Identification of a new caspase homologue: caspase-14

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Abstract

Caspases are cysteinyl aspartate-specific proteinases, many of which play a central role in apoptosis. Here, we report the identification of a new murine caspase homologue, viz. caspase-14. It is most related to human/murine caspase-2 and human caspase-9, possesses all the typical amino acid residues of the caspases involved in catalysis, including the QACRG box, and contains no or only a very short prodomain. Murine caspase-14 shows 83% similarity to human caspase-14. Human caspase-14 is assigned to chromosome 19p13.1. Northern blot analysis revealed that mRNA expression of caspase-14 is undetectable in all mouse adult tissues examined except for skin, while it is abundantly expressed in mouse embryos. In contrast to many other caspase family members, murine caspase-14 is not cleaved by granzyme B, caspase-1, caspase-2, caspase-3, caspase-6, caspase-7 or caspase-11, but is weakly processed into p18 and p11 subunits by murine caspase-8. No aspartase activity of murine caspase-14 could be generated by bacterial or yeast expression. Transient overexpression of murine caspase-14 in mammalian cells did not elicit cell death and did not interfere with caspase-8-induced apoptosis. In conclusion, caspase-14 is a member of the caspase family but no proteolytic or biological activities have been identified so far. The high constitutive expression levels in embryos and specific expression in adult skin suggest a role in ontogenesis and skin physiology.

Keywords: caspase; cloning; apoptosis; ICE; CED-3; cell death

Abbreviations: DD, death domain; d.p.c., days post coitus; EST, expressed sequence tag

Introduction

Members of the caspase protein family are key mediators in the execution of apoptotic cell death. Specific inhibition of one or more caspases by CrmA, p35 or the peptide derivatives Ac-YVAD-CHO and Ac-DEVD-CHO revealed that several caspases are involved in apoptosis mediated by stimulated Fas or 55-kDa tumor necrosis factor receptor (Los *et al*, 1995; Bertin *et al*, 1997; Nagata, 1997). Both receptors transduce the death signal through a cytoplasmic sequence motif called 'death domain' (DD). After receptor trimerization this domain rapidly associates with a similar DD in the adapter molecules TRADD and/or FADD. The N-terminal end of FADD exhibits significant sequence homology to two similar regions within the prodomain of caspase-8 and caspase-10, referred to as death effector domains, which allow heterodimerization of these caspases with FADD (Boldin *et al*, 1996; Muzio *et al*, 1996; Vincenz and Dixit, 1997). In the case of Fas, it has been demonstrated that receptor occupation results in FADD-mediated recruitment of caspase-8 into the receptor complex. Receptor-associated caspase-8 is then proteolytically cleaved to generate active caspase-8 (Yang *et al*, 1998). Once caspase-8 has become activated, it might initiate a proteolytic caspase activation cascade, since it is able to process, at least *in vitro*, all known caspases into their active subunits (Srinivasula *et al*, 1996). Activated caspases are believed to be the executors of cell death by aspartate-specific proteolysis of substrates, which results in the characteristic features of apoptosis, such as DNA degradation, nuclear condensation and membrane blebbing (Villa *et al*, 1997).

Here we report the identification of a new member of the caspase gene family, viz. caspase-14, which might be involved in ontogenesis and skin physiology.

Results and Discussion

Identification of murine and human caspase-14

TBLASTN homology searches in the expressed sequence tag (EST) database, with human caspases as a query, revealed two murine ESTs coding for an unidentified caspase-like protein, viz. the 15.5 days post coitus (d.p.c.), embryonal-derived cDNA clone 555962, and the 11-week adult mouse whole skin-derived cDNA clone 607978. Sequencing of these cDNA clones revealed that both encode the same open reading frame and contain an in-frame stop codon 5' of the start codon (data not shown). This suggests that these cDNAs span the full open reading frame. Considering that the prodomain of caspases is removed from the p20 subunit by cleavage after Asp, murine caspase-14 may contain either no prodomain or a prodomain of 7 (D₇) or 17 (D₁₇) amino acids. However, based on multiple sequence alignment (data not shown) the Tyr residue at position 16 (Y₁₆) is conserved in the large subunit of all known mammalian caspases. If there is a

