Cleavage and Shedding of E-cadherin after Induction of Apoptosis*

Ulrike Steinhusen‡§, Jörg Weiske‡§, Volker Badock¶, Rudolf Tauber§, Kurt Bommert‡, and Otmar Huber**

From the Departments of ‡Medical Oncology and Tumourimmunology and ¶Protein Chemistry, Max Delbrück Center of Molecular Medicine, Robert-Rösle-Strasse 10, D-13092 Berlin and the §§University Hospital Benjamin Franklin, Institute of Clinical Chemistry and Pathobiochemistry, Hindenburgdamm 30, D-12200 Berlin, Germany

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Apoptotic cell death induces dramatic molecular changes in cells, becoming apparent on the structural level as membrane blebbing, condensation of the cytoplasm and nucleus, and loss of cell-cell contacts. The activation of caspases is one of the fundamental steps during programmed cell death. Here we report a detailed analysis of the fate of the Ca2+-dependent cell adhesion molecule E-cadherin in apoptotic epithelial cells and show that during apoptosis fragments of E-cadherin with apparent molecular masses of 24, 29, and 84 kDa are generated by two distinct proteolytic activities. In addition to a caspase-3-mediated cleavage releasing the cytoplasmic domain of E-cadherin, a metalloproteinase sheds the extracellular domain from the cell surface during apoptosis. Immunofluorescence analysis confirmed that concomitant with the disappearance of E-cadherin staining at the cell surface, the E-cadherin cytoplasmic domain accumulates in the cytosol. In the presence of inhibitors of caspase-3 and/or metalloproteinases, cleavage of E-cadherin was almost completely blocked. The simultaneous cleavage of the intracellular and extracellular domains of E-cadherin may provide a highly efficient mechanism to disrupt cadherin-mediated cell-cell contacts in apoptotic cells, a prerequisite for cell rounding and exit from the epithelium.

The crucial role of apoptosis during development and for tissue homeostasis of multicellular organisms is well established (1). Malfunctions of the death program and its control mechanisms often result in prenatal death during development and contribute to immune and neuronal diseases or cancer in the adult organism (2–4). The central mechanism of this cell death machinery is a proteolytic cascade mediated by the caspase family of cysteine proteinases (5, 6), which specifically cleave their substrates after aspartate residues. Caspases are members of the death machinery is a proteolytic cascade mediated by the caspase family of cysteine proteinases (5, 6), which specifically cleave their substrates after aspartate residues. Caspases are members of the death machinery is a proteolytic cascade mediated by the caspase family of cysteine proteinases (5, 6), which specifically cleave their substrates after aspartate residues. 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inhibitor Z-DEVD-FMK, whereas the other proteolytic event, shedding the E-cadherin extracellular domain into the cell culture medium, was mediated by a metalloprotease. According to these data both proteases are required for the efficient cleavage of E-cadherin during apoptosis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—MDCK (Madin-Darby canine kidney) cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) with 10% (v/v) fetal calf serum in the presence of 100 units/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Inc.) at 5% CO₂. The human breast epithelial cell line H184A1 was grown in Dulbecco's modified Eagle's medium/Ham's F12 (1:1) (Biochrom, Berlin, Germany) supplemented with 5% (v/v) fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml transferrin (Sigma), 10 μg/ml insulin (Biochrom), and 1.8 μg/ml hydrocortisone (Biochrom). The human breast carcinoma cells MCF-7/3.28 (transfected with caspase-3) and MCF-7/Vector (17) (kindly provided by Dr. A. Porter) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.8 mg/ml G418 (Life Technologies, Inc.).

