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Proteolytic processing of the adherens junctions components β-catenin and γ-catenin/plakoglobin during apoptosis

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Abstract

Apoptotic cells undergo specific morphological changes that include loss of cell-cell interactions. Cellular adhesiveness is dependent on members of the cadherin family of adhesion receptors and on the cytoplasmic adaptor proteins α-catenin, β-catenin and γ-catenin/plakoglobin. The caspase family of cystein proteases play a key role during the execution phase of the apoptotic program. These proteolytic enzymes, once activated, cleave cellular proteins which are important for the maintenance of cell integrity. Here we report that γ-catenin is cleaved at different sites during apoptosis in various cell lines. The major apoptotic product of γ-catenin still retains the ability to bind α-catenin but loses the carboxy-terminal region. We also show that γ-catenin is cleaved by caspase-3 *in vitro* although with lower affinity when compared to PARP or β-catenin. These findings indicate that multiple proteolytic events regulate the dismantling of the cell-cell junctional complexes during apoptosis .

Keywords: caspases; cadherins; PARP; UV

Abbreviations: PARP, poly(ADP ribose) polymerase; TCF/LEF T Cell Factor/Lymphocyte Enhancing binding Factor

Introduction

The cystein proteases specific for aspartic residues, known as caspases, play a critical role in apoptosis from *Caenorhabditis elegans* to humans (Ellis *et al*, 1991; Alnemri *et al*, 1996; Salvesen and Dixit, 1997). Each caspase is synthesized as inactive precursor and during activation of the apoptotic program it is converted, by proteolytic processing, into an active heterodimer composed of four subunits (Cohen 1997). A model for caspase-3 activation has been recently proposed based on *in vitro* reconstituted assays using purified components. Apaf-1, the human homolog of CED-4 (cell-death abnormal), cytochrome c and dATP/ATP trigger caspase-9 activation which in turn cleaves and activates caspase-3 (Zou *et al*, 1997; Li *et al*, 1997).

The existence of a hierarchically ordered caspase cascade has also been suggested by studies on the CD95/FAS/APO-1 dependent apoptotic pathway. Caspase-8 is initially activated after interaction with the death receptor at the cell surface (Boldin *et al*, 1996; Muzio *et al*, 1996) and a later activity related to caspase-3 has been reported (Enari *et al*, 1996). Based on these observations the caspases regulating apoptosis might be divided into initiators and executioners (Salvesen and Dixit, 1997). The initiator caspases should be responsible for activating the executioner caspases which in turn cleave specific cellular proteins known as death substrates.

Different death substrates have been identified whose coordinate proteolysis probably leads to the specific morphological changes characterizing apoptosis (Wyllie *et al*, 1980). A large class of them shows as a common primary recognition sequence the DEXD tetrapeptide (Porter *et al*, 1997; Tan and Wang, 1998). *In vitro* studies have demonstrated that this tetrapeptide is the optimal recognition sequence for caspase-3 and caspase-7 (Thornberry *et al*, 1997).

We have recently identified a new substrate for caspase-3 in β-catenin (Brancolini *et al*, 1997). β-catenin is a multifunctional protein, regulating both the organization of intercellular adhesion and cell signalling during development (Miller and Moon, 1996). β-catenin forms complexes linking, the actin cytoskeleton to the cell-cell adhesion receptor cadherins by binding α-catenin (Gumbiner, 1996). In addition, complexes between β-catenin and the TCF/LEF family of transcription factors are critical for the determination of cell fate during early development of *Xenopus* and *Drosophila* (Beherens *et al*, 1996; Molenaar *et al*, 1996). β-catenin accumulates inside the cells in response to Wingless/Wnt signals which possibly act by suppressing the degradative pathway normally regulated by glycogen synthase kinase-3 and APC (adenomatous polyposis coli) (Miller and Moon, 1996; Peifer, 1997; Willert and Nusse, 1998).

β-catenin processing during apoptosis *in vivo* or after incubation with caspase-3 *in vitro* removes both the amino and the carboxy-terminal regions of the protein, thus suggesting that different β-catenin functions could be modulated during cell death (Brancolini *et al*, 1997). The central region comprising 13 *arm* repeats is 85% homologues to a corresponding segment of γ-catenin/plakoglobin, a protein that in addition to a role in Wingless/Wnt signalling is a component of the adherens junctions and of the intracellular plaque of desmosomes (Koch and Franke, 1994; Sacco *et al*, 1995; Miller and

Moon, 1996; White *et al*, 1998). In the present work we demonstrate that plakoglobin is cleaved during apoptosis *in vivo* and after incubation with caspase-3 in an *in-vitro* proteolytic assay. In addition, we have compared β-catenin and γ-catenin/plakoglobin proteolytic patterns in various cell lines treated with different apoptotic stimuli and find that their processing was impaired in UV treated MCF-7 cells. Furthermore *in vitro* proteolytic studies suggest that β-catenin is preferentially processed by caspase-3 with respect to γ-catenin/plakoglobin.

In conclusion these results indicate that complex proteolytic events regulate adherens junction organization during apoptosis.