A

mCASP-14	1	MESEBMSDPQPLQERY...DMSG
hCASP-14	1	MDDEFRENITEKY...DMSG
hCASP-1	81	SYLAGTLGLSADQTSQVLSFFPAPQAVQDNFAMPTSSGSEGNVLCSEAEQRINRQKSAETYPIMQKSS
mCASP-14	21	ARLALLG.....VTKAREGSEVDMALERMFRNKKFESTMKRDPTAQOFLFELDFFOOTIDNNEEPVSCAFVVLMAH
hCASP-14	17	ARLALLIG.....VTKAREGSEVDMALERMFRNKKFESTMKRDPTAQOFLFELDFFOOTIDNNEEPVSCAFVVLMAH
hCASP-1	161	RTRLALLIQNEEFDSIPRRTISAEVDITGIMMLONTISVSVLTKNLTASDMTTELEPAHRRPHNTSD.SSTFLVEMSH
mCASP-14	94	GFEGLL...K.GEDLKKVRLLEDLFVLINNKNCALRQKPKVYIIQACRGEDPGEELRGNEELGGDEELGGDEVAVLN
hCASP-14	90	GFEGLL...K.GEDLKKVRLLEDLFVLINNKNCALRQKPKVYIIQACRGEDPGEELRGNEELGGDEELGGDEVAVLN
hCASP-1	238	GIREGICGKHHSEVPEITLGLNATINMLNTEKNCESLKKPKVYIIQACRGD...SPG.VVWFKQSVGSGNLSLPTTEEFE
mCASP-14	169	NNFQSITYTDTLHIYSTVEGYLSYRHDEKSGSEFIQTLDVETHKKGSILELLEETITRLMA.NTEVMQEGKPRKVNPEV
hCASP-14	154	DSPQOTITYTDTLHVYSTVEGYIAYRHDKSGSEFIQTLDVETHKKGSILELLEETITRLMA.EAEVLQEGKARKNTDEI
hCASP-1	315	DDAKKAHLERDIAAFCSSTPDNVSWRHPTMGSMFISRLIE.HMQEYACSDVVEELERKVRFSSEFQPDGRAQMPTE
mCASP-14	247	QSTLRKKLYLQ*~
hCASP-14	232	QSTLRKKLYLQ*~
hCASP-1	391	RVTLRCEYLFPGH

B

Figure 1 Analysis of caspase-14 sequences and their relationship with the caspase family. **(A)** Alignment of the amino acid sequence of murine and human caspase-14 with human caspase-1. The murine caspase-14 sequence was obtained by sequencing the EST clones 555962 and 607978. The sequence of human caspase-14 is predicted from cosmid R31973 (Ac004699) by the GENSCAN computer program. Identical and similar amino acids are boxed in black and gray, respectively. ♦, amino acids aligning with the residues in human caspase-1 which form the binding pocket for P1 Asp. ●, amino acids involved in catalysis. ■, Y₁₆ believed to belong to the p18 subunit. ▼, D₇ and D₁₇ define a putative prodomain and a less likely alternative, respectively. Brackets indicate both EELGGDE heptapeptides containing the presumed cleavage sites between p18 and p11 subunits of murine caspase-14. In human caspase-14 only one of these potential cleavage sites is conserved. **(B)** Phylogenetic comparison of human and murine caspases. The amino acid sequences were aligned using the Genetics Computer Group (Madison, WI, USA) PILEUP algorithm. This alignment was inserted into the CLUSTAL W program (Thompson *et al*, 1994) to generate the dendrogram. Bootstrap values are indicated at the fork of each branch. The well-defined caspase-1 and caspase-3 subfamily members are encircled. Note that murine caspase-11 is presumably the homologue of human caspase-4 (TX) (Van de Craen *et al*, 1997a)

B

Figure 2 Polypeptide length verification of murine caspase-14. (A) *In vitro* transcription and translation of murine caspase-14 clones in reticulocyte lysate in the presence of ^{35}S -methionine for 90 min at 30°C . $1\ \mu\text{l}$ of this mixture was loaded on a 15% SDS-polyacrylamide gel. M, M_r marker. (B) Detection of E-tagged caspase-14 in HEK293T lysates. HEK293T cells were transiently transfected with E-tagged murine caspase-1 or murine caspase-14. The lysates were prepared 30 h later, fractionated by SDS-PAGE, electroblotted and revealed with an anti-E-tag antibody