**Reagents and Antibodies**—The monoclonal antibody directed against the human E-cadherin extracellular domain (clone 36) was purchased from Transduction Laboratories (Lexington, KY); antibody HECD-1 against the human E-cadherin extracellular domain was obtained from R & D Systems (Wiesbaden, Germany); and DECA-1 was kindly provided by Dr. Rolf Kemler (Max Planck Institute of Immunobiology, Freiburg, Germany). Horseradish peroxidase-labeled anti-mouse and anti-rabbit antibodies were purchased from Dianova (Hamburg, Germany). Alexa Fluor™ 488 goat anti-mouse IgG and Alexa Fluor™ 594 phalloidin were obtained from Molecular Probes (MoBiTec, Gottingen, Germany). Caspase-3 inhibitor Z-DEVD-FMK and MMP inhibitor I were purchased from Calbiochem; TAPI (N-(L-Asp(methoxy)-Glu(L-Leu)-L-Leu)-L-leucine, 2-aminoethyl amide) was kindly provided by Dr. Roy Black (Immunex, Seattle, WA). Staurosporine, camptothecin, ALLN (N-acety-Leu-Leu-Nor-Leu-CHO) and TAPI were obtained, as indicated, from Calbiochem; TAPI (N-(L-Asp(methoxy)-Glu(L-Leu)-L-Leu)-L-leucine, 2-aminoethyl amide) was kindly provided by Dr. Roy Black (Immunex, Seattle, WA). Staurosporine, camptothecin, ALLN (N-acety-Leu-Leu-Nor-Leu-CHO) and TAPI were obtained from Calbiochem (Eschweiler, Germany); and DECMA-1 was kindly provided by DECMA-1 was kindly provided by Biozyn, Hess, Oldendorf, Germany) and transferred onto polyvinylidene difluoride membranes (Immobilon™-P, Millipore). 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 primary antibody at a concentration of 1 μg/ml in TBST for 1 h. After three washes, membranes were incubated with horseradish peroxidase-conjugated secondary antibody diluted 1:10,000 in TBS. After washing, chemoluminescence detection was performed by exposure of Lumi-Light Western blotting substrate (Roche Molecular Biochemicals)-treated membranes to Biomax MR films (Kodak, Rochester, NY). For quantification of the chemoluminescence signals, membranes were scanned with a FujiFilm LAS-1000 system and analyzed with the Image Gauge version 3.2 software. Molecular weights of fragments were determined using the BenchMark™ Prestained Protein Ladder (Life Technologies, Inc.).

**RESULTS**

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**Immunoprecipitation**—To detect the 84-kDa E-cadherin fragment released from the cell surface, cell culture supernatants were collected at different time points after induction of apoptosis. After centrifugation for 10 min at 20,800 × g, 1 ml of the supernatant was pre-cleared by incubation with 30 μl of protein A-Sepharose for 30 min at 4 °C under constant agitation. Protein A beads were removed by centrifugation at 20,800 × g for 10 min at 4 °C. For immunoprecipitation, 0.5 μg of HEC-1D antibody or 5 μg of DECA-1 antibody were added. After 30 min of incubation at 4 °C, supernatant fluid was collected by gentle centrifugation at 3100 g for 10 min. After three additional washes with PBS, 50 μl of anti-E-cadherin antibody HECD-1 antibody or 5 μg of anti-MMP antibody I were incubated with Protein A-Sepharose beads were added and incubated for 1 h as described above. Protein A beads were washed three times with TBS, 0.1% (v/v) Tween 20, 8000 g for 1 h. After washing, proteins were separated by 7.5% SDS-polyacrylamide gel electrophoresis, and Western blotting was performed as described above.

**Immunofluorescence**—Cells were grown on glass coverslips. 3 h or 6 h after induction of apoptosis, cells were briefly washed with PBS and fixed in ice-cold methanol for 10 min. Subsequently, cells were washed in PBS, and after incubation for 10 min at room temperature, cells were incubated with anti-E-cadherin antibody HECD-1 for 30 min at room temperature (0.5 μg/ml for anti-E-cadherin clone 36 and 2 μg/ml for anti-HECD-1). After three washes in PBS, cells were incubated with Alexa Fluor™ 488 goat anti-mouse IgG for another 30 min and washed again before mounting in elvanol. For double staining, cells were washed twice with prewarmed PBS, fixed in 3% paraformaldehyde for 20 min, and incubated with blocking buffer (PBS + 25 mM glycine) for 5 min. After two additional washings with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 3 min and subsequently blocked with 0.1% (v/v) goat serum in PBS for 30 min at room temperature. E-cadherin staining was performed as described above, and subsequent phallolidin staining was performed with Alexa Fluor™ 594 phallolidin according to the manufacturer's instructions. Analysis and photography were performed on a Zeiss LSM510 confocal microscope with ×100 magnification at excitation wavelengths of 453 and 488 nm. Details on the microscopy setup are available on request. In Vitro Caspase Cleavage—GST-ECT was expressed in Escherichia coli as described (41). 5 μg of recombinant protein were digested with 50 ng of recombinant caspase-3, -6, or -7 in 50 mM HEPES (pH 7.4), 0.1% (v/v) CHAPS, 5 mM dithiothreitol, and resuspended in 20 mM Tris/HCl (pH 7.4), 3 mM MgCl₂, 0.5% (v/v) Triton X-100, and resuspended in 20 μl of 2 × SDS sample buffer. After boiling, proteins were separated by 7.5% SDS-polyacrylamide gel electrophoresis, and Western blotting was performed as described above.

