Mutations dislocate caspase-12 from the endoplasmatic reticulum to the cytosol

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Abstract Mouse AKR-2B cells express two forms of caspase-12: the full-length form coding for a protein of 47.8 kDa and a new splice variant of 40.2 kDa which is devoid of the CARD domain. In addition, three point mutations were disclosed: I/L-15, E/D-46 and P/L-105. A major portion of the two protein variants was found in the cytosol. Immunofluorescence studies showed an even distribution of caspase-12 within the cell, indicative for a cytoplasmatic localization. Transfection of AKR-2B cells with wild-type caspase-12 showed a colocalization of this protein with the endoplasmic reticulum (ER). Unlike mouse embryonal fibroblasts (MEF) which contain wild-type caspase-12, AKR-2B cells were largely resistant against treatment with the endoplasmatic reticulum stressing reagents brefeldin and tunicamycin. In AKR-2B cells, cytoplasmatic caspase-12 is bound to high molecular weight complexes of >1000 kDa [Cell Death Differ. 9 (2001) 125] and serum depletion leads to cleavage and detachment of caspase-12 from this high molecular weight complex. Cleavage of caspase-12 and -3 occurred almost simultaneously reaching a maximum 3–5 h after serum deprivation at which time also maximum apoptosis is found. Analysis of caspase-12 cleavage in vitro in comparison with fragmentation in vivo suggests that during death in AKR-2B cells induced by starvation, cleavage was brought about by caspase-3 at positions D24 and D94. Thus, mutated caspase-12 is differently integrated in signaling pathways of cell death and has lost its function as initiator caspase upon ER-stress. Instead, it is turned into a substrate of effector caspases. The implication of these findings in the pathological phenotype of ARK-2B mice is discussed.
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1. Introduction

The caspase protease family plays a central role in the implementation of apoptosis in vertebrates [1,2]. Caspases are activated upon processing of a precursor into ∼20-kDa (p20) and ∼10-kDa (p10) mature fragments forming heterotetramers [3]. Alternatively, for caspase-9, the formation of dimers is sufficient for activation [4]. The caspase family is broadly divided into two groups: initiator caspases (caspase-8, -9, and -12) and effector caspases (caspase-3, -6, and -7), which can be activated via sequential processing by other caspase family members [1,5]. The implication of different organelles has been reviewed in [2].

Caspase-12 has been described to function as an initiator caspase in response to a toxic insult of the endoplasmic reticulum (ER) [6], such as by treatment with tunicamycin (an inhibitor of glycosylation), thapsigargin (an inhibitor of the ER-specific calcium ATPase), or calcium ionophores [7–11]. Furthermore, caspase-12-deficient cells are resistant to inducers of ER stress, suggesting that caspase-12 is involved in ER stress-induced apoptosis. Caspase-12-deficient mice show a reduced sensitivity to tunicamycin-induced renal tubular epithelial cell death and to amyloid-β-induced neuronal cell death. Based on the latter finding, caspase-12 was suggested to play an important role in the pathogenesis of Alzheimer's disease and to represent a potential target for its treatment [8].

However, in humans different splice variants of caspase 12 are expressed leading to a premature termination. A small population of black Africans express a full-length caspase-12, which due to a point mutation is catalytically inactive. Detailed studies have excluded the proposed pathogenic role in the development of Alzheimer's disease in humans [12,13].

Murine caspase-12, which has a CARD domain and is specifically localized on the cytoplasmic side of the ER, is processed within its intersubunit region at amino acids D318 and D341 [7,10,14,15]. Several possible molecular mechanisms for the processing of caspase-12 have been postulated: (1) caspase-12 is initially processed at the N-terminal region after K158 by calpain activated by ER stress, then activated and autoprocessed at D318 [7], (2) caspase-12 is released from TRAF2 complexes by ER stress and is then autoprocessed via homodimerization [16,17], (3) caspase-12 is processed at D94 by caspase-7 and then autoprocessed at D341 [10]. Thus, the molecular mechanism by which caspase-12 is activated seems to vary within different model systems. Once activated, caspase-12 can specifically cleave procaspase-9 in vitro and in vivo [18,19], which in turn can then activate the effector caspase-3.

Density-arrested AKR-2B fibroblasts die rapidly after serum deprivation exhibiting typical morphological changes including membrane blebbing and chromatin condensation, but no DNA fragmentation. Furthermore, dead cells are rapidly lysed. We have shown previously that all caspases are expressed in AKR-2B cells with the exception of caspase-7. Caspase-3 is the main executioner caspase during apoptosis

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induced by serum deprivation or after anisomycin treatment [20–26].