Figure 3 Expression pattern of murine caspase-14. (A) Mouse multiple tissue Northern blot hybridized sequentially with murine caspase-14 or murine caspase-7 cDNA. Each lane contains $2\ \mu\text{g}$ poly(A) $^+$ RNA. (B) Whole mouse embryo Northern blot hybridized with murine caspase-14, -3 or -8 cDNA. Each lane contains $2\ \mu\text{g}$ poly(A) $^+$ RNA of whole embryos in different stages of development, as shown on top. (C) Reverse transcriptase PCR on total RNA derived from murine adult testis, 12 d.p.c. murine embryo, 16 d.p.c. murine embryo and murine adult skin. At the top, amplifications performed with caspase-14 specific primers. At the bottom, amplifications performed with actin specific primers

prodomain, D₇ is the most likely candidate. Three Asp residues (D₁₄₂, D₁₅₆ and D₁₆₂) are potential cleavage sites between a p18 and a p11 subunit. His₉₃, Gly₉₄ and Cys₁₃₆, homologous to the catalytically important residues of human

caspase-1, are conserved (Walker *et al*, 1994; Wilson *et al*, 1994) (Figure 1A). The amino acids Arg₃₃, Gln₁₃₄, Arg₂₉₄ and Ser₂₀₀, constituting the Asp-binding pocket, are the same as in human caspase-1 (Walker *et al*, 1994; Wilson *et al*, 1994)

Figure 4 *In vitro* cleavage of ³⁵S-methionine-labeled His₆-tagged murine caspase-14 by murine caspase-8. (A) 5 μ l of *in vitro* labeled, purified His₆-tagged murine caspase-14 was incubated with 30 μ g lysate of *Escherichia coli* transfected with pLT-mCASP-8 plasmid. Lysate of bacteria transfected with empty pLT10TH served as a control. The resulting cleavage products are shown on the right. ■, His₆ tag of 2 kDa; T, theoretically calculated *M_r*; Exp, experimentally found *M_r*. (B) A 1 : 5 serial dilution was made starting with 30 μ g lysate of bacteria expressing pLT-mCASP-8 (= 1). Each dilution was incubated with 5 μ l of labeled, purified His₆-tagged murine caspase-14 or 1 μ l of radiolabeled murine caspase-3. Reaction products were separated on 15% SDS-PAGE, followed by autoradiography to reveal cleavage fragments. Uncleaved products are indicated by open arrowheads; resulting cleavage fragments are shown by closed arrowheads. M, *M_r* marker

(Figure 1A) and other murine and human caspases (Van de Craen *et al*, 1997a). These features suggest that caspase-14, like caspase-1, exerts a cysteine protease activity upon activation.

A TBLASTN homology search in the EMBL database, with murine caspase-14 as a query, revealed that human cosmid R31973 (Ac004699) contains a gene with high homology to murine caspase-14. Assembly of the exons of this gene disclosed a putative coding sequence of human caspase-14. The theoretical open reading frame of human caspase-14 shows 72% identity and 83% similarity to murine caspase-14 (Figure 1A and B). Human cosmid R31973 was assigned to chromosome 19p13.1 (Ac004699). Figure 1B shows that murine and human caspase-14 are relatively more related to human caspase-9 and human/murine caspase-2, and that they are no members of the caspase-3 or caspase-1 subfamilies. This suggests that, besides the well-defined caspase-1 and caspase-3 subfamilies, a third group of proteins (human caspase-9, human/murine caspase-2 and human/murine caspase-14) is emerging, with relatively low homology to each other. Therefore, it is doubtful whether they can be considered one subfamily.

In order to verify whether the theoretical length of the murine open reading frame corresponds with the M_r of the protein coded for by the cDNA, both clones 607978 and 555962 were *in vitro* transcribed and translated in the presence of ^{35}S -methionine. The resulting products were analyzed on 15% SDS-PAGE. As shown in Figure 2A, both murine caspase-14-encoding clones generated a product of approximately 31 kDa (the theoretical calculated M_r is 29.5), indicating that the presumed start codon is used. In order to confirm that murine caspase-14 is correctly expressed in eukaryotic cells, it was elongated at its C-terminus with an E-tag (pCDNA-mCASP-14E). This expression plasmid was transiently transfected in HEK293T cells and the product length of the heterologously expressed protein was verified by Western blotting with an anti-E-tag antibody. E-tagged murine caspase-1 expression was included as a positive control. As expected, murine caspase-14 and murine caspase-1 were expressed as proteins of 33 kDa and 46 kDa, respectively (Figure 2B).