**E-cadherin Cleavage during Apoptosis of Epithelial Cells**—To investigate the fate of E-cadherin during programmed cell death, lysates of MDCK cells were examined by Western analysis upon induction of apoptosis by staurosporine treatment. Changes in morphology, fragmentation of the nucleus, and detachment from the substrate indicated that MDCK cells responded to the apoptotic stimulus. Nearly all full-length E-cadherin was cleaved 24 h after addition of staurosporine (Fig. 1A). As determined by quantitative chemoluminescence imaging, 50% of E-cadherin was proteolytically processed during the first 11–13 h of staurosporine treatment (Fig. 1B). Distinct cleavage products of E-cadherin with apparent molecular masses of about 24 kDa and 29 kDa, designated fragment 1 and fragment 2, respectively, were detectable by 3 h after induction of apoptosis with an antibody (clone C20) directed against the cytoplasmic domain of E-cadherin. A 35-kDa polypeptide band reacting with the anti-E-cadherin antibodies was present in nonapoptotic cells, indicating that this band is not related to the apoptotic cleavage of E-cadherin. Furthermore, by comparing Ltk− cells and E-cadherin-transfected Ltk− cells, it could be shown that this band reflects nonspecific cross-reactivity of the antibody with an unknown polypeptide. The 35-kDa band was detected in Ltk− and E-cadherin-transfected Ltk− cells,
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whereas a 120-kDa cadherin band was only present in E-cadherin-transfected Ltk°C cells (data not shown). The accumulation of fragments 1 and 2 peaked at about 6 h and subsequently declined over the next 6 h, indicating that these fragments were further degraded (Fig. 1, A and B). This might explain why the intensity of the signals for the cleavage products is considerably weaker than the signal for full-length cadherin at all time points following induction of apoptosis. In the presence of ALLN, fragment 1 was stabilized, suggesting that further degradation of fragment 1 might be a proteasome-mediated process (Fig. 1C). In contrast, the amount of fragment 2 was not affected by ALLN, suggesting that this fragment might be an intermediate product. Similar results were obtained after induction of apoptosis with camptothecin, independent of whether MDCK or the human mammary epithelial cell line H184A1 was used (Fig. 1D), confirming that the described fragmentation of E-cadherin is not cell-line specific.

To find out whether fragments 1 and 2 were generated by caspase cleavage, the membrane-permeable irreversible caspase inhibitor Z-DEVD-FMK was added to the cultured cells prior to staurosporine treatment, and cell lysates were analyzed for E-cadherin 6 h later. In the presence of Z-DEVD-FMK, formation of fragment 1 was completely blocked, whereas formation of fragment 2 was not affected (Fig. 2). This observation explains why, despite the presence of caspase-inhibitor, full-length E-cadherin still was proteolytically processed (data not shown) and suggests that E-cadherin is targeted by a distinct second cleavage event.

In Vitro Cleavage of the E-cadherin Cytoplasmic Domain with Recombinant Caspase-3, -6, and -7—Because fragment 1 was detected with an antibody directed against the cytoplasmic domain of E-cadherin, and generation of fragment 1 was blocked by Z-DEVD-FMK, it was expected that this fragment resulted from a caspase-mediated intracellular cleavage event. To determine the cleavage site and the caspase(s) responsible for this cleavage, recombinant mouse E-cadherin cytoplasmic tail expressed as a GST fusion protein (GST-ECT) was digested \textit{in vitro} with recombinant caspase-3, -6, and -7, respectively. GST-ECT was efficiently digested by caspase-3, resulting in the formation of 30- and 24-kDa cleavage products (Fig. 3A). Caspase-7 generated fragments of identical molecular mass, however, with a markedly reduced efficiency and thus were barely detectable. Caspase-6 treatment did not generate detectable cleavage products at all. Treatment with the caspase inhibitor Z-DEVD-FMK blocked the generation of the 24-kDa cleavage product (Fig. 3A). Edman degradation revealed that the 30-kDa fragment represented the N terminus of the GST moiety. The amino acid sequence obtained from the 24-kDa fragment precisely defined the caspase-3 cleavage site C-terminal to Asp\textsubscript{752} in an Asp-Asp-Asp-Thr-Arg-Asp\textsubscript{752} Asn-Val-Tyr-Tyr motif. This site is highly conserved in different species, located next to the transmembrane domain, and represents the only caspase-3 consensus sequence in the cytoplasmic tail (Fig. 3C).