Results

γ-catenin proteolytic processing during apoptosis

To determine whether γ-catenin/plakoglobin is cleaved during apoptosis, MDCK cells were UV irradiated and levels of apoptosis were assessed by FACS analysis using propidium iodide staining. Analysis of DNA content (Figure 1a) revealed a significant increase of cells with sub G1 DNA content, indicative of apoptosis (Israeli *et al*, 1997), which parallels the intensity of the UV irradiation. The γ-catenin expression pattern was next analyzed by Western blot combining both adherent as well as non-adherent (floating dead cells) UV treated MDCK cells. As shown in Figure 1b UV treatment of MDCK cells induced the appearance of faster migrating forms of γ-catenin which parallels the appearance of apoptosis. When the same extracts were probed with an antibody specific for the carboxy-terminal of γ-catenin (aa 724-743) the faster migrating forms of γ-catenin were undetectable, thus suggesting that they represent proteolytic fragments lacking the carboxy-terminal region. Actin, used as control, failed to be processed under the same conditions (Brancolini *et al*, 1997).

To confirm that γ-catenin was proteolyzed during apoptosis we used as alternative genotoxic stress the alkylating agent methylmethane sulphonate (MMS). MDCK were treated for 4 h with increasing amount of MMS and 24 h later Western analysis was performed on adherent and non-adherent cells. Proteolytic processing of γ-catenin was detected also when apoptosis was induced by MMS treatment (Figure 1c). Again the relative amount of the processed forms parallel the intensity of the genotoxic insult and the appearance of the apoptotic cells in the medium (Brancolini *et al*, 1997). When the same extracts were probed with an anti-CT γ-catenin antibody only the unprocessed form was detected (Figure 1c).

To further characterize γ-catenin processing during apoptosis both MDCK and NIH3T3 cells were grown in serum free medium and adherent (C) and non-adherent apoptotic (A) cells were harvested separately. Western analysis revealed that deprivation of survival signals in both MDCK and NIH3T3 induced proteolytic processing of γ-catenin (Figure 1d). It is interesting to note that in apoptotic NIH3T3 cells three different bands at approximately 75, 66 and 55 kDa showing similar intensity were observed. A different pattern was evident in apoptotic MDCK cells where a major band of approximately 75 kDa was detected, while the 66 and 55 kDa bands were present at reduced levels. γ-catenin was detected only in non-apoptotic MDCK cells when the same extracts were probed with an antibody specific for its carboxy-terminal.

In the adherens junctions γ-catenin is associated with cadherin and α-catenin, which provides anchorage to F-actin or α-actinin (Sacco *et al*, 1995; Aberle *et al*, 1996; Nieset *et al*, 1997). Therefore we analyzed whether the apoptotic processed forms of γ-catenin could still bind α-catenin in a co-immunoprecipitation assay. Apoptosis was induced in MDCK by UV irradiation, adherent and apoptotic floating cells were harvested separately and the same amount of proteins derived from apoptotic or non-apoptotic cell lysates were immunoprecipitated using anti-γ-catenin antibody. After electrophoretic separation, the immunoblots were revealed with anti-α-catenin antibody.

Figure 1e shows that a similar amount of γ-catenin was present in the lysates of apoptotic and non-apoptotic cells and that the same amount of α-catenin could be detected in complexes with the apoptotic processed form of γ-catenin or with the γ-catenin in viable cells. This result indicates that the major proteolytic product of γ-catenin in apoptotic cells is still able to associate with α-catenin. The same blot was also probed with an antibody against γ-catenin to demonstrate that the same amount of γ-catenin was immunoprecipitated under the different experimental conditions.

Processing of adherence junctions components during apoptosis in different cell lines

The analysis of γ-catenin processing during apoptosis suggests that γ-catenin is processed at different sites. Complex proteolytic processing during apoptosis has been reported also in the case of the related protein β-catenin. In fact both the amino and the carboxy-terminal of β-catenin are removed during apoptosis, moreover *in vitro* studies suggest that within its amino terminus different aspartic residues could be target for caspase activity (Brancolini *et al*, 1997). These data indicate that multiple proteolytic events could regulate adherences junctions dynamic during apoptosis. Therefore we decided to analyze in detail the proteolytic pattern of the different components of the adherens junction during apoptosis induced by UV irradiation. Different cell lines were UV irradiated (120 J/m2) and non-adherent apoptotic (A) cells were harvested. Western blot analysis was performed using lysates from untreated or UV treated, apoptotic cells. As shown in Figure 2, β-catenin cleavage was detected when apoptosis was induced by UV treatment in all the cell lines tested with the exception of MCF-7 where a decrease in the amount of the protein was observed without specific cleavages. It could be noted that at least three different forms of β-catenin can be detected in the apoptotic cells which probably represent intermediate proteolytic products. The relative abundance of the three forms differed among the cell lines analyzed as exemplified in NIH3T3 cells were the low migrating ~65 kDa, was the most prominent form, and in A-549 where the same form was barely detectable. This result suggests that caspase activity responsible for β-catenin processing might fluctuate among different cell lines.