Expression pattern of murine caspase-14

In two independent experiments a multiple adult tissue Northern blot was hybridized with the 1719 bp *EcoRI* fragment of clone 607978. No hybridization was observed with this caspase-14 probe (Figure 3A). The quality of the blot was checked using a murine caspase-7 cDNA probe. A murine caspase-7 transcript of 2.5 kb was clearly detectable (Figure 3A) and the expression pattern was similar to that found previously (Van de Craen *et al*, 1997a). It can be concluded that murine caspase-14 transcripts, if any, were below the detection limit in these adult tissues examined. Since clone 555962 was isolated from a mouse embryo cDNA library, we examined whether murine caspase-14 mRNA was expressed in mouse embryos. A Northern blot was hybridized containing poly(A)⁺ RNA from 7, 11, 15 and 17 days old, whole embryos (Figure 3B). The murine caspase-14 probe

clearly recognized a transcript of 2.5 kb. The murine caspase-14 mRNA expression increased during maturation of the embryo (Figure 3B). Remarkably, the 11-day old embryo did not show expression of caspase-14 mRNA. Hybridization of the same blot with a caspase-3 or a caspase-8 probe suggests that all lanes contain equal amounts of poly(A)⁺ RNA (Figure 3B). Because the caspase-14 transcript size, which includes a large poly(A)⁺ tail, was consistent with the length of both cDNA clones (2203 and 2213 bp), the latter presumably contained an insert corresponding to the major caspase-14 mRNA species. A weaker band was detected at 4.5 kb and possibly represents incompletely or alternatively processed mRNA. Since clone 607978 was isolated from a mouse skin-derived cDNA library, the expression of caspase-14 transcripts was examined in adult mouse skin by reverse transcriptase PCR (RT-PCR). Figure 3C demonstrates that a caspase-14 cDNA fragment could be amplified from RNA prepared from adult mouse skin (lane 4), while RT-PCR performed on RNA from adult mouse testis did not result in amplification of the caspase-14 cDNA fragment (lane 1). To confirm the apparent absence of caspase-14 transcripts around day 11 of development, additional amplifications were performed on RNA isolated from 12 and 16 d.p.c. mouse embryos. Indeed, caspase-14 could be amplified from RNA derived from 16 d.p.c. embryos but not from RNA derived from 12 d.p.c. embryos (Figure 3, lanes 2 and 3). Amplification of an actin cDNA fragment was identical for all RNA preparations (Figure 3C). In summary, the expression of caspase-14 transcripts increases during maturation of a mouse embryo but drops temporarily to an undetectable level around 11–12 d.p.c. Additionally, the expression pattern in adult tissues is very restricted since only skin expressed caspase-14 transcripts among the adult tissues examined.

Murine caspase-14 is a weak substrate of caspase-8, but is no substrate of caspase-1, caspase-2, caspase-3, caspase-6, caspase-7, caspase-11 or granzyme B

Members of the caspase family are activated by cleavage at Asp residues to generate p20 and p10 subunits which constitute the active tetrameric enzyme (p20₂/p10₂) (Walker *et al*, 1994; Wilson *et al*, 1994; Nicholson *et al*, 1995). Since proforms of caspases are often substrates for active p20₂/p10₂ caspases (Srinivasula *et al*, 1996) or granzyme B (Darmon *et al*, 1995; Van de Craen *et al*, 1997b), we examined whether caspase-14 was proteolytically cleaved by these proteases. Therefore, ^{35}S -methionine-labeled caspase-14 was incubated with purified murine caspase-x (x = 1, 2, 3, 6, 7 or 11) or purified murine granzyme B. However, none of these active enzyme preparations was able to cleave caspase-14 (results not shown).

Because purified, enzymatically active murine caspase-8 was not available, a lysate of *Escherichia coli*-expressing pLT-ΔpromCASP-8 was used to examine whether murine caspase-8 exhibited proteolytic activity on *in vitro* radio-labeled and partially purified His₆-tagged murine caspase-14. The His₆-tagged murine caspase-14 migrated about 4 kDa slower on SDS-PAGE as compared to non-tagged caspase-14 (the epitope tag adds 2 kDa, resulting in a

theoretical 31.4 kDa). Figure 4A shows that murine caspase-8 cleaved this 35.5 kDa murine caspase-14 weakly into 24 kDa (p18) and 11 kDa (p11) fragments. These products suggest cleavage at D₁₅₆ and/or D₁₆₂. Since these Asp residues are both embedded in an identical, repeated EELGGDE heptapeptide (Figure 1A), it is quite possible that cleavage occurs at both sites. In human caspase-14 only one of these sites is conserved (Figure 1A). It should be noted that the slower migration of the full-length His₆-tagged murine caspase-14 was largely due to the charged His₆ tag, since the p18 fragment also runs approximately 4 kDa slower than theoretically expected (Figure 4A).