The 24-kDa \textit{in vitro} cleavage product and fragment 1 produced \textit{in vivo} in apoptotic cells comigrated on SDS-polyacrylamide gels, indicating that both fragments are identical (Fig. 3B). The discrepancy in the apparent (24 kDa) and calculated molecular masses (15 kDa) for this E-cadherin cytoplasmic domain fragment might be explained by an unusual migration behavior of the cytoplasmic domain of E-cadherin during SDS-polyacrylamide gel electrophoresis or because of migration differences of molecular mass standards.

Further evidence that caspase-3 is the major caspase responsible for cleavage of the E-cadherin cytoplasmic tail in apoptotic cells was given by analyses of MCF-7 cells that have been previously shown to be deficient in caspase-3 (17). Induction of apoptosis in these cells did not result in the generation of detectable amounts of fragments 1 and 2, respectively. In con-
contrast, in MCF-7 cells that have been stably transfected with caspase-3, both fragments were generated (Fig. 4).

Shedding of the E-cadherin Extracellular Fragment from the Cell Surface during Apoptosis—The inhibitor studies and the mapping of the caspase-3 cleavage site to Asp 752 proximal to the transmembrane segment of E-cadherin suggested that fragment 2 was generated by an extracellular cleavage event. This, however, should result in an extracellular domain fragment that is released from the cell surface into the culture medium. Indeed, in immunoprecipitation experiments with antibodies directed against the extracellular domain of E-cadherin, increasing amounts of an 84-kDa polypeptide (fragment 3) were precipitated from cell culture supernatants of apoptotic cells with time after staurosporine addition (Fig. 5).

Next we wanted to characterize the enzyme(s) responsible for the formation of the 84-kDa extracellular fragment of E-cadherin. There is clear evidence now that shedding of the extracellular domain of a number of cell surface proteins is a regulated process that can be blocked by metalloproteinase inhibitors (42). To test whether the formation of the 84-kDa extracellular fragment of E-cadherin is mediated by this type of shedding protease, inhibitor studies were performed. In the presence of the matrix metalloproteinase inhibitor I, no significant inhibitory effect was detected (data not shown). In contrast, TAPI, a metalloproteinase inhibitor that was shown to block tumor necrosis factor- \( \alpha \) convertase (43), markedly inhibited formation of E-cadherin fragment 3 in a concentration-dependent manner (Fig. 6A). Consistent with this observation, generation of fragment 2 was also reduced, whereas formation of fragment 1 was unaffected (Fig. 6B, lane 3). In the presence of both TAPI and caspase inhibitor Z-DEVD-FMK, formation of both fragments was significantly inhibited (Fig. 6B, lane 4). Moreover, in cells treated with both inhibitors the amount of full-length E-cadherin was nearly comparable with nonapoptotic control cells (Fig. 6B, lane 4). In consequence, TAPI treatment should result in the formation of a new cleavage product that contains the E-cadherin transmembrane domain and thus remains attached to the membrane. Indeed, with an antibody directed against the extracellular domain of E-cad-
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Localization of E-cadherin in Apoptotic Cells—Caspase-3 cleavage of E-cadherin cytoplasmic domains proximal to the transmembrane region was expected to release fragment 1 into the cytosol. To analyze this, E-cadherin and E-cadherin fragments were localized in apoptotic cells by confocal immunofluorescence microscopy. Staining of E-cadherin at the plasma membrane of MDCK cells with anti-E-cadherin C36 antibody directed against the cytoplasmic domain of E-cadherin was reduced and became diffuse after onset of apoptosis. Cells

started to shrink, and E-cadherin cell surface staining was finally undetectable in cells that had lost their cell-cell contacts. Concomitant with cell rounding and disintegration of cell nuclei, E-cadherin staining became detectable in the cytoplasm, confirming the release of the E-cadherin fragment 1 into the cytosol (Fig. 6A, a–c). Cells treated with Z-DEVD-FMK retained staining of E-cadherin at the plasma membrane, and in consequence cytoplasmic E-cadherin staining was reduced (Fig. 7A, d). Moreover, in the presence of the caspase inhibitor, STS-treated cells exhibited significantly less rounding, and morphological integrity was less affected.