The same lysates were next analyzed for γ-catenin processing. Even though expression of γ-catenin was heterogeneous, showing higher levels in the epithelial cell lines, proteolytic processing was evident in the different cell lines used. The previously described major proteolytic product of ~75 kDa was detected in all the tested cell

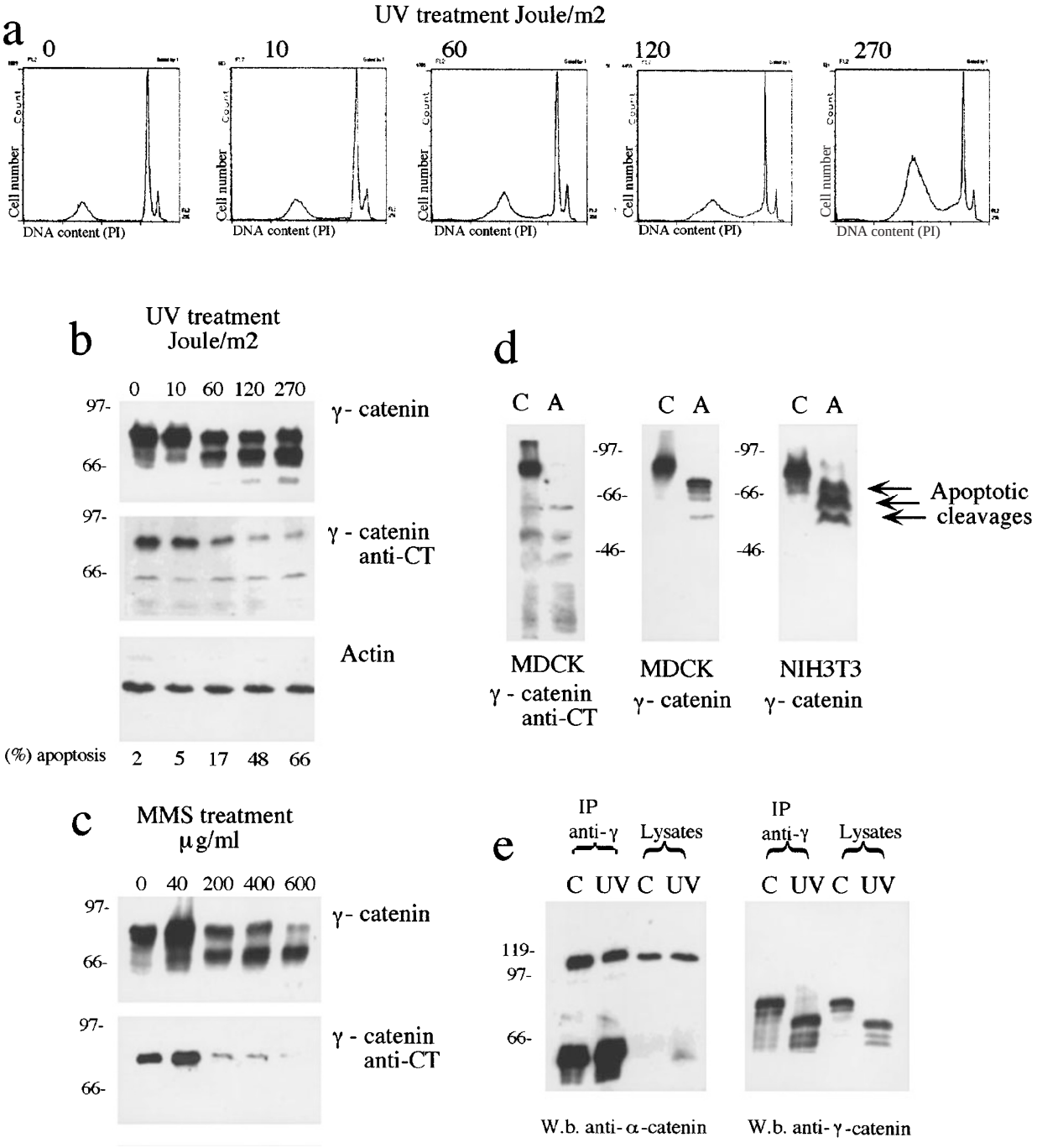

Figure 1 γ-catenin proteolytic processing during apoptosis. (**a**) Flow cytometric analysis of apoptosis in UV treated MDCK cells after propidium iodide staining. (**b**) MDCK cells grown for 4 d in 10% FCS were UV irradiated as indicated. After 24 h, lysates from both adherent viable cells and non-adherent apoptotic cells were combined and Western blot analysis was performed. (**c**) MDCK cells grown for 4 d in 10% FCS were treated for 4 h with the indicated amount of MMS. After 24 h, lysates from both adherent viable cells and non-adherent apoptotic cells were combined and Western blot analysis was performed. (**d**) MDCK and NIH3T3 cells were incubated in serum free medium for 24 h, adherent (C) and non-adherent apoptotic cells (A) were processed separately and Western analysis was performed. (**e**) MDCK cells grown for 4 d in 10% FCS were UV treated. Untreated (C) and UV treated (120 J/m^2) apoptotic cells were processed separately. Immunoprecipitations using anti-γ-catenin were performed as described in Materials and Methods. The immunocomplexes were resolved in SDS–PAGE and processed for Western analysis with anti-α-catenin or anti-γ-catenin as indicated

lines while the relative amount of the ~66 and 55 kDa forms was different among them. Similar to β-catenin, proteolytic processing of γ-catenin was substantially impaired in apoptotic MCF-7 cells where only reduced levels of the ~75 kDa form were detected. When α-catenin expression was analyzed proteolytic processing was undetectable as previously described (Brancolini *et al*, 1997).

