Proteolytic cleavage of β-catenin by caspases: an in vitro analysis

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Abstract Cleavage of structural proteins by caspases has been associated with the severe morphological changes occurring during the apoptotic process. One of the proteins regulating the connection of the actin filament with cadherins in a cell-cell adhesion complex is β-catenin. During apoptosis, both an N-terminal and a small C-terminal part are removed from β-catenin. Removal of the N-terminal part may result in a disconnection of the actin filament from a cadherin cell-cell adhesion complex. We demonstrate that caspase-8, -3 and -6 directly proteolyse β-catenin in vitro. However, the β-catenin cleavage products generated by caspase-8 were different from those generated by caspase-3 or caspase-6. Caspase-1, -2, -4/11 and -7 did not or only very inefficiently cleave β-catenin. These data suggest that activation of procaspase-3, -6 or -8 by different stimuli in the cell might result in a differential proteolysis of β-catenin.

Key words: Caspase; Catenin; Cleavage; Proteolysis

1. Introduction

The execution of cell death during ontogeny, homeostasis and cellular differentiation is associated with characteristic morphological and biochemical changes [1]. Programmed cell death or apoptosis is associated with dramatic morphological changes of the entire cell. These changes include decreased adhesion and intercellular contacts, increased cell membrane blebbing, chromatin condensation along the periphery and overall shrinkage of the cell. As a result of these modifications, the dying cell disintegrates into apoptotic bodies [2]. Cleavages of important structural proteins during apoptosis are at least partially responsible for these morphological changes. Some of those cleavages can be achieved by caspases [3–7]. Caspases are cysteinyl aspartate-specific proteases, which are conserved during evolution and which are part of the highly controlled pathways leading to cell death [8–10]. So far, 13 members of the mammalian caspase family have been identified [11–13].

In accordance with the significant morphological changes during apoptosis, some important proteins with structural functions, such as Gas2 [4], keratin 18 [6], gelsolin [3] and β-catenin [5,7], are substrates for caspases. The latter do not randomly cleave all structural proteins since at least actin, α-catenin, tubulin, vimentin and talin have been shown not to undergo proteolytic degradation in apoptotic cells [4,5,14].

β-Catenin is not only a protein which plays an important role in cell-cell adhesion, it is also involved in the Wnt/wingless growth factor-signaling pathway [15,16]. Consistent with these two functions, β-catenin is localized primarily in two intracellular pools: a membrane-associated pool involved in cadherin-mediated cell-cell adhesion and a cytoplasmic pool important for signaling. To exert its multiple functions, β-catenin associates with a wide panel of proteins, including α-catenin [17], cadherins [18], epidermal growth factor receptor [19], c-erbB-2 [20], the tight junction zonula occludens-1 protein [21], fascin [22], leukocyte antigen-related protein tyrosine phosphatase [23], PTP-κ [24], the adenomatous polyposis coli tumor suppressor protein [25,26] and members of the T-cell factor/lymphoid enhancer factor subfamily [27–29]. Recently, it has been demonstrated that β-catenin is proteolysed during apoptosis [5,7]. Both caspase-3 and -6 can induce proteolysis of β-catenin in fragments comparable to those detected in apoptotic cells [5,7]. β-Catenin is proteolysed at several positions both at its N-terminal and C-terminal parts. Since the N-terminal part is responsible for interaction with α-catenin, proteolysis of β-catenin may destroy the connection between the actin cytoskeleton and cadherin complexes at the points of cell-cell contacts [5,7]. The putative effect of caspase-mediated proteolysis of β-catenin on its transcriptional activation function is still unclear.

We analyzed the proteolytic properties of a set of recombinant murine caspases on β-catenin. We demonstrate that not only caspase-3 and caspase-6 activity [5,7,30] but also caspase-8 activity can proteolyse β-catenin in a direct way. However, caspase-8 activity clearly results in different products compared to caspase-3 or caspase-6 activity. Among all the other caspases tested, none was able to induce clear β-catenin cleavage, which argues for proteolytic specificity of the caspases.