We have demonstrated the formation of two different-sized complexes of caspase-3 in response to serum deprivation. The 600- and 250-kDa multimeric apoptosome-like complexes contain activated caspase-3, but are devoid of apoptosis protease activating factor-1 (apaf-1) and cytochrome *c* [25,26].

The proenzyme of caspase-12 was found to be bound to cytosolic high molecular weight complexes from which it was released after activation by serum deprivation as well as anisomycin treatment [25,26]. Here, we demonstrate that this activation is not coupled to ER stress. Sequence analyses of caspase-12 from AKR-2B cells revealed mutations that are responsible for its different subcellular localization. This different localization alters largely its implication and position in

the pathway of cell death. Instead of being an initiator caspase, caspase-12 in AKR-2B cells is rather a substrate for caspase-3.

2. Materials and methods

2.1. Materials
The antibody against caspase-12 was from Cell Signaling Technology (CST, Frankfurt, Germany), against Grp-78 from Stressgene/Biomol (Hamburg, Germany) and against the HA-tag from Roche (Mannheim, Germany), against full-length caspase-3 from BD Biosciences (Heidelberg, Germany). ER-tracker Blue-White DPX was from Molecular Probes (Mobitec, Hamburg, Germany). Tunicamycin, brefeldin, thapsigargin, A23187 and ProteoExtract (S-PEK) were from Calbiochem (Merck Bioscience, Bad Soden, Germany). Cytobuster was from Novagen (Merck Bioscience, Bad Soden, Germany). Cy3-labeled goat-anti-rabbit antibody was from Dianova (Hamburg, Germany). The plasmids pIRES2-EGFP and pECFP-ER were from Clontech (Heidelberg, Germany).

2.2. Cell culture
Stock cultures of AKR-2B mouse fibroblasts were propagated in antibiotic-free McCoy-5A medium (Life Technologies, Eggenstein, Germany) with 5% Hyclone calf serum for less than 3 months to minimize fluctuations. Cultures were seeded at a density of 5×10^3 cells/cm^2 into plastic dishes (Falcon, Becton Dickinson, Heidelberg, Germany) and grown for 5 days without medium change. For the determination of cell numbers, 12 well plates were used. After the addition of the indicated compounds, cells were detached by trypsinization at the indicated time and the number of viable cells was determined by the Casy I cell counter. MEF cells (mouse embryo fibroblasts transformed with the tet-repressor) were purchased from Clontech, kept in DMEM containing 5% Hyclone calf serum and were propagated similar to AKR-2B cells.

2.3. Gel electrophoresis and immunoblotting
For gel electrophoresis, cell lysates were prepared either by treatment with Cytobuster (Novagen) or fractionated using the subcellular fractionation kit S-PEK from Calbiochem (Merck Bioscience, Soden, Germany). Blotting and detection by chemiluminescence were done as previously described [27]. Loading control was done by staining the nitrocellulose sheets with Amido Black. Bands representing a prominant 47 kDa protein are shown in the individual Western blots (control).

2.4. cDNA cloning
The cDNA coding for the full-length mouse caspase-12 was obtained by PCR by using the forward primer GCA CGA GCA CAG CCA

Fig. 1. Caspase-12 is upregulated in confluent cells. (A) Total RNA was isolated from confluent (c) or proliferating AKR-2B cells (p) and equal amounts were used for RT-PCR. GAPDH was used as control. (B) Lysates were prepared by treatment of the cells with Cytobuster. Blots were probed with the caspase-12 antibody. Control: staining with Amido Black (47 kDa protein).

Fig. 2. Amino acid sequence of caspase-12. The amino acid sequence of caspase-12 was deduced from the cDNA sequence. The alignment of the published murine sequence [28], and the sequences from MEF, AKR-2B full length and splice variant are shown.

TGG (Casp12-For2) and the reverse primer CAT GAG AGT TGC CTG TGC TA (Casp12-Rev) from a Marathon-Ready cDNA library from mouse 15-day embryo (Clontech, Heidelberg, Germany) using the Advantage HF 2 polymerase (Clontech). DNA-sequencing confirmed that the obtained DNA did not contain any mutation [28]. cDNAs for caspase-12 from AKR-2B and MEF cells were obtained by RT-PCR starting from whole RNA by using the primer GAG TCC TGC ACA GCC ATG G (Casp12g-For2) and the reverse primer (Casp12-Rev). To exclude mutations caused by PCR, at least four clones were completely sequenced.