We next asked whether the pattern of expression of members of the cadherin superfamily of cell adhesion receptors, could be regulated during apoptosis. The cadherin family includes different members (Takeichi, 1995). We analyzed the expression patterns of N-cadherin, E-cadherin, and P-cadherin both in untreated and UV irradiated apoptotic cells. The different cadherins analyzed showed a cell line specific pattern of expression (see Figure 2), however the relative amounts decreased dramatically in the apoptotic cells. Actin, used as control, failed to be processed under the same experimental conditions (Brancolini *et al*, 1997).

β-catenin and γ-catenin proteolytic patterns were also analyzed when apoptosis was induced by deprivation of survival signals. Cells were grown for 24 h in serum free medium and non-adherent apoptotic cells were harvested for Western analysis. Figure 3 shows β-catenin proteolytic processing during apoptosis in the different cell lines analyzed. β-catenin intermediate proteolytic products can be detected in the apoptotic cells, and here again NIH3T3 cells contained mainly the ~65 kDa form, while in the A-549 the same form was barely detectable. In MCF-7 cells a faint band of approximately 66–67 kDa was present together with the unprocessed form. When the same extracts were analyzed for γ-catenin processing a picture resembling the response to the UV treatment was obtained. Actin failed to be processed under the same experimental conditions (Brancolini *et al*, 1997).

Analysis of the interaction between the β-catenin and α-catenin during apoptosis

We have recently demonstrated that when apoptosis was induced both in NIH3T3 and MDCK cells by serum starvation the induced ~65 kDa form of β-catenin was unable to bind α-catenin that is responsible for actin filament binding (Brancolini *et al*, 1997). We decided to analyze if the intermediate proteolytic products of β-catenin could still bind α-catenin. We choose UV treated MDCK cells as model system since the three different forms were equally represented.

Adherent and apoptotic floating MDCK cells were harvested separately and the same amount of proteins derived from apoptotic or non-apoptotic cell lysates were immunoprecipitated using anti-α-catenin or anti-β-catenin antibodies. After electrophoretic separation, the immunoblots were revealed with anti-β-catenin antibody.

Figure 4 shows that three forms of β-catenin were immunoprecipitated from apoptotic cells using the anti-β-catenin antibody. The two higher molecular weight forms of β-catenin present in apoptotic MDCK cells were immuno-

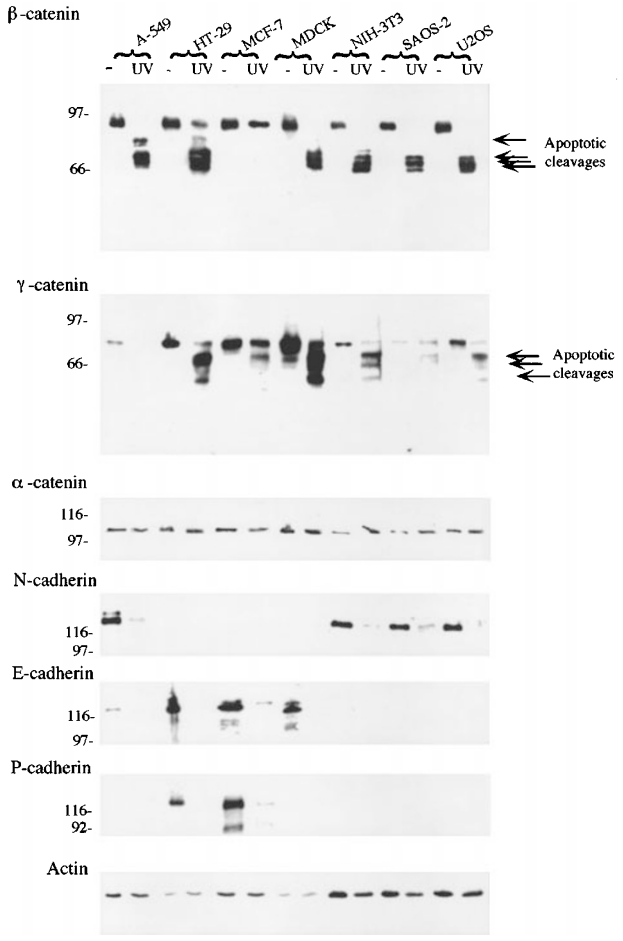

Figure 2 Processing of adherence junctions components during apoptosis induced by UV irradiation. Different cell lines grown for 3 d in 10% FCS were UV irradiated (120 J/m^2) or untreated. After 20 h untreated (−) and apoptotic floating cells (UV) were harvested separately. Western analysis was performed using the indicated antibodies