2. Materials and methods

2.1. In vitro transcription and translation

Human β-catenin cDNA was excised with a *Sal*I/*Xba*I restriction enzyme digest from a pBATβcat plasmid (a generous gift from Dr J. Behrens, Max-Delbruck-Center for Molecular Medicine, Berlin, Germany), blunted by Pfu DNA polymerase (New England Biolabs, Beverly, MA, USA) and cloned into an *Eco*RV-opened pCDNA3 vector (Invitrogen, San Diego, CA, USA). The resulting plasmid is referred to as pCDNA-β-catenin. For the generation of in vitro transcribed and translated β-catenin, pCDNA-β-catenin was applied as template for T7-RNA polymerase in a coupled transcription/translation kit from Promega Biotech (Madison, WI, USA) according to the manufacturer's recommendations. In vitro transcription and translation was performed in the presence or absence of [^{35}S]methionine to obtain radiolabelled or non-radiolabelled β-catenin, respectively. [^{35}S]-methionine-labelled β-catenin was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stored at −70°C until needed. Poly(ADP-ribose) polymerase (PARP) cDNA (kindly provided by Dr Z. Wang, Research Institute for Molecular Pathology, Vienna, Austria) was subcloned into pGEM11zf(+)

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(Promega Biotech) to generate pGEM-PARP. The latter plasmid was used for the generation of radiolabelled PARP by coupled in vitro transcription and translation.

2.2. In vitro cleavage by caspases

Murine caspases devoid of the prodomain were purified to homogeneity from *Escherichia coli* MC1061 lysates (M.V.D.C., unpublished). During bacterial expression, the caspases are autoprocessed into ~20 and ~10 kDa fragments. The ~20 kDa fragments were used to estimate the amount of active enzyme. Of every caspase, an equal amount (120 ng) of ~20 kDa fragment, indicative of active caspase, was used in the assay. The caspases were co-incubated for 1.5 h at 37°C with 4 μl [^{35}S]methionine-labelled β-catenin in a total of 50 μl caspase buffer (50 mM HEPES, pH 7.5, 2 mM EDTA, 10 mM DTT, 50 μM leupeptin, 1 mM PMSF and 20 μg/μl aprotinin). The mixture was analyzed by SDS-PAGE, followed by autoradiography. To compare the enzymatic activities of caspase-3, -6 and -8 of β-catenin more accurately, a 1/8 serial dilution of the enzymes starting with 60 ng (~20 kDa fragment) was made and incubated with radiolabelled β-catenin in a total volume of 25 μl. The cleavage of PARP by caspase-3 was taken as a reference for caspase-3 activity. The caspases had the following activities on the peptide caspase substrate acetyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-aminomethylcoumarin: 11 726 U/mg (caspase-1), 42 000 U/mg (caspase-2), 8 004 500 U/mg (caspase-3), 568 180 U/mg (caspase-6), 312 800 U/mg (caspase-7), 911 950 U/mg (caspase-8), 17 969 U/mg (caspase-11). One unit of purified caspase represents the amount of enzyme necessary to generate 1 pmol of aminomethylcoumarin/min from 50 μM of the peptide caspase substrate at 30°C.

2.3. Western blot analysis

Non-radiolabelled transcribed and translated β-catenin (4 μl) or recombinant GST-β-catenin fusion protein (500 ng) was incubated for 1.5 h at 37°C with 120 ng (~20 kDa fragment) caspase-3, -6 or -8 in a total volume of 50 μl caspase buffer. To reveal the cleavage products, the mixture was separated on 8% SDS-PAGE, electroblotted on a nylon membrane and visualized by Western blot analysis with an antibody directed against β-catenin (Transduction Laboratories, Lexington, KY, USA) or anti-GST (Pharmacia Biotech).

3. Results

In order to test which caspases are able to induce β-catenin cleavage, an in vitro test system was set up. Human β-catenin was [^{35}S]methionine-labelled by in vitro transcription and translation. The resulting product had an apparent molecular mass of ~97 kDa on SDS-PAGE (Fig. 1), which is identical to endogenously expressed β-catenin [31,32]. Radiolabelled human β-catenin was incubated with active murine caspases. Caspase-3, -6 and -8 efficiently induce processing of β-catenin in this assay, generating major bands of ~70 kDa (Fig. 1). The ~70 kDa fragments obtained with caspase-3 or caspase-6 incubation resemble those of the endogenous β-catenin fragment, which are generated after apoptosis induction in NIH3T3 cells following serum deprivation [5] or in HUVEC cells after growth factor deprivation [7]. Caspase-7 induced only very weak processing of β-catenin. This lower activity of caspase-7 is not due to low intrinsic enzymatic activity of this particular preparation, since serial dilutions of caspase-3 and caspase-7 proteolysed equally well the prototype substrate PARP (data not shown). Caspase-1, -2 and -11 did not exert any in vitro proteolytic activity on β-catenin.