The vector pIRES2-EGFP was modified to contain the sequence coding for an HA-tag (*Sal*I and *Bam*HI) at the C-terminal site of an integrated protein. The full-length caspase-12 cDNA from the Marathon library was cloned by PCR into the *Bgl*II/*Sal*I sites of the modified expression vector pIRES2-EGFP. Cystein 298 was mutated into alanine by PCR according to the QuikChange protocol from Stratagene (Amsterdam, The Netherlands) yielding the plasmid pC12-WT-P2-HA.

2.5. Transfection and microscopy

18 hours prior to transfection, 4×10^6 AKR-2B fibroblasts from a confluent culture were seeded into 14.5 cm plates. Cells were then detached with trypsin and 2×10^6 cells were resuspended in hypoosmol-aric buffer (0.3 mM KH_2PO_4, 0.85 mM K_2HPO_4, 25 mM myo-inositol and 100 mOsmol). Transfection with 10 μg of plasmid DNA was done using an electroporator (Eppendorf, Hamburg, Germany) with 400 V, 100 μs, and two pulses. Transfection efficiency was 30–50% as estimated by the expression of the EGFP or ECFP. Cells were seeded onto coverslips and were analyzed 24 h later. After fixation in 3% paraformaldehyde, cells were permeabilized by treatment for 5 min in 0.2% Triton X-100 in PBS. Non-specific binding was blocked by incubation with 0.2% gelatine in PBS. Staining with anti-caspase-12 antibody from CST (10 μg/ml) was done overnight at 4 °C. Counterstaining was done with Cy3-labeled anti-rabbit antibody (1 μg/ml). The coverslips were then processed as described. Filters A, I3, N1 (Leica, Bensheim, Germany) or XF114-2 (Omega, Vermont, USA) were used to monitor blue, cyan, green or red fluorescence, respectively. Photographs were recorded using a cooled digital camera (Kappa, Gleichen, Germany). No staining was observed when the primary antibody was omitted. If indicated, ER-tracker (1μg/ml) was added to living cells for 1 h.

2.6. RT-PCR

5 μg of total RNA, prepared according to Chomczynski and Sacchi [29] were taken for RT-PCR by using the primers Casp12-For2 and Casp12-Rev and HotStarTaq polymerase from Qiagen (Hilden, Germany).
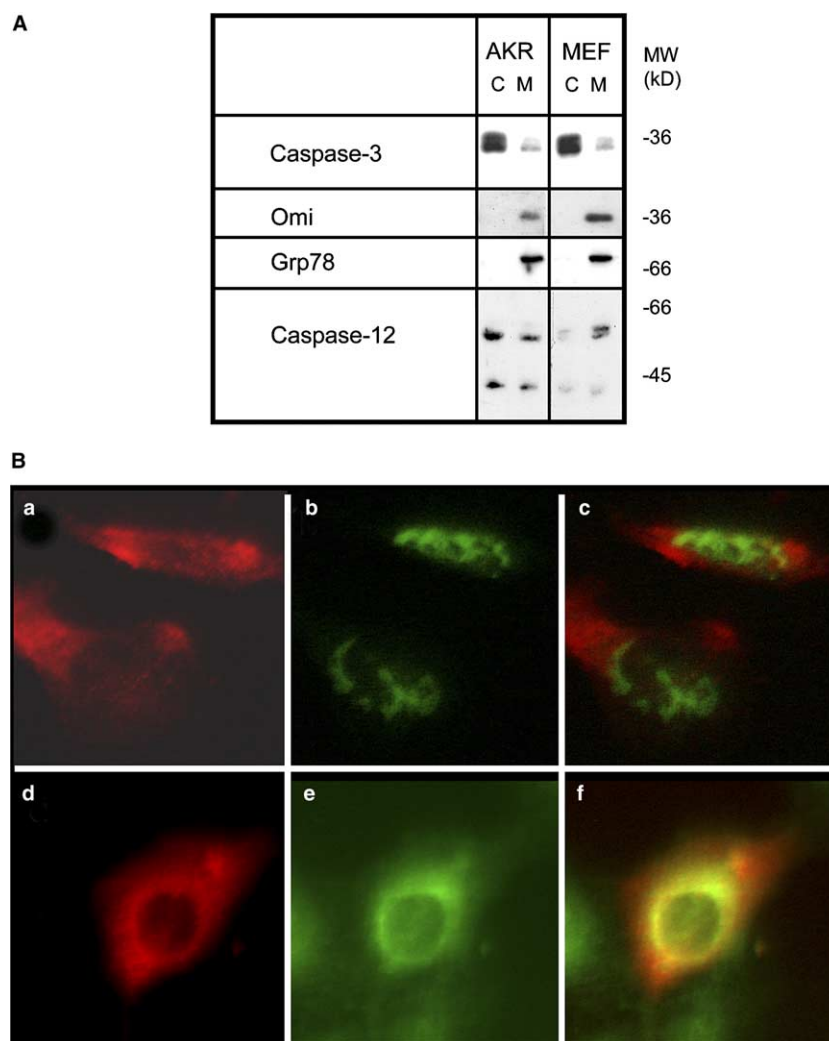
Fig. 3. Subcellular localization of caspase-12. (A) Subcellular localization. The cellular content was fractionated using the S-PEK cell fractionation kit. Equal amounts of protein (15 μg) were analyzed by Western blotting. Blots were probed with the polyclonal antibody against caspase-12, an antibody against Grp-78 (ER), an antibody against omi (mitochondria), and an antibody against full-length caspase-3 (cytosol). Only the cytosolic (C) and the membrane/organelle (M) fractions are shown. (B) Immunofluorescence and transfection (a–c) AKR-2B cells were transfected with the plasmid pECFP-ER and stained with the polyclonal antibody for caspase-12; left: caspase-12, middle: ECFP, right: merge. Pictures were taken 24 h after transfection. (d–f) AKR-2B cells were transfected with plasmid pC12-WT-P2-HA and subsequently stained with ER-tracker and the anti HA-antibody; left stain with anti-HA antibody, middle ER-tracker, right merge.

