocal microscopy (Fig. 6A, panel a). Nuclear plakophilin-1 staining was rapidly lost within 3 h after induction of apoptosis, and a diffuse staining all over the cytoplasm became detectable. A weak staining at sites of cell contacts remained detectable (Fig. 6A, panel b). After 6 h the cytoplasmic staining was strongly reduced, whereas plakophilin-1 staining was still detectable at cell-cell contacts (Fig. 6A, panel c). In the presence of Z-DEVD-FMK, however, the nuclear and cell-cell contact staining pattern was comparable with the pattern seen at time point 0 h (Fig. 6A, panel d). Immunofluorescence analysis with anti-DP1&2–2.15 antibody directed against desmoplakin showed a strong punctate staining at sites of cell-cell contacts (Fig. 6B, panel a). After 3 h the punctate pattern was lost and appeared as an intense and less punctate lining at cell contacts (Fig. 6B, panel b). The intensity of desmoplakin staining then strongly recedes within the next 3 h concomitant with the morphological changes of the cells (Fig. 6B, panel c). After 6 h in the presence of Z-DEVD-FMK both loss of desmoplakin and cell rounding could be blocked consistent with results of the Western blotting experiments. Moreover, the cell borders showed more continuous and intense staining and have more lateral projections (Fig. 6B, panel d).

DISCUSSION

Among the substrates targeted by effector caspases during apoptosis, a number of proteins involved in the regulation of cell contacts and of the cytoskeleton have been identified, e.g., focal adhesion kinase 32 (10–12), E-cadherin (14, 16), β-catenin (39, 40), plakoglobin (32), fodrin (7), and Gas 2 (9). Here we show that desmosomal cadherins and desmosomal plaque proteins are efficiently cleaved during apoptosis of epithelial cells. The observed cleavage pattern suggests that the human Dsg3 cytoplasmic tail is cleaved by caspases at two distinct sites and that, in addition, the extracellular domain is released from the cell surface by a metalloproteinase activity. Interestingly, the sheddase activity releasing the Dsg3 extracellular domain during apoptosis is different from the activity releasing the E-cadherin extracellular domain as shown by inhibitor studies. Although E-cadherin shedding was inhibited by TAPI, a metalloproteinase inhibitor initially shown to block tumor necrosis factor-α convertase (41), neither Dsg3 nor Dsc3 shedding was blocked by TAPI. On the other hand, the MMP-1 was found to act as an efficient inhibitor of desmosomal cadherin shedding during apoptosis. At present the physiological consequences of these differences in the substrate specificity of the shedding proteases specificity for classical and desmosomal cadherins during apoptosis are unknown. It will be interesting to find out why E-cadherin is cleaved by a member of the ADAM (a disintegrin and metalloproteinase) family of metalloproteinases, whereas desmosomal cadherins are targeted by specific matrix metalloproteinases during apoptosis. As shown for stromelysin during involution, E-cadherin in principle can also be cleaved by matrix metalloproteinases (42). Moreover, it is interesting to speculate whether misregulated shedding of desmosomal cadherins might contribute to the pathogenesis of epithelial diseases, e.g., blistering skin disease. Recently the *Staphylococcus aureus* exfoliative toxin A was shown to act as a protease cleaving the extracellular domain of Dsg1 and and to be responsible for the molecular pathology of the blistering diseases staphylococcal scalded skin syndrome and bullous impetigo (43).

Cleavage of desmosomal cadherins by metalloproteinase generates fragments that include a small part of the extracellular domain, the transmembrane segment and the cytoplasmic tail is cleaved by caspases at two distinct sites and over, the cell borders showed more continuous and intense staining and have more lateral projections (Fig. 6B, panel d).
proteins are cleaved by caspases during apoptosis. Desmosomal plaque proteins provide interaction sites for cytokeratin filaments as shown for the C terminus of desmolakin (46, 47) and the head domain of plakophilin (25–27). Thus proteolytic fragmentation of these proteins prevents binding of intermediate filaments and in consequence results in remodeling of the intermediate filament cytoskeleton as confirmed by immunofluorescence microscopy. Desmolakin staining changed from a punctate to uniform membrane distribution, indicating that the desmosomal structure is disrupted. Plakophilin-1 staining rapidly changed within the first hours after induction of apoptosis from a membrane and strong nuclear staining to a predominant diffuse cytoplasmic staining. This suggests that plakophilin-1 is impaired in supporting the formation and maintenance of desmosomes. Moreover, the observed rapid nuclear exclusion of plakophilin-1 or the plakophilin-1 cleavage product detected by the antibody suggests that also the putative signaling function of plakophilin-1 might be affected. Recently, it was shown that enhanced expression of plakoglobin up-regulates the expression of the anti-apoptotic protein Bcl-2 (48); however, the ability of plakoglobin to exert an anti-apoptotic effect appears not to be universal (49). In this context it will be interesting to analyze whether overexpression of plakophilin-1 also results in inhibition of apoptosis by up-regulation of anti-apoptotic protein(s). In summary, cleavage of proteins such as plakoglobin or plakophilin-1 appears to be a highly efficient mechanism in cells destined for death to simultaneously inactivate a potential anti-apoptotic activity and to break down cell contacts.

The proteolytic cleavage of junctional proteins affects both the integrity of cell-cell contacts and the structure of the cytoskeleton and thus contributes to the dramatic morphological changes observed during apoptosis. Consistent with our data, human colonic epithelial cells analyzed during Fas-mediated apoptosis showed dramatic rearrangements of tight junctions, adherens junctions, and desmosomes (50), a situation found in inflammatory bowel disease and ulcerative colitis when Fas ligand-expressing lymphocytes in the lamina propria induce apoptosis in colonic crypt epithelial cells. Apoptotic cells lose their contact to neighboring cells and detach, whereas the remaining cells establish new contacts to maintain barrier function. Thus the coordinated disruption and establishment of cell contacts during apoptosis appears to be an important mechanism for the homeostasis of cells in an epithelial cell layer.

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