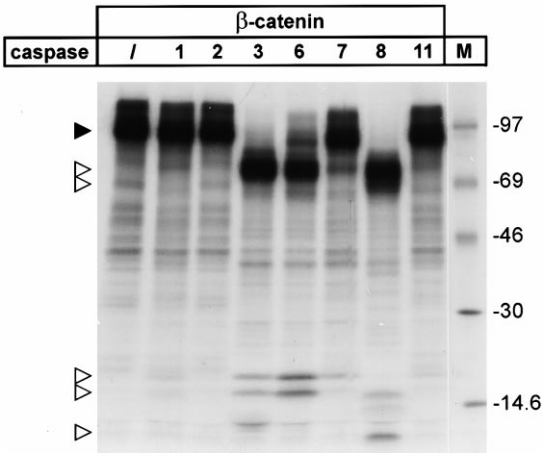

Fig. 1. Cleavage of in vitro labelled β-catenin by caspases. β-Catenin (4 μl) was incubated for 1.5 h at 37°C in a total volume of 50 μl caspase buffer with an amount of murine caspases corresponding to 120 ng of large subunit (~20 kDa). Reaction products were separated on 4–20% SDS-PAGE; closed arrowhead, full-length product.

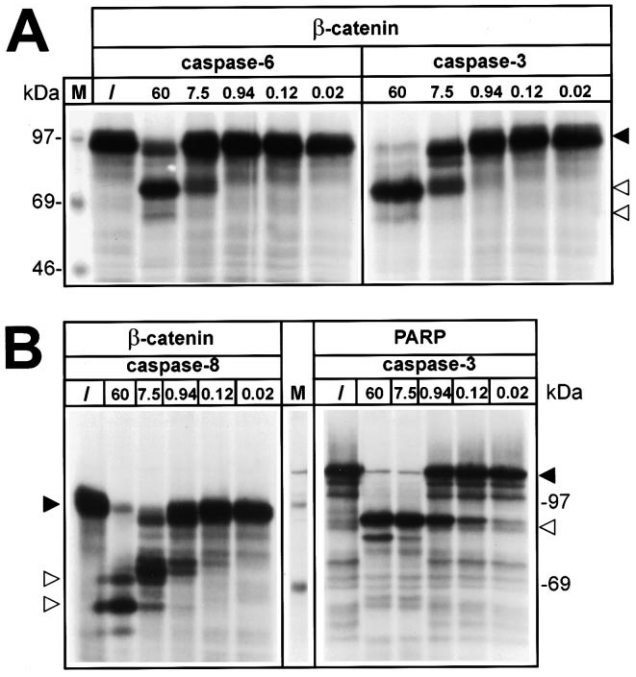

Fig. 2. Comparison of caspase activities on β-catenin. [^{35}S]methionine-labelled β-catenin (2 μl) was incubated without or with different ng amounts of purified caspase-3 or -6 (A) or caspase-8 (B) in 25 μl caspase buffer for 1.5 h at 37°C. As an internal control for caspase activity, caspase-3 was additionally incubated with [^{35}S]methionine-labelled PARP (B). Reaction products were separated on 8% SDS-PAGE, followed by autoradiography. Open arrowhead, cleavage fragment; closed arrowhead, full-length product.

To evaluate the difference in activity of caspase-3, -6 and -8 on β-catenin more extensively, a 1/8 dilution series of these caspases was incubated for 1.5 h with radiolabelled β-catenin. Fig. 2 demonstrates that caspase-8 was about 10 times more efficient than caspase-3 in inducing cleavage of β-catenin. Caspase-3 was only slightly more active on β-catenin than caspase-6. Since PARP is considered a prototype substrate of caspase-3 [33], it was used as a reference for caspase-3 activity. Caspase-3 was about 10–50 times more efficient in PARP cleavage as compared to β-catenin cleavage (Fig. 2), indicating that caspase-8 might be a good candidate for β-catenin proteolysis in the cell. These serial dilutions also indicate that caspase-8 cleaved β-catenin at multiple sites and

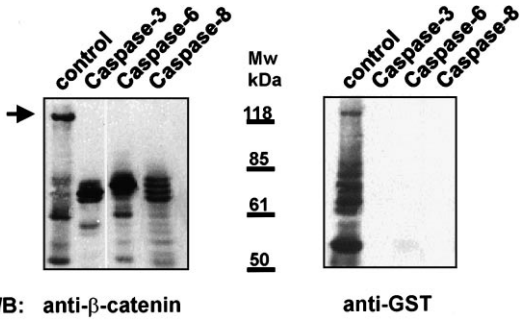

Fig. 3. Direct cleavage of β-catenin by caspases. GST-β-catenin fusion protein was incubated with recombinant caspase-3, -6 or -8 for 1.5 h at 37°C. Samples were loaded on a 8% SDS-PAGE, electroblotted and developed with anti-β-catenin or anti-GST antibodies using ECL (Amersham). The arrowhead indicates the position of the full-length GST-β-catenin fusion protein.