2.7. Caspase-3 preparation

For the expression in *Escherichia coli*, a cDNA coding for the full-length mouse caspase-3 was amplified from a Marathon-Ready cDNA library from 15-days old mouse embryo (Clontech, Heidelberg, Germany) using the forward primer CGC TAA GAA AAG TGA CCA TGG AG and the reverse primer ACC CCC AAT CAT TCC TCT AGT GA. Expression was done as described in [30].

2.8. In vitro translation

Various fragments of caspase-12 were cloned either into the *Nco*I / *Bam*HI sites of the vector pET-3d or the *Nde*I/*Xho*I sites of the vector pET-21b with a stop codon before the His-tag. Site-directed mutagenesis was done according to the QuikChange protocol from Stratagene (Amsterdam, The Netherlands). Mutations were verified by DNA-sequencing. In vitro translation was done using the Rapid Translation System from Roche (Mannheim, Germany) according to the manufacturer's instructions.

For treatment with caspase-3, 1.5 μg recombinant caspase-3 was added to 10 μl in vitro translation mixture.

3. Results

3.1. The expression of caspase-12 in AKR-2B cells is growth regulated

The expression of caspase-12 was investigated by RT-PCR and Western blotting (Fig. 1) RT-PCR showed a prominent band of 1290 bp corresponding to the expected size of the PCR-product and a minor band of about 1000 bp. Proliferating cells express only about 50% of both mRNA species of caspase-12 compared to confluent cells (Fig. 1A). As control, the expression of the house keeping gene GAPDH was analyzed showing equal amounts of mRNA in both cases. Two bands of 50- and 40-kDa, respectively, were detected by Western blotting (Fig. 1B). A 50% decreased expression in proliferating cells was also observed by Western blotting for both the 50- and the 40-kDa bands. The expression of caspase-12 was previously reported to be regulated during the development in mouse embryos and was inducible by IFN-γ in mouse fibrosarcoma and melanoma cells [17]. To investigate the nature of the 1000-bp cDNA fragment and the corresponding 40 kDa protein, the PCR products were cloned into the Topo-vector (Invitrogen) and sequenced (Fig. 2). Indeed, the 1290-bp insert codes for the full-length caspase-12. Interestingly enough, the 1000-bp PCR product codes for a so far unknown splice variant. Remarkably, almost the entire CARD-domain is missing. The insert codes for a protein of 40.2 kDa (Fig. 2), which is in perfect agreement with the results from Western blots. But surprisingly, the full-length sequence of caspase-12 from AKR-2B cells contained three mutations compared with the wild type obtained from the Marathon-Ready cDNA library: I/L at position 15, E/D at position 46 and P/L at position 105. No mutation was found for caspase-12 in MEF-cells (Fig. 2).