To investigate the efficiency of caspase-8-mediated cleavage of caspase-14, a 1/5 serial dilution of lysate from murine caspase-8-expressing bacteria was incubated with radiolabeled His₆-tagged murine caspase-14 or radiolabeled murine caspase-3 for 1.5 h at 37°C. The resulting products were analyzed by SDS-PAGE and autoradiography. Figure 4B clearly demonstrates that proteolytic activity of caspase-8 was at least 1000 times more efficient on caspase-3 than on caspase-14. Consequently, it is quite possible that other proteases may process murine caspase-14 more efficiently than caspase-8 or that caspase-14 activation involves unknown mechanisms.

Bacterial expression of murine caspase-14

Bacterial expression of caspases lacking their prodomain (p30) usually results in the generation of p20 and p10 domains in the soluble fraction of the bacterial extract (Molineaux *et al*, 1993; Kamens *et al*, 1995). Three different pLT10TH constructs were made to generate active murine caspase-14 enzyme. One plasmid contained the full-length murine caspase-14 coding sequence (pLT-mCASP-14). Furthermore, two constructs were generated in which a putative prodomain was removed (pLT-Δ₁₋₇mCASP-14 and pLT-Δ₁₋₁₇mCASP-14). To facilitate the purification of the proteins, an N-terminal polyhistidine sequence (His₆ tag) was fused to the murine caspase-14 sequences. The same conditions for bacterial expression were used as for the generation of other prodomain-deleted murine caspases. These conditions generated active caspase-1, caspase-2, caspase-3, caspase-6, caspase-7, caspase-8 and caspase-11 (Van de Craen *et al*, in preparation). All caspase-14 constructs were well expressed, but the product was predominantly present in insoluble inclusion bodies. No p18 or p11 subunits were detectable after bacterial expression; neither in the soluble nor in the insoluble fractions was aspartase activity detected using Ac-DEVD-AMC (100 μM), Ac-YVAD-AMC (100 μM) or z-VAD-AFC (100 μM) fluorogenic peptide caspase substrates. This was also the case after 30 min preincubation of the soluble fraction at 37°C, which might have allowed an autocatalytic generation of caspase activity (Ramage *et al*, 1995). Also denaturation and refolding of the insoluble fractions of murine caspase-14 or separate expression of the subunits did not generate activity on the fluorogenic tetrapeptide substrates (data not shown).

Murine caspase-14 does not autoprocess in yeast

We have previously successfully used a yeast expression system to demonstrate autoprocessing of murine caspase-1 (Van Crielinge *et al*, 1996). Since bacterial expression of murine caspase-14 suggests the inability of this caspase to autoprocess, the autocleavage activity of murine caspase-14 was tested in a yeast expression system. pAS2 plasmids encoding the Gal4 DNA-binding domain (Gal4DB) fused to murine caspase-1, murine caspase-1(C285S), murine caspase-14 or murine caspase-14(C136S) were transformed in *Saccharomyces cerevisiae* strain YRG-2. Single yeast colonies were used to prepare lysates for Western blot analysis with antibodies against Gal4DB. However, also in yeast no autoprocessing activity could be detected for wild-type murine caspase-14 (Figure 5). Transformations with caspase-1 or the inactive Cys mutant CASP-1(C285S) fused to Gal4DB were used as controls and confirmed that the p10 subunit of caspase-1 was cleaved off without involvement of endogenous yeast proteases (Figure 5). These results indicate that murine caspase-14, in contrast to murine caspase-1, is not able to autoprocess in *S. cerevisiae*, which might contain eukaryotic helping factors. However, it should be kept in mind that caspase-14 contains no large prodomain that can be involved in oligomerization-induced autoactivation, as has been demonstrated for caspase-1 (Van Crielinge *et al*, 1996) and caspase-8 (Yang *et al*, 1998).