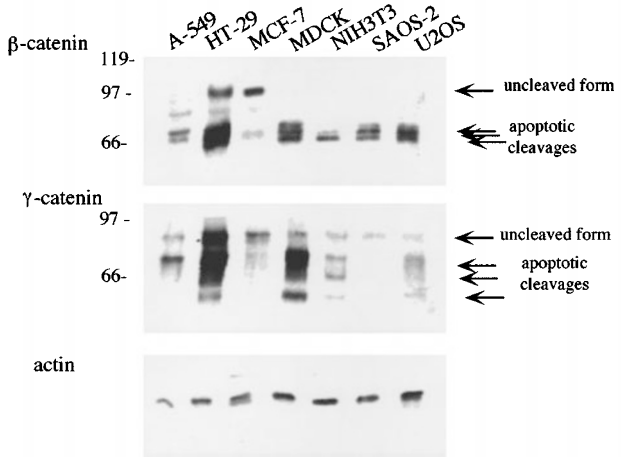

Figure 3 Processing of adherence junctions components during apoptosis induced survival signals deprivation. Different cell lines after 3 d in 10% FCS were grown for further 24 h in serum free medium. Apoptotic floating cells were processed and Western analysis was performed using the indicated antibodies

precipitated using the anti-α-catenin antibody while the third form, which should represent the final proteolytic product, was present at reduced levels, thus indicating that only the final proteolytic product of β-catenin lacks the domain responsible for efficient interaction with α-catenin (Aberele *et al*, 1994; Oyama *et al*, 1994). Similar results were obtained in the HT29 cell line (data not shown).

These results demonstrate that the intermediate proteolytic products of β-catenin as detected in different cell lines and in relation with the apoptotic stimuli can still bind α-catenin, thus suggesting that they could represent incomplete digestion of its amino-terminal region.

γ-catenin is proteolytically processed by caspase-3 *in vitro*

To determine whether γ-catenin could serve as substrate of caspases, *in vitro* translated γ-catenin was incubated with increasing amount of bacterial lysates expressing caspase-3. Treatment with caspase-3 specifically cleaved γ-catenin and this cleavage was inhibited by Ac-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO), a specific peptide inhibitor of caspase-3-like proteases (Nicholson *et al*, 1995) (Figure 5). It is interesting to note that while *in vivo* apoptotic proteolysis of γ-catenin resulted in at least three different bands of approximately 75, 66 and 55 kDa, *in vitro* the 55 kDa form was the most evident and the 66 almost undetectable. These differences could reflect both distinct cleavage efficiency or accessibility of the substrate target sequences between the *in vitro* and *in vivo* conditions. PARP (poly-ADP-ribose polymerase), a well defined substrate of caspase-3 (Nicholson *et al*, 1995; Cohen 1997; Porter *et al*, 1997) was incubated with increasing amounts of the same lysates over the same time course. The same caspase-3 activity that was responsible for full PARP processing was unable to process γ-catenin, thus suggesting that *in vitro*, PARP is a preferential substrate for caspase-3.

γ-catenin and β-catenin show differential sensitivity to caspase-3 proteolytic activity

Having demonstrated that both γ-catenin and β-catenin can be processed by caspase-3 we next analyzed if they showed differential sensitivity to caspase-3. *In vitro* translated β-catenin and γ-catenin were incubated with increasing amounts of caspase-3 expressing bacterial lysates and their processing was analyzed. As shown in Figure 6a a caspase-3 activity that was responsible for full processing of β-catenin could only partially cleave the *in-vitro* translated γ-catenin.

The classical cadherins are the transmembrane components of the cell-cell junctions and through their cytoplasmic domain indirectly associate with the actin cytoskeleton via the catenins (Takeichi, 1995; Gumbiner, 1996). The catenins were identified by their ability to co-immunoprecipitate with the cadherins (Nagafuchi and Takeichi, 1988; Ozawa *et al*, 1989). In order to study caspase-3 mediated γ-catenin and β-catenin processing *in vitro* in a condition resembling the *in-vivo* processing, we decided to isolate by immunoprecipitation the complexes formed by cadherin/α-β-γ-catenins and to test the susceptibility of their components to caspase-3 processing in a system where the protein-protein interactions should be maintained.

MDCK cells were labeled for 12 h with ^{35}S-methionine and after cell lysis immunprecipitations were performed using antibodies against E-cadherin, α-catenin, β-catenin and γ-catenin. In Figure 6b it can be seen that the antibody against E-cadherin co-immunoprecipitated the α-/β-/γ-catenins and that cell-cell adherence complexes containing β-catenin do not contain γ-catenin and vice versa as previously demonstrated (Hinck *et al*, 1994). To isolate the cell-cell complexes immunoprecipitations were performed using anti-E-cadherin antibody. When the immunopurified complexes were incubated with an amount of purified caspase-3 sufficient to process β-catenin at different sites

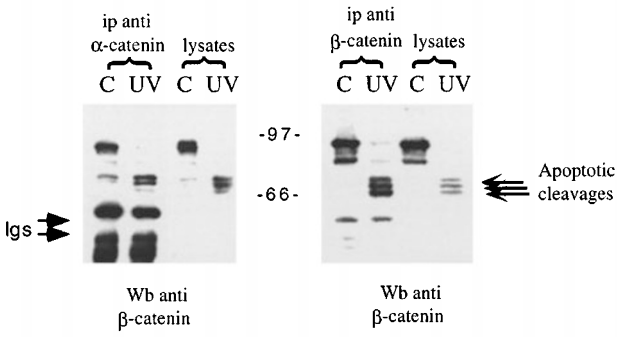

Figure 4 γ-catenin processing during apoptosis. MDCK cells grown for 4 d in 10% FCS were UV treated. Untreated (C) and UV treated (120 J/m^2) apoptotic cells were processed separately. Immunoprecipitations using anti-α-catenin or anti-β-catenin were performed as described in Materials and Methods. The immunocomplexes were resolved in SDS–PAGE and processed for Western analysis with anti-β-catenin as indicated