Confocal immunofluorescence analysis of H184A1 cells with antibody HECD-1 directed against the extracellular domain also showed a reduction of E-cadherin staining at the cell membrane. TAPI treatment inhibited this decrease in E-cadherin staining, showing that by blocking the extracellular cleavage event, fragment 4 containing the transmembrane domain remained attached in the cell membrane. Furthermore, cell morphology appeared to be less affected in these cells. E-cadherin staining in cells treated with both TAPI and Z-DEVD-FMK was highly similar to that of nonapoptotic cells; however, cells appeared flattened and the cell surface was more ruffled (Fig. 7B). To visualize the reorganization of the actin cytoskeleton during apoptosis, we performed costaining with Alexa Fluor™ 594-phalloidin. In untreated cells strong staining at sites of cell-cell contact and beyond the cell surface was detectable. STS treatment resulted in a nearly complete disruption of actin filaments within 3–6 h. Residual staining could often be detected in irregularly shaped structures especially prominent in rounded cells, similar to those shown by Brancolini et al. (35) (data not shown). TAPI treatment did not block actin fiber destruction. In the presence of both inhibitors cell size remained comparable with nonapoptotic control cells, and phalloidin staining was detectable at sites of cell-cell contacts. This emphasizes the importance of the cadherin-catenin system for the establishment and maintenance of the submembran-

Fig. 5. The E-cadherin extracellular domain is shed from the cell surface during apoptosis. The E-cadherin extracellular domain (fragment 3) released from H184A1 cells was immunoprecipitated from the cell culture supernatant with anti-HECD-1 antibody at different time points after induction of apoptosis and analyzed on Western blots with anti-HECD-1 antibody. The strong signal at a molecular mass of 50 kDa represents the heavy chain of the precipitating antibody. MW, molecular weight.

Fig. 6. TAPI inhibits shedding of the E-cadherin extracellular domain (fragment 3) during STS-induced apoptosis. A, increasing concentrations of TAPI were added to H184A1 cells prior to induction of apoptosis by STS. After 6 h of STS treatment fragment 3 was immunoprecipitated from the cell culture supernatants and analyzed on Western blots with anti-HECD-1 antibody. Lane 1, control; lane 2, STS; lanes 3–6, STS + 6.25, 12.5, 25, or 50 µM TAPI, respectively. B, lysates of cells treated with TAPI or TAPI + Z-DEVD-FMK were analyzed by Western blotting with anti-E-cadherin antibody C96. Lane 1, MeSO control; lane 2, STS; lane 3, STS + TAPI; lane 4, STS + TAPI + Z-DEVD-FMK. *, not identified. C, H184A1 cells were treated as described in B, and cell lysates were analyzed on Western blots with anti-HECD-1 antibody. In the presence of TAPI, a new fragment (fragment 4) with a slightly higher molecular mass of 88 kDa was generated (lane 1) compared with fragment 3 (lane 2) immunoprecipitated from the cell culture supernatant. In control cell lysates, fragment 4 was not present (lane 3). D, fragment 4 generated in TAPI-treated MDCK cells was detected with anti-DECMA-1 antibody. Lanes 1 and 2, 0 and 6 h after induction of apoptosis; lane 3, 6 h after induction of apoptosis in the presence of TAPI. MW, molecular weight.
A

B

FIG. 7. Confocal immunofluorescence microscopy of apoptotic cells. A, MDCK cells were analyzed for E-cadherin by immunofluorescence staining with anti-E-cadherin C36 antibody at different time points after induction of apoptosis with STS. a, 0 h; b, 3 h; c, 6 h; d, 6 h in the presence of Z-DEVD-FMK. The inset in c shows completely rounded cells with diffuse E-cadherin staining in the cytoplasm (arrow). E, H184A1 cells analyzed by indirect immunofluorescence double-staining with anti-HECD-1 antibody for E-cadherin (green) and Alexa Fluor® 594-phalloidin (red) for F-actin. a, d, g, and j, E-cadherin; b, e, h, and k, phalloidin; c, f, i, and l, merged images of E-cadherin and phalloidin stainings. a–c, 0 h; d–f, 6 h STS; g–i, 6 h STS + TAPI; j–l, 6 h STS + TAPI + Z-DEVD-FMK.