3.2. Different subcellular localization of caspase-12 in AKR-2B and MEF cells

Subcellular distribution was done by using the kit S-PEK from Calbiochem, which is based on the differential solubility of certain subcellular compartments in special reagent mixtures. Control proteins caspase-3, HtrA2 (omi), and Grp-78 showed the normal expected distribution, but for caspase-12 the distribution between cytosol and membrane/organelle fraction was almost inverted between AKR-2B cells and MEF (Fig. 3A). Whereas the major portion of caspase-12 was membrane/organelle-bound in MEF, for AKR-2B cells it was predominantly found in the cytosol. These findings were supported by immunofluorescence studies (Fig. 3B). AKR-2B cells were transfected with the plasmid pECFP-ER to label specifically the ER. For AKR-2B cells (Fig. 3B(a)–(c)), there was no colocalization between ER-stain and immuno stain. To substantiate these findings, AKR-2B cells were transfected with a wild-type HA-tagged caspase-12 construct. ER was stained with the specific ER-tracker. Fig. 3B(d)–(f) shows a strong overlap of transfected wild type caspase-12 with the ER, indicative for an ER-localization of wild type caspase-12 in AKR-2B cells.

3.3. Activation without ER-stress

We have previously shown that in AKR-2B cells, fragmentation and possibly activation of caspase-12 can be induced either by serum starvation or by treatment with 10 μM anisomycin [25,26]. Concerning serum starvation, our results are
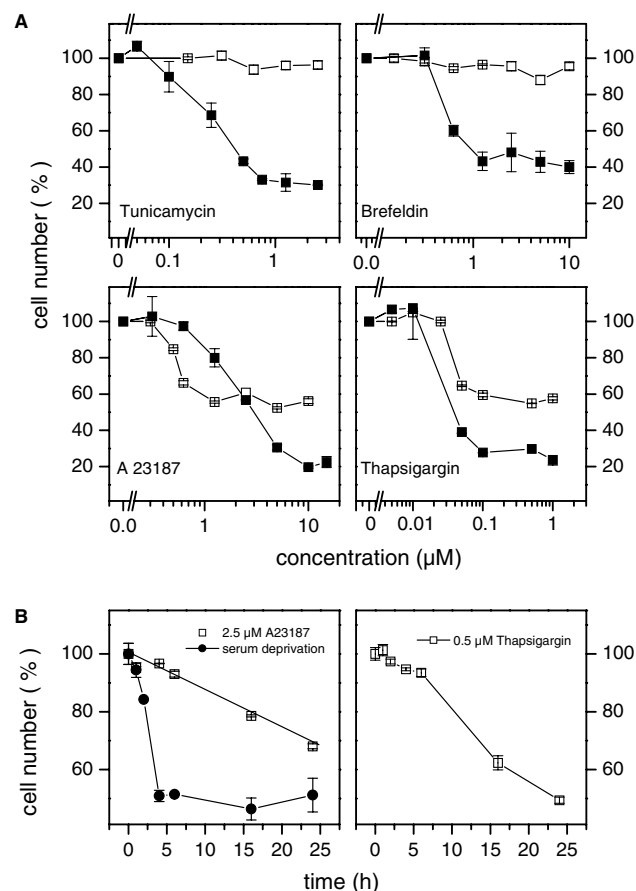

Fig. 4. Effect of ER-stressing reagents on cell integrity. (A) Various concentrations of tunicamycin, brefeldin, A23187 or thapsigargin were added to confluent cells kept in medium containing 5% Hyclone calf serum. The number of viable cells was determined after 24 h. □; AKR-2B cells, ■; MEF cells. (B) Time-dependent loss of viable cells. To confluent AKR-2B cells, the indicated amounts of A23187 or thapsigargin, respectively, which evoked maximum loss of viable cells, were added. For comparison, the kinetic of cell death induced by serum starvation is included. After the indicated time intervals, the number of viable cells was determined.

in contradiction with various previous reports using PC12 cells, where no fragmentation of caspase-12 was observed after serum deprivation [8].