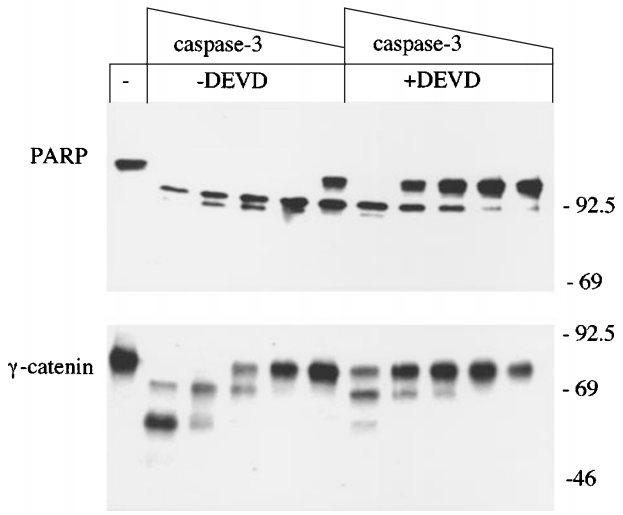

Figure 5 *In vitro* protease assays. ^{35}S methionine labelled *in vitro* translated γ-catenin and PARP were incubated for 1 h at 37°C with caspase-3 buffer alone, with increasing amounts of caspase-3 expressing bacteria lysates or in the presence of 0.2 μM of Ac-DEVD-CHO a specific caspase-3 inhibitor

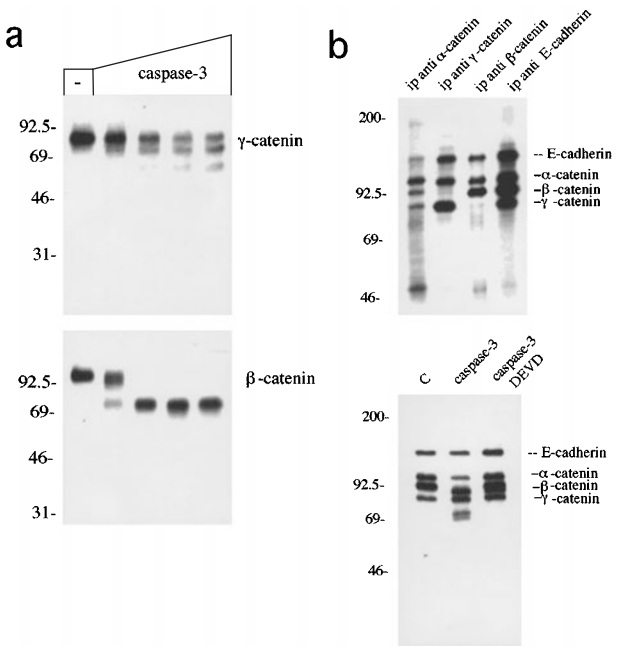

Figure 6 *In vitro* protease assays. (**a**) ^{35}S methionine labelled in vitro translated γ-catenin and β-catenin were incubated for 1 h at 37°C with caspase-3 buffer alone or with increasing amounts of caspase-3 expressing bacteria lysates. (**b**) MDCK were labelled with ^{35}S methionine after cell lysis immunoprecipitations were performed as described in Material and Methods. Immunocomplexes were resuspended in caspase-3 buffer and incubated for 30 min with purified caspase-3, caspase-3 in the presence of 0.2 μM Ac-DEVD-CHO, or buffer alone

as previously described, γ-catenin was uncleaved. It is possible that the observed lack of γ-catenin processing could be due to the masking of the caspase cleavage sites by specific protein-protein interactions. Caspase-3 treatment also induced a reduction in the amount of α-catenin (Figure 6b). Such reduction might be a consequence of the release of α-catenin from the adhesion complex, following the caspase-3 dependent cleavage of its binding site on β-catenin.

The cleavage of β-catenin was inhibited by addition of DEVD-CHO.

In conclusion these results indicate that β-catenin is a better substrate for caspase-3 than γ-catenin and that it can be processed even though presents in the junctional complex.

Discussion

A number of different proteins cleaved during apoptosis by caspases have been identified. Known also as death substrates, they include protein kinases, transcription factors, DNA repair enzymes, cytoskeletal proteins, oncogenes and anti-oncogenes (Porter *et al*, 1997; Enari *et al*, 1998; Tan and Wang, 1998). Cleavage of death substrates can either activate or inactivate essential functions. The existence of multiple substrates supports the idea that the execution phase of apoptosis is dependent on the proteolytic modulation of a defined number of key death substrates, instead of a single critical death substrate.