Figure 5 Murine caspase-14 does not autoprocess in *S. cerevisiae*. Lysates of *S. cerevisiae* strain YRG-2, transformed with pAS2-encoding Gal4DB fusions with full-length murine caspase-1, murine caspase-1(C285S), murine caspase-14 or murine caspase-14(C136S), were subjected to SDS-PAGE, electroblotted and revealed with anti-Gal4DB antibody. In the lane at the right hand side, the cleavage product of the Gal4DB fusion with murine caspase-1 is derived from proteolysis at the C-terminal p10 domain of murine caspase-1

Transiently transfected murine caspase-14 exhibits no evident apoptotic-related activity in mammalian cells

Caspase-14 was examined for its capacity to induce cell death by transient overexpression in HeLaH21 cells. These cells were transfected with three different vectors expressing caspase-14, *viz.* pCDNA-mCASP-14E (E-tagged caspase-14), pCAG-mCASP-14 or pCAG-mCASP-14C136S. The extent of cell death in the transfected population was measured by the decrease in number of adherent β Gal-expressing cells as revealed using an XGal substrate. A pCDNAI-derived expression vector coding for FADD was used as a positive control. Figure 6A shows that transient overexpression of the FADD-containing plasmid caused a profound reduction in number of β Gal-expressing HeLaH21 cells as compared to the negative control (pCDNAI). Transient overexpression of the different murine caspase-14-encoding constructs did not result in a clear reduction in number of adherent blue cells (Figure 6A), suggesting that murine caspase-14 itself is not capable to induce apoptosis. Similar results were obtained in HEK293T cells.

Figure 6 Murine caspase-14 has no clear function in apoptosis. (A) HeLaH21 cells were cotransfected with pUT651, pCDNA-FADD, pCDNA-mCASP-14E, pCAG-mCASP-14, pCAG-mCASP-14C136S or empty pCDNAI. After 24 h, cells were stained with XGal and counted. No clear apoptotic morphology was observed after transfection with a caspase-14-expressing construct. (B) HEK293T cells were transfected with 200 ng pUT651 (for visualization of transfected cells), 50 ng pCDNA-mCASP-8 (for induction of cell death) and 600 ng pCAG-mCASP-14, pCAG-mCASP-14C136S, pCAG-CrmA or empty pCDNAI. Controls included experiments with 50 ng pCDNAI instead of pCDNA-mCASP-8. After 24 h, cells were stained with XGal. Since HEK293T cells were very efficiently transfected, the percentage of blue apoptotic cells was determined

With FLIP_L (Irmeler *et al*, 1997) as an example, it is conceivable that also caspase-14 exerts apoptosis-inhibiting features. Another link to a potential caspase-14-interfering activity is the low protease activity of caspase-8 on caspase-14 as revealed by *in vitro* cleavage assays. Since Western blot analysis revealed that murine caspase-14 is well expressed in HEK293T cells, and since murine caspase-8 induced clear apoptosis in this cell line (Van de Craen *et al*, unpublished observations), the potential effect of murine caspase-14 on murine caspase-8-induced apoptosis was examined in HEK293T cells by transient cotransfection experiments. Cotransfection of caspase-8 with CrmA was applied as a positive control for inhibition. Figure 6B demonstrates that caspase-8-induced cell death was not influenced by cotransfection with caspase-14 or caspase-14(C136S), while CrmA inhibited cell death significantly. Also dilution series of a murine caspase-8-encoding plasmid, cotransfected with caspase-14-expressing constructs, did not reveal any effect of caspase-14 (data not shown). These data indicate that caspase-14 does not interfere negatively with apoptosis, at least when cell death is induced by caspase-8.

In conclusion, we have identified a new caspase homologue, caspase-14. Although the amino acid sequence would classify murine caspase-14 as a *bona fide* cysteine aspartase, no aspartase activity could be revealed. Further experiments, such as generation of caspase-14-deficient mice and *in situ* hybridizations, will be needed to assign proper biological functions to this new caspase homologue. Nevertheless, the expression of caspase-14 mRNA in whole embryos and in adult skin suggests a role in ontogenesis and skin physiology.

Materials and Methods

Cloning of murine caspase-14

In order to identify new murine caspases, TBLASTN searches for homology with known human caspases were performed on an EST database. Two new murine cDNA clones with significant caspase homology were identified, *viz.* clones 555962 and 607978. The physical cDNA clones were obtained from the I.M.A.G.E. consortium (Washington University School of Medicine, St. Louis, MO, USA; Lennon *et al*, 1996). Sequencing of the entire inserts on an ABI373A sequencer (Applied Biosystems, Foster City, CA, USA) revealed that clones 555962 and 607978 encoded the same full open reading frame, called caspase-14.

Identification of human caspase-14

A TBLASTN search in the EMBL database, with murine caspase-14 as a query, revealed that cosmid R31973 (Ac004699) contains the human caspase-14 gene. The GENSCAN computer program was applied to deduce the coding sequences. GENSCAN predicts that the human caspase-14-coding sequence is 729 bp long and encodes a protein of 242 amino acids. The theoretical *M_r* is 27.7.