We used various reagents known to induce ER-stress, to tackle the question whether in AKR-2B cells the fragmentation of caspase-12 is linked to ER-stress. Fig. 4 shows the effect of tunicamycin, brefeldin, ionophore A23187 or thapsigargin, respectively, on the survival of AKR-2B cells. All reagents were used over a wide concentration range covering those concentrations applied in previous publications. Surprisingly, neither tunicamycin nor brefeldin had any effect on the number of viable cells (Fig. 4A). Treatment with A23187 or thapsigargin was ineffective after 6 h (data not shown) but led to a cells loss of about 50% after 24 h (Fig. 4A). ED_{50}-values were 0.4 μM for A23187 and 0.03 μM for thapsigargin, respectively. Kinetic measurements for these two reagents were done at concentrations effecting maximum efficiency (2.5 μM for A23187 or 0.5 μM for thapsigargin (Fig. 4B)). For comparison, kinetic data for serum starvation are included, which show maximum cell death after 2.5 h. For A23187 and thapsigargin much slower kinetics were observed. Remarkably, there was no significant cell death after 6 h, whereas at this time point apoptosis induced by serum starvation was complete. Thus, these data show that ER-stress- and serum deprivation-induced cell death exhibit quite different characteristics. Fig. 5 shows the effect of ER-stressing reagents in AKR-2B cells on the expression of Grp-78, an ER-specific chaperone which is induced under ER-stress and on caspase-3 or -12. After treatment with brefeldin but not with tunicamycin, there was a considerable loss of caspase-12 but no fragments were detected. As expected there was a high increase in Grp-78. Thus, both tunicamycin and brefeldin induced effectively ER-stress which is accompanied with an unspecific digestion of caspase-12 in the case of brefeldin without any loss of viable cells (Figs. 4A and 5A). There was no substantial loss in the content of procaspase-3 induced by either of these reagents (shorter exposure times led to the same result (data not shown)). Furthermore, the antibody against full-length caspase-3 did not detect any fragments in the range of the large subunit (19 kDa), though there were strong signals after serum deprivation (Fig. 5), indicating that no major cleavage had occurred. A23187 and thapsigargin behaved similar to brefeldin regarding caspase-12 and Grp-78, but traces of a 19 kDa cleavage product of caspase-3 could be detected. Remarkably, serum starvation led to a substantial cleavage of caspase-12 (indicated by a star; Fig. 5C). Fragmentation of caspase-12 was maximum between 3 and 5 h, matching the kinetic data of cell loss in Fig. 4. In parallel, there was a strong activation of caspase-3 (Fig. 5C) [25]. Importantly, there was no increase in Grp-78-expression.
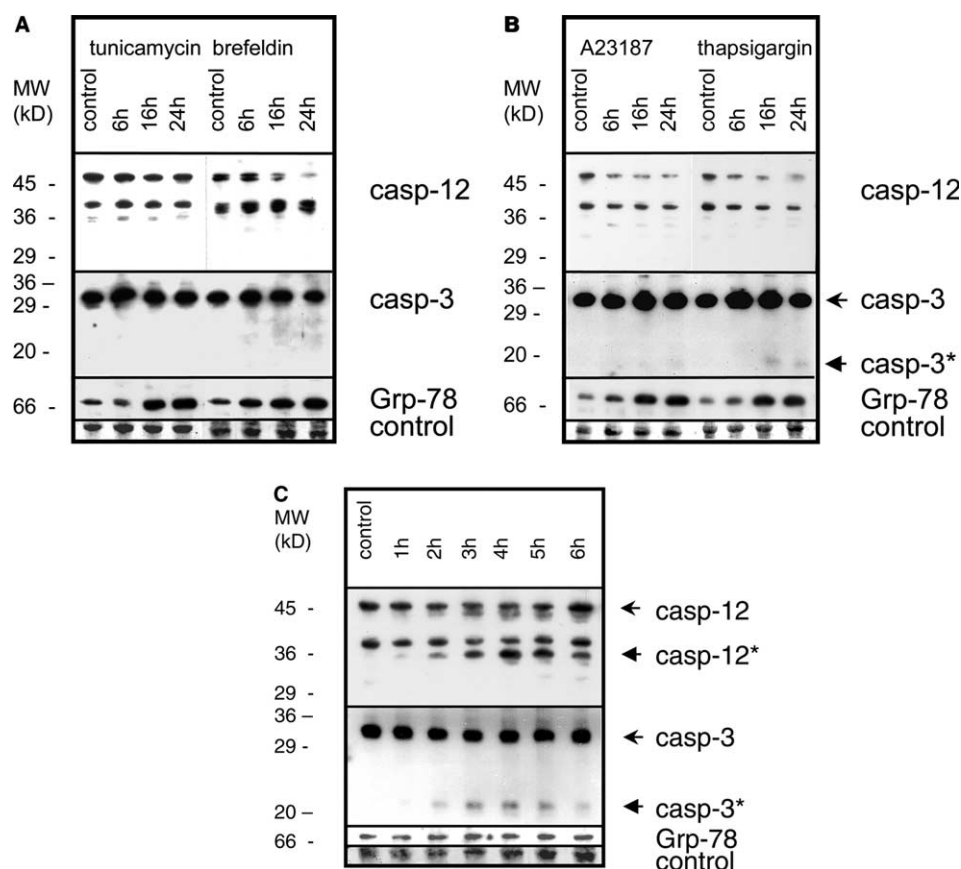