We have recently demonstrated that a caspase dependent processing of β-catenin, trimming both the amino and carboxy-terminal regions could be relevant in dismantling cell-cell contacts during apoptosis. *In vitro* the β-catenin amino-terminal region is cleaved at multiple sites by caspase-3 (Brancolini *et al*, 1997). Here we report that also *in vivo* the amino-terminal of β-catenin is cleaved at multiple sites and that the pattern of cleavage is different depending both on the apoptotic stimuli and the cellular context. This evidence suggests that the caspase activity responsible for β-catenin processing is differentially regulated in various cell lines. In MCF-7 cells apoptosis induced by UV treatment was characterized by an impaired β-catenin proteolytic processing. An atypical behaviour, in terms of morpholological changes and DNA cleavage was also observed in this cell line during cell death induced by serum starvation (Oberhammer *et al*, 1993). More recently it has been reported that MCF7 cells are deficient in caspase-3 expression (Scaffidi *et al*, 1998; Srinivasan *et al*, 1998; Janicke *et al*, 1998) thus possibly explaining the lack of β-catenin processing during apoptosis in this cell line.

β-catenin shares about 65% identity with γ-catenin/plakoglobin. Both proteins play a role in Wingless/Wnt signalling and in adherens junctions formation, although *in vitro* binding of γ-catenin to the adherens junction components E-cadherin and α-catenin shows lower affinity than β-catenin (McCrea and Gumbiner, 1991; Aberle *et al*, 1994). In addition γ-catenin is a components of the desomosomes where it mediates the interaction between desmosomal cadherins and intermediate filaments (Koch and Franke, 1994; Gumbiner, 1996). Here we report that also γ-catenin/plakoglobin is proteolytically processed at different sites during apoptosis. This processing generates three major bands of approximately 75, 66 and 55 kDa, which relative levels are different among the various cell lines. *In vitro* γ-catenin was cleaved by caspase-3 but the cleavage efficiency, when compared to the relevant caspase-3 substrate PARP was lower. Caspase-3 also shows higher affinity for β-catenin compared to γ-catenin in an *in vitro* proteolytic assay, however γ-catenin processing, similar to β-catenin, was impaired in apoptotic MCF-7 cells, which have lost caspase-3 expression. Therefore this evidence indicates that *in vivo* caspase-3 directly or indirectly, by activating other caspases, could be responsible for processing γ-catenin.

In vitro different aspartic residues within the amino-terminal domain of β-catenin are targets for caspase activity. Among them the aa 144-145 and 162-164 are conserved between γ-catenin and β-catenin. Why the cleavage efficiency was different? It has been suggested that to remove the amino-terminal region of β-catenin, caspases act by sequential cleavages (Brancolini *et al*, 1997). In this context the initial critical aspartic residues in γ-catenin and β-catenin could present different residues at the P4′ position which is a critical determinant of caspases specificities (Thornberry *et al*, 1997). To answer this question will be important to define if the amino-terminal

region of γ-catenin is cleaved during apoptosis and to map the cleaved aspartic residues.

It is possible that dismantling of the cell-cell contacts during apoptosis occurs also through proteolytic processing of other junctional components. In fact we have observed that the level of the cadherin receptors detectable in apoptotic cells was dramatically reduced, possibly due to a proteolytic degradation. Moreover it has been recently reported that, during endothelial apoptosis, the extracellular domain of VE-cadherin is lost from the cell surface by metalloproteinase-sensitive mechanism (Herren *et al*, 1998).

Dismantling of the cell-cell contacts during apoptosis may be dependent on the proteolytic processing of β-catenin, γ-catenin/plakoglobin and of cadherins. This concerted proteolysis could represent a selected strategy to efficiently disassemble the adherens junctions. In this context the imperfect proteolytic processing of the amino-terminal domain of β-catenin and therefore the partial retention of α-catenin binding, as detected in some cell lines, and the fact that the major proteolytic product of γ-catenin in apoptotic cells can still bind to α-catenin, should not influence the dismantling of the cell-cell contacts since alternative proteolytic activities could act, for example, on the cadherin receptors. However further studies will be necessary to confirm that a metalloproteinase-mediated shedding of cadherins is a common event during apoptosis.

By using specific antibodies we have demonstrated that γ-catenin, similarly to β-catenin, is cleaved at its carboxy terminal region during apoptosis. β-catenin and γ-catenin interact with the transcription factor TCF/LEF (Behrens *et al*, 1996; Molenaar *et al*, 1996), but it is still unclear if in the case of plakoglobin this interaction is functionally significant (White *et al*, 1998). The interaction with TCF/LEF is mediated by the central repeat region of the proteins while, the carboxy-terminal domains which is dispensable for cell adhesion, seems to be necessary to fully activate transcription of target genes (Orsulic and Peifer, 1996; van de Wetering *et al*, 1997). Removal of the carboxy-terminal of β-catenin and γ-catenin was a common event during apoptosis induced by different stimuli, therefore further studies will be necessary to understand if such cleavage could be important to regulate their transactivation properties.

In conclusion, by trimming either the amino and carboxy terminal parts of β-catenin and γ-catenin, caspases could modulate both their cell adhesion and signalling functions.

Material and Methods

Cells lines and culture conditions

Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml). For induction of apoptosis the culture medium was replaced with serum free DMEM medium. In the case of genotoxic-dependent apoptosis, density inhibited cells were treated with MMS for 4 h. For UV treatment culture medium was removed, dishes were washed once with PBS, UVC irradiated and fresh medium, containing 10% FCS was added to the cells (Brancolini *et al*, 1995).