Plasmid constructions

Clone 555962 was used as a template to amplify caspase-14 with the PCR primers GCGAAGCTTCCACCATGGAGTCAGAGATGAGT-

GATCCT and GGGAGAAGCGGCCGCTTGCAAATAGAGCTTCTTCC. The *HindIII/NotI*-digested amplicon was provided with a C-terminal E-tag in a modified pCDNAI vector (Invitrogen, San Diego, CA, USA) to generate pCDNA-mCASP-14E. To construct pCAG-mCASP-14, murine caspase-14 was amplified with the primers GCGGATATCCAC-CATGGAGTCAGAGATGAGTGATCCT and GCGGATATCTTATTG-CAAATAGAGCTTCTTCC. Then the *EcoRV*-digested amplicon was ligated in a *Ball*-opened pCAGGS expression vector (Niwa *et al*, 1991). The cowpox CrmA gene (a generous gift from Dr. D. Pickup, Durham, NC, USA) was *EcoRI*-cloned in pCAGGS, resulting in pCAG-CrmA. The bacterial expression vectors for caspase-14 were constructed by PCR with GCGGATATCCATGGAGTCAGAGATGAGTGATCCT (F1), GCGGATATCACCTCAGCCATTGCAAGAGGAAAGA (F2), GCGGATATCCATGTGAGGTGCCCGCCTGGCCCTGACG (F3) and GCGGATATTCGATATCTTATTGCAAATAGAGCTTCTTCC (R). Primers F1 and R amplified full-length caspase-14, primers F2 and R created the putative prodomain-deleted Δ_{1-17} mCASP-14, and primers F3 and R amplified Δ_{1-17} mCASP-14. The products were *EcoRV*-cloned into a pLT10TH vector (Mertens *et al*, 1995), resulting in pLT-mCASP-14, pLT- Δ_{1-17} mCASP-14 and pLT- Δ_{1-17} mCASP-14, respectively. The caspase-14 inserts were N-terminally fused to a His₆ tag to facilitate purification. The same cloning strategy with the primers GCGGATATC-CAGTGAGTCACGGACTTCAGACAAAG and GCGGATATCGAATTC-TATTAGGGAGGGAAGAAGAGCTTC (and EST clone 533745 as template DNA) was applied to generate the bacterial expression vector for prodomain-deleted murine caspase-8, viz. pLT- Δ promCASP-8.

Yeast murine caspase-14 expression plasmids were also constructed by PCR. The primers used were: AAAGAGATC-GAATTCGCCATGGAGTCAGAGATGAGTGATCCTC (YF), ATAGATCTCTGCAGGTGACGTTATTGCAAATAGAGCTTCTTCCG-GAG (YR), CATCCAGGCTAGTAGAGGAGAGCACAGAG (YCSF) and CTCTGTGCTCTCTCTACTAGCCTGGATG (YCSR). Primers YF and YR were used to generate wild-type caspase-14. The four primers were applied to mutate the active Cys residue to Ser (C136S). All amplicons were *NcoI/SalI*-inserted in pAS2, to result in pASmCASP-14 and pASmCASP-14C136S. The Gal4DBp45ICE-C285S plasmid (Van Crielinge *et al*, 1996) was renamed pASmCASP-1C285S.

Northern blot analysis

A multiple tissue Northern blot of eight different mouse adult tissues and a Northern blot containing poly(A)⁺ RNA from four mouse embryos (7, 11, 15 and 17 days old) were purchased from Clontech Laboratories (Palo Alto, CA, USA). Sequential hybridization and stripping were performed according to the manufacturer's instructions.

Reverse transcriptase PCR

Reverse transcriptase reactions on total RNA isolated from murine adult skin, murine adult testis, 12 d.p.c. murine embryos and 16 d.p.c. murine embryos were performed by the superscript preamplification system for first-strand cDNA synthesis (Gibco BRL, Rockville, MD, USA). The primers GCGGATATCTGAGGTTGCTGTGCTCAAGAA-CAACC and GCGGAATTCGATATCTTATTGCAAATAGAGCTTCT-TCC were used to amplify a caspase-14 cDNA fragment of 331 bp, while the primers GCTACCATGGATGATGATATCGCC and GGATGCCTCTCTTGTCTGGGCTC were applied for amplification of an actin cDNA fragment of 199 bp.