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Specific cell-cell and cell-matrix contacts regulate cell growth in epithelial cells, and disruption of these contacts induces apoptotic cell death (44, 45). In the course of apoptosis the activation of caspases is the central step driving cells into the execution phase of programmed cell death. Among the substrates of the effector caspses, a number of proteins involved in formation and/or regulation of cell-matrix and cell-cell contacts were identified, including FAK (22–24), PAK2 (46), fodrin (17), β-catenin (35–37, 47, 48), and plakoglobin (36, 47).

Here we show that during apoptosis, in addition to β-catenin and plakoglobin, E-cadherin is efficiently cleaved in epithelial cells. Three fragments with apparent molecular masses of 24 kDa (fragment 1), 29 kDa (fragment 2), and 84 kDa (fragment 3) were generated after induction of apoptosis by staurosporine or camptothecin. These cleavage products resulted from two distinct proteolytic activities. From inhibitor studies and in vitro cleavage reactions we assume that caspase-3 is predominantly responsible for the generation of the 24-kDa fragment. Caspase-7 was also able to generate fragment 1, however, with a much lower efficiency, whereas caspase-6, another effector caspase, did not cleave recombinant GST-ECT. Mapping of the caspase-3 cleavage site C-terminal to Asp752 showed that fragment 1 represents nearly the entire cytoplasmic domain of E-cadherin. These observations were confirmed by indirect immunofluorescence microscopy, revealing that the E-cadherin intracellular domain was lost at the plasma membrane and accumulated in the cytoplasm after induction of apoptosis. Phalloidin staining revealed disintegration of the actin microfilament system within 3 h after induction of apoptosis. Disruption of actin fibers could not be prevented by the addition of Z-DEVD-FMK or TAPI alone. In the presence of both inhibitors strong phalloidin staining at cell membranes suggests that the cadherin-based adhesion system is retained and allows formation of a subcortical actin filament network, whereas in the presence of either one of the inhibitors, affected clustering of cadherins or absence of the cytoplasmic domains appears to impair linkage of cadherin molecules to the actin cytoskeleton.

Because fragment 1 still contains the binding sites for β-catenin and p120, the question arises whether this fragment has a physiological role during apoptosis, especially in respect to the signaling function of β-catenin in association with lymphocyte enhancer factor-1/T cell factor transcription factors (49, 50) where β-catenin provides transcriptional activation domains (51–53). Previous studies have shown that ectopic expression of the E-cadherin cytoplasmic domain can block the lymphocyte enhancer factor-1/T cell factor–β-catenin-mediated transactivation process (54, 55), indicating that generation of fragment 1 might be a mechanism to block the signaling function of β-catenin. This assumption is in line with the finding that β-catenin is also fragmented during apoptosis (35–37) and that the resulting apoptotic β-catenin fragments exhibit reduced transactivation potential (48). In this context it is interesting to note that recently the adenomatous polyposis coli tumor suppressor protein APC involved in the regulation of the proteasome-mediated degradation of β-catenin was reported to be another target of caspase-3 during apoptosis (56). All these data indicate that the Wnt-signaling pathway is affected during apoptosis, although the molecular consequences remain to be unraveled.

The generation of the 29- and 84-kDa fragments could not be blocked by caspase inhibitors but was efficiently blocked by TAPI, an inhibitor originally used to study tumor necrosis...
factor-α-converting enzyme (43, 57), showing that these two fragments are generated by a metalloproteinase. This observation and the finding that the soluble 84-kDa fragment was released to the cell culture medium demonstrated that the cleavage site generating the 84- and 29-kDa fragments is localized in the extracellular domain of E-cadherin. Tumor necrosis factor-α-converting enzyme is a member of the growing ADAM (a disintegrin and metalloproteinase) family of metalloproteinases (58, 59). Because TAPI also blocks other metalloproteinases, it is intriguing to figure out whether an ADAM family member or even tumor necrosis factor-α-converting enzyme itself might shed the 84-kDa fragment into the cell culture supernatant. Unfortunately, up to now we have not been able to precisely determine the cleavage site in the extracellular domain of E-cadherin. When we compared the mobility of immunoprecipitated E-cadherin fragments released to the cell culture supernatant after induction of apoptosis or by ionomycin-induced Ca²⁺ influx, both fragments perfectly aligned on Western blots (data not shown). This suggests that the metalloproteinase(s) activated during apoptosis have the same substrate specificity reported for the metalloproteinase(s) induced by Ca²⁺ influx, which was mapped C-terminal to Asp⁷⁵₂ close to the transmembrane domain. Inhibition of caspase-3 activity with Z-DEVD-FMK results in the formation of fragments 2 and 3 (B). The extracellular cleavage is mediated by a metalloprotease activity that releases the E-cadherin extracellular domain (fragment 3) to the cell culture supernatant. This shedding is blocked by TAPI, resulting in the formation of fragments 1 and 4 (C). PM, plasma membrane.