Fig. 5. Effects of ER-stressing reagents or serum starvation on the expression of Grp-78 and the cleavage of caspase-12 and -3. Confluent AKR-2B cells were treated with (A) 1 μM tunicamycin or 3 μM brefeldin (B) 3 μM A23187 or 0.5 μM thapsigargin or (C) were starved in MCDB 402 medium without serum. Cells were lysed by treatment with cytobuster. Western blotting and detection using antibodies against caspase-12, Grp-78 or caspase-3 were done as described in Section 2. * indicates cleavage products; ← uncleaved caspases and ◄, fragments of caspases. Control: staining with Amido Black (47 kD protein).

3.4. Assignment of cleavage sites in caspase-12

Fig. 6A shows a site by site comparison of caspase-12 fragments found in the cytosol of AKR-2B cells starved for 5 h, fragments generated by cleavage with caspase-3 in vitro in cytosolic extracts from non-starved cells and in vitro translated caspase-12 from AKR-2B cells. As markers, in vitro translated caspase-12 fragments G95-end and T159-end were included. Remarkably, in all instances a fragment comigrating with caspase-12 G95-end (37 kDa) and a faint band with a molecular weight of 48 kDa were observed. As shown in Fig. 6A, the full-length caspase-12 from AKR-2B cells is largely degraded, whereas the splice variant is less affected. In lysates, caspase-3 effectively digested the splice variant leading to an identical 37 kDa fragment (Fig. 6A), indicating that the cleavage site DEED is still accessible. Our data suggest that the full-length caspase-12 was more efficiently cleaved than the splice variant during serum starvation. There was no indication for a cleavage after K158, indicative for an action of calpain [7]. Only for in vitro translated caspase-12 treated with caspase-3, there was a low molecular weight fragment of 28 kDa indicative for an autoprocessing. In order to further assign the different fragments, caspase-12 and various mutants were expressed in vitro in an *E. coli*-based in vitro translation system. The advantage of this system is the high protein yield and the absence of endogenous caspases. Caspase-12 was detected by Western blotting. Besides the expected 50-kDa band, a 31-kDa species of variable intensity was seen in all experiments which most likely represents a fragment of caspase generated by using a downstream methionine-169 as translation starter (Fig. 6B). When in vitro synthesized caspase-12 was treated with active caspase-3, three new bands were observed (48, 37 and 28 kDa). Mutation analysis by replacing D24 or D94 into alanine identified D94 and D24 as cleavage sites for caspase-3 (Fig. 6B). In the mutant D94A, the fragment of 28 kDa was missing, suggesting that this fragment was generated by autocleavage of caspase-12. Therefore, a mutant C298A was generated. After treatment of the mutant protein with caspase-3, the 28-kDa band almost disappeared, indicating a predominant autocleavage. Importantly, in the mutant D94A caspase-3 treatment led only to a cleavage at D24 (48 kDa) without the generation of the 28-kDa fragment. Thus, for autoprocessing the removal of the entire N-terminal CARD-domain up to D94 appears to be necessary. Mutation analyses in the linker region between the large an the small subunit (D318A/D320A and D341A) revealed that D341 was the target for autocleavage. These data suggest that caspase-12 was indeed cleaved at D24 and D94 by caspase-3 in vivo in AKR-2B cells.