Immunoblotting

For Western blotting, proteins were transferred to 0.2 μm pore sized nitro-cellulose (S.&S.) using a semidry blotting apparatus (Biorad) (transfer buffer: 20% methanol, 48 mM Tris, 39 mM glycine and 0.0375% SDS). After staining with Ponceau S, the nitro-cellulose sheets were saturated for 1 h in Blotto-Tween 20 (15) (50 mM Tris-HCl pH 7.5, 500 mM NaCl 5% non-fat dry milk and 0.1% Tween 20) and incubated over night at room temperature with the specific antibody: anti-β-catenin and anti-α-catenin anti-γ-catenin (aa 553-738), anti-P-cadherin anti E-cadherin (Transduction Lab.), anti-γ-catenin carboxy-terminal aa 724-743 (Santa Cruz) anti-pan-cadherin (Sigma). Blots were then rinsed three times with Blotto-Tween 20 and reacted with peroxidase conjugated rabbit anti-goat (Southern B.) or goat anti-mouse (Sigma) for 1 h at room temperature. The blots were then washed four times in Blotto-Tween 20, rinsed in phosphate buffer saline and developed with an ECL kit, as recommended by the vendor (Amersham).

Expression of caspase-3 (CPP32) in bacteria and *in vitro* protease assay

Caspase-3 was expressed in bacterial using the pQE-12 expression system (Qiagen). Cells were grown to an A_{600} of 0.6 and expression of caspase-3 was induced by adding isopropyl-β–D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After 2 h cells were collected by centrifugation at 3000 g for 5 min, and then resuspended in 5 vol of caspase-3 buffer as previously described (Brancolini *et al*, 1997). Cells were lysed by sonication and debris were sedimented by centrifugation at 14 0000 $\times$ *g* for 20 min. Caspase-3 was purified using Ni-NTA resin.

PARP, β-catenin and γ-catenin were labelled with [^{35}S] using the TNT-coupled reticulocyte lysate system (Promega). The same number of TCA precipitable counts for each *in vitro* translated protein was incubated with the appropiate dilution of the bacterial lysates in caspase-3 buffer (final volume of 10 μl) for 1 h at 37°C. Reactions were terminated by adding one volume of SDS gel loading buffer and boiling for 3 min. The specific caspase-3 inhibitor Ac-Asp-Glu-Val-Asp-CHO was obtained from Bachem Bioscience.

Immunoprecipitation

Analysis of γ-catenin/α-catenin complexes in apoptotic and non apoptotic cells was performed as previously described (Brancolini *et al*, 1997). Cells were extracted with lysis buffer (140 mM NaCl, 4.7 mM KCl, 0.7 mM $MgSO_4$, 1.2 $CaCl_2$ 20 mM Tris HCl pH 7.5, 5% glycerol, containing 1% Triton X-100 and 1 mM PMSF and 10 mg/ml each of aprotinin, leupeptin, antipain, and pepstatin) and centrifugation at 14 000 $\times$ *g* for 15 min monoclonal antibody anti-γ-catenin (Transduction Lab) was added. After 3 h on ice protein A-Sepharose (Pharmacia) was added and incubation was prolonged for 1 h on ice. After a brief centrifugation in Eppendorf centrifuge, immunoprecipitates were washed with 100 mM NaCl, 5 mM EDTA, 20 mM Tris HCl pH 7.5, 1% Triton X-100, containing 1 mM PMSF and 10 mg/ml each of aprotinin, leupeptin, antipain, and pepstatin. Immunocomplexes were released by boiling 5 min in SDS sample buffer separated in a 10% SDS–PAGE, and Western blots were performed as described. For methionine labelling MDCK cells were labelled for 12 h in 1 ml of DMEM methionine-free, containing 400 mCi /ml ^{35}S methionine (ICN; Trans^{35}S label 1133 Ci/mmole 492 Tbq/mmole).

After washing with cold PBS, cells were lysed on the dish by addition of 0.5 ml lysis buffer. The lysates were cleared by centrifugation in an Eppendorf centrifuge for 2 min. The supernatants were incubated with 5μl of normal mouse serum for 1 h at 4°C and transferred to a new eppendorf tube containg 20 μl wet volume pellet of prewashed Staph A. After resuspension of the Staph A, the lysates were incubated by continuous rocking at 4°C, for 30 min and then centrifuged for 2 min in Eppendorf centrifuge. This was repeated once more and the lysates were finally centrifuged for 10 min in a Eppendorf centrifuge. The resulting supernatants were then used for immunoprecipitation by incubation with anti-β-catenin, anti-α-catenin, anti-E-cadherin and anti-γ-catenin (Transduction Lab) for 3 h at 4°C with rocking. Finally 80 μl of protein A-Sepharose (10% wt/wl) suspension was added and the incubation was continued for 1/2 h by rocking at 4°C. Protein A-Sepharose was recovered by centrifugation, washed three times in wash buffer (20 mM TEA pH 7.5, 150 mM NaCl, 0.5% Triton-X 100 e 1 mM PMSF) and finally resuspended in caspase-3 digestion buffer.

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