Consistent with our observations, it was recently reported that during apoptosis of endothelial cells, the extracellular domain of the endothelium-specific VE-cadherin is shed from the cell surface by a TAPI-inhibited activity (39). However, for VE-cadherin no caspase-mediated cleavage of the cytoplasmic tail was shown. This may be due to the antibody used in this study, which was directed against the VE-cadherin extracellular domain. VE-cadherin fragments A and B described in this study might correspond to E-cadherin fragments 3 and 4 described in our study. Therefore, it is also likely that the VE-cadherin cytoplasmic tail is a caspase target in endothelial cells. In another recent report, E-cadherin and P-cadherin were shown to be subjected to cleavage during early stages of apoptosis (38). E-cadherin cleavage generated a 48-kDa cleavage product, and formation of this fragment was reported to be blocked by the caspase inhibitor Z-VAD-FMK. This cleavage event was assigned to residue Asp⁴⁷⁹ in the extracellular domain of E-cadherin. However, this is contradictory to the localization of caspases in the cytoplasm. The 104-kDa P-cadherin fragment might correspond to fragment 4 described in our study. Furthermore, in this report shedding was not analyzed for both cadherins. These discrepancies might be explained by the different cell lines used in both studies. Nevertheless, it would be interesting to analyze the supernatant of these cells for cadherin fragments.

During recent years it became evident that membrane protein secretases (often named sheddases) play an important regulatory role in the activation/inactivation of transmembrane proteins as seen for tumor necrosis factor-α-converting enzyme, transforming growth factor-α-secretase, Notch-activating enzyme Kuzbanian, or FasL-secretase (42). In this respect, soluble forms of the N-cadherin extracellular domain were reported to be generated during embryonic retinal histogenesis by proteolysis, representing a novel functional form of N-cadherin involved in retinal development (60). Soluble E-cadherin fragments were detected in the urine, in the blister fluid of cutaneous diseases, and in the circulation of cancer.
patients (61–63). Interestingly, expression of a stromelysin transgene in a mammary epithelial cell line led to an epithelial-mesenchymal transition phenotype concomitant with a disappearance of E-cadherin and shedding of minor amounts of E-cadherin to the cell culture supernatant, suggesting that either stromelysin itself or a stromelysin-activated secondary product might be involved in this process (64). At present the mechanisms generating these fragments are unknown, and it is an open question how these sheddase activities are regulated.

The following mechanisms can be considered. 1) the sheddase cytoplasmic tail itself might represent a substrate for caspases, and cleavage might induce a conformational change that subsequently allows extracellular cleavage of E-cadherin. 2) the shedding protease might be activated as a secondary target during programmed cell death. 3) cleavage of β-catenin and plakoglobin normally bound to the cytoplasmic domain might be a prerequisite for the release of a sterical block, subsequently allowing metalloproteinase access to its substrate, or might induce a change in the E-cadherin conformation that leads to the exposure of the metalloproteinase target site. Results obtained in kinetic experiments with caspase-negative MCF-7 cells indicate that their metalloproteinase activity is markedly reduced but not completely absent, because we could detect minor amounts of soluble fragment 3 in the cell culture supernatant (data not shown). Retransfection of caspase-3 coincided with a higher metalloproteinase activity in the transfected cells. This observation suggests that caspase-3-mediated cleavage of β-catenin or plakoglobin might enhance metalloproteinase activity on E-cadherin. A similar observation has been reported for L-selectin shedding by a metalloproteinase activity on E-cadherin. A similar observation has been reported for L-selectin shedding (65). Consistent with these results, it was reported that disruption of E-cadherin function by anti-E-cadherin antibodies leads to complete disruption of cadherin-mediated cell-cell adhesion. The following mechanisms can be considered. 1) the sheddase cytoplasmic tail itself might represent a substrate for caspases, and cleavage might induce a conformational change that subsequently allows extracellular cleavage of E-cadherin.

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