that the fragments generated were different from those generated by caspase-3 or caspase-6. To exclude that the observed processing might be an indirect effect due to caspase-dependent activation of proteases in the rabbit reticulocyte lysate, we incubated the bacterially expressed purified β-catenin N-terminal GST fusion with the caspases that scored positively in the previous tests, viz. caspase-3, -6 and -8. Western blotting using an antibody raised against the 571–781 fragment of mouse β-catenin revealed identical fragments as those represented in Fig. 2, which is based on in vitro translated, labelled β-catenin. The heterogeneity of the cleavage fragments is most probably due to differential cleavage at various potential caspase-specific sites in the N- and C-terminal region of β-catenin [5]. As a control, these blots were developed with anti-GST antibody, but no cleaved bands were revealed. Hence, the protein fragments detected with the anti-β-catenin antibody were indeed N-terminally cleaved. The results clearly demonstrate that the previously reported caspase-3- and caspase-6/Mch-2-dependent cleavage of β-catenin [7] is direct and that caspase-8 is also able to proteolyse directly.

4. Discussion

Multiple apoptosis-inducing stimuli lead to the activation of caspases [34,35]. One of the recently identified caspase substrates is β-catenin [5,7,30]. However, so far, only a limited number of caspases, viz. caspase-3, -6 and -7, has been tested for their ability to induce proteolysis of β-catenin in vitro [5,7]. Here, we report the activity of seven murine caspases on human β-catenin in an in vitro assay. The primary amino acid sequence of human and murine β-catenin is identical [36], which allowed for the use of murine caspases on this human substrate. Co-incubation of purified caspase-1, -2 or -11 with β-catenin did not result in hydrolysis of the substrate, while caspase-7 was only very weakly able to induce cleavage of β-catenin in vitro (Fig. 1). On the other hand, caspase-3, -6 and -8 were clearly able to generate β-catenin cleavage products (Fig. 1). The proteolytic fragments are similar, but not completely identical, for caspase-3 and -6, both at high (Figs. 1–3) and low enzyme concentrations (Fig. 2). Incubation of β-catenin with caspase-8 results in a different cleavage pattern as clear from serial dilutions (Fig. 2) and detection of the cleavage products by Western analysis (Fig. 3). Incubation of in vitro transcribed and translated β-catenin with caspase-3, -6 or -8 resulted in multiple cleavage sites in β-catenin. Serial dilutions of caspase-8, -6 and -3 also indicate that caspase-8 was most efficient in inducing in vitro cleavage of β-catenin, followed by caspase-3 and -6 (Fig. 2). Using recombinant, purified β-catenin and caspases as substrate and enzymes, respectively, we demonstrated that caspase-3, -6 and -8 proteolyse β-catenin in a direct way, independent of any reticulocyte lysate-associated proteases. In case of caspase-6/Mch-2 cleavage of β-catenin, it has been proposed that this might be due to a reticulocyte lysate artefact used for in vitro translation [7]. In summary, caspase-3, -6 and -8 are the main candidates to proteolyse β-catenin directly, while caspase-1, -2, -7 and -11 might be excluded.

Proteolysis of β-catenin has not only been described to occur in the course of apoptotic cell death processes [5,7,30] but also during the disorganization of endothelial adhesion junctions [37–39]. Although the initial recruitment of polymorphonuclear leukocytes (PMNs) on activated endothelium has been intensively studied, trans-endothelial migration is poorly understood. Transmigration suggests modifications in the architecture of endothelial cell to cell junctions. Recently, it has been demonstrated that adhesion of PMNs to endothelial cells (ECs) leads to disorganization of the vascular endothelial cadherin-dependent adherence junctions [37,39]. Endothelial β-catenin and plakoglobin are proteolysed upon PMN attachment to ECs. The destruction, especially of β-catenin, is probably partially responsible for the disassembly of the adherence junctions [7]. It is not yet clear how PMNs induce the proteolysis in ECs but a firm adhesion of the PMNs to the EC surface appears to be a prerequisite. A same anti-β-catenin antibody raised against a peptide encompassing amino acids 571–781 of mouse β-catenin was previously used to examine cleavage of β-catenin during apoptosis and to examine proteolysis of β-catenin in the case of PMN adhesion to ECs [5,38]. Both systems displayed clear proteolysis of β-catenin, but only in the apoptotic system, the β-catenin cleavage products were detected using this antibody. This suggests that β-catenin is differently cleaved in both systems and that the generated fragments might be differentially degraded further. Most probably, β-catenin is proteolysed during apoptosis by activation of caspase-3 and/or caspase-6, which are believed to be downstream executionary caspases [10], while caspase-8 appears to be a good candidate to be involved in β-catenin cleavage induced by PMN adhesion to ECs. This would imply that caspase-8 activation in ECs does not always lead to apoptosis and to activation of caspase-3. These hypotheses are currently under investigation.

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