4. Discussion

Caspase-12 from AKR-2B cells contains three point mutations compared with the wild type (Fig. 2). As a consequence, a significant part of caspase-12 is localized in the cytoplasma. There was no indication for an attachment to the ER and ER stress induced by tunicamycin, thapsigargin, brefeldin and A23187 did not lead to a cleavage of caspase-12 in AKR-2B cells. In contrast, as shown previously, serum deprivation or anisomycin treatment induced cleavage of caspase-12 [25,26] and importantly, this cleavage paralleled the kinetic of apoptosis and caspase-3 activation (Figs. 4 and 5). During these both types of treatment the ER was not stressed, since there was no increase in the protein content of Grp-78 and the $[Ca^{2+}]$ homeostasis was not affected [26]. An obvious reason for this discrepancy is the binding of caspase-12 to high molecular weight complexes located in the cytosol, first described by our group, in AKR-2B cells. Similar to activation involving the ER, caspase-12 is released from this complex and low molecular weight fragments are recovered after gel chromatography in the region corresponding to a heterotetrameric protein [25,26]. The analysis of cleavage sites indicates a processing by caspase-3 (Fig. 6) which is the main executioner caspase in AKR-2B cells, since caspase-7 is not detectable in these cells [25]. Possibly, caspase-3 action leads to a displacement of caspase-12 from the high molecular weight complex, which is not consistent with a role of caspase-12 as an initiator for caspase-3. Also, the fact that caspase-3 and-12 are cleaved at the same time point does not favor a role as initiator caspase. On the contrary, in AKR-2B cells caspase-12 seems to be downstream of caspase-3 since in starved cells caspase-12 is apparently cleaved at postion D-94, which is the cleavage site for caspase-3. But on the other hand, there was no further processing in the intersubunit region leading to fragments ~28 kDa, though in vitro translated caspase-12 was able to autoprocess at D-341 after cleavage at D-94 by caspase-3. One explanation is the much higher concentration of caspase-12 in the in vitro translation assay. It has been proposed, based on in vitro and in vivo data, that caspase-12 initiates a caspase cascade by cleaving caspase-9 which then cleaves caspase-3 [9,18]. This pathway induced by ER-stress seems to be largely excluded in AKR-2B cells, since we did not observe cleavage of caspase-9 during apoptosis induced either by serum starvation or by treatment with anisomycin nor a recruitment into ap-
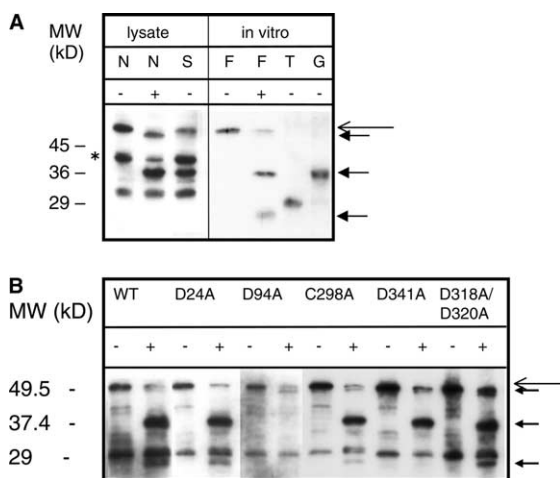

Fig. 6. Comparison of in vivo and in vitro caspase-12 fragmentation. (A) Lysates from non-starved cells (N) and from cells starved for 5 h (S) were separated on 12% SDS–polyacrylamide gels. If indicated (+), recombinant caspase-3 was added. For comparison, full-length caspase-12 (F), caspase-12 G95-end (G) or caspase-12 T159-end (T) was translated in vitro and, if indicated (+), treated with caspase-3. The 40-kDa band indicated by a star is the caspase-12 splice variant. (B) The cDNA coding for caspase-12 was cloned into the pET-3d or pET-21b vector, respectively, starting with a methionine. No tags were added. Mutated residues are indicated. The addition of recombinant caspase-3 is indicated by a +. Aliquots of about 3 μg of protein were subjected to SDS gel electrophoresis using a 12% gel. ←, uncleaved caspase-12 and ◄, fragments of caspase-12.

optosomes [25,26]. These cells have a peculiar pathway for the activation of caspase-3, which does not require the formation of an apaf-1/caspase-9/cyt c complex [25,26]. Interestingly, the existence of this pathway has been confirmed recently by using apaf-1$^{-/-}$ and caspase-9$^{-/-}$ cell lines [31]. But so far, AKR-2B cells are the only example where such a pathway is utilized though both apaf-1 and caspase-9 are present in the cell.

AKR mice show a high incidence for the development of leukemia [32–35]. Although this phenomenon has been attributed to the spontaneous expression of murine leukemia virus in these mice [36]], the altered location of caspase-12 described here may also contribute to the establishment of the above mentioned phenotype. AKR mice thus may serve as a mouse model to test the involvement of caspase-12 in various diseases. Interestingly enough, like in knockout mice [8] the effect of a different location of caspase-12 in AKR-2B cells was not lethal for the respective animals.

The nucleotide sequences are deposited under the number AY675224 for full-length caspase-12 and AY675223 for the splice variant from AKR-2B fibroblasts.

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