Cell lines and transient transfections

HeLaH21 is a human cervix carcinoma derived from HeLa cells. HEK293T is a human embryonal kidney carcinoma stably transfected

with the SV40 T-antigen, having a transfection efficiency of >40% (a generous gift of Dr. M. Hall, University of Birmingham, UK; DuBridge *et al*, 1987). In order to assess the size of epitope-tagged murine caspase-14, 5×10^5 HEK293T cells/6-well were transiently transfected using the calcium phosphate precipitation method (O'Mahoney and Adams, 1994). A total amount of 1 μ g plasmid DNA was used. Cell lysates were prepared with lysis buffer containing 50 mM Tris pH 8.0, 300 mM NaCl, 5 mM EDTA, 15 mM MgCl₂, 1% NP-40, 1 mM PMSF, 50 μ M leupeptin and 20 μ g/ml aprotinin. 90 μ g of total protein was used for Western analysis with anti-E-tag antibody (Pharmacia Biotech, Uppsala, Sweden).

In order to evaluate the apoptotic activity of caspases, 5×10^5 HeLaH21 cells/6-well were transiently transfected with DOPE transfection reagent according to the manufacturer's instructions (Eurogentec, Seraing, Belgium). A total of 2 μ g DNA, 50% pUT651 (Cayla, Toulouse, France) and 50% vector of interest [pCAG-mCASP-14, pCAG-mCASP-14C136S, pCDNA-mCASP-14E, pCDNA-FADD or empty pCDNAI vector] was used. After 24 h, the cells were tested for cell death by a decrease in number of positive cells using XGal substrate. This β Gal assay was performed as described previously (Van de Craen *et al*, 1997a).

In order to assay the effect of murine caspase-14 on murine caspase-8-induced apoptosis, 5×10^5 HEK293T cells/6-well were transiently transfected using the calcium phosphate precipitation method (O'Mahoney and Adams, 1994). Three plasmids were transfected simultaneously: 200 ng pUT651, 50 ng pCDNA-mCASP-8 (which contains full-length murine caspase-8; Van de Craen *et al*, unpublished observations) and 600 ng pCAG-mCASP-14, pCAG-mCASP-14C136S, pCAG-CrmA, or empty pCDNAI. Controls included experiments with empty pCDNAI instead of pCDNA-mCASP-8.

In vitro cleavage assays

³⁵S-methionine-labeled substrates were generated by *in vitro* transcription and translation as previously described (Van de Craen *et al*, 1997a). A lysate of bacteria expressing murine caspase-8 was used as a source for this enzyme. 30 μ g total lysate was incubated with 5 μ l purified, labeled His₆-tagged murine caspase-14 in 20 μ l caspase buffer for 90 min at 37°C. Radiolabeled His₆-tagged murine caspase-14 was obtained by *in vitro* transcription/translation of the pLT-mCASP-14 plasmid and purified using a Co²⁺ resin (Talon; Promega Biotec, Madison, WI, USA). The substrate specificity of murine caspase-8 proteolytic activity was determined by making a 1/5 serial dilution of total lysate of murine caspase-8-expressing bacteria starting with 30 μ g protein. These dilutions were incubated with 5 μ l purified, radiolabeled, His₆-tagged caspase-14 or 1 μ l radiolabeled caspase-3 in a total volume of 20 μ l for 90 min at 37°C.

Bacterial expression

pLT10-mCASP-14, pLT- Δ_{1-17} CASP-14 and pLT- Δ_{1-17} CASP-14 were transformed in MC1061 bacteria containing a pICA2 plasmid (Mertens *et al*, in preparation). A 50 ml Luria broth culture of A₆₀₀=0.5 was induced overnight with 1 mM IPTG at 20°C. The cells were harvested by centrifugation. MC1061 cell pellets were suspended in 1 ml bacterial lysis buffer (20 mM Tris-HCl pH 7.5, 10% glycerol, 1 mM oxidized glutathione, 1 mM PMSF, 50 μ M leupeptin and 20 μ g/ml aprotinin) and lysed by sonication. The soluble and insoluble fractions were separated by centrifugation for 20 min at 16 000 $\times$ g. The expressed proteins were analyzed by 12.5% SDS-PAGE and Coomassie blue staining. The same procedure was followed for pLT- Δ promCASP-8.

Autocleavage in yeast

These experiments were performed as previously described (Van Criekeing *et al*, 1996), except that the YRG-2 yeast strain (Stratagene Cloning Systems, La Jolla, CA, USA) was used instead of HF7c.

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