Characterization of seven murine caspase family members

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Abstract Seven members of the murine caspase (mCASP) family were cloned and functionally characterized by transient overexpression: mCASP-1 (mICE), mCASP-2 (Ich1), mCASP-3 (CPP32), mCASP-6 (Mch2), mCASP-7 (Mch3), mCASP-11 (TX) and mCASP-12. mCASP-11 is presumably the murine homolog of human CASP-4. Although mCASP-12 is related to human CASP-5 (ICE_{rel}-III), it is most probably a new CASP-1 family member. On the basis of sequence homology, the caspases can be divided into three subfamilies: first, mCASP-1, mCASP-11 and mCASP-12; second, mCASP-2; third, mCASP-3, mCASP-6 and mCASP-7. The tissue distribution of the CASP-1 subfamily transcripts is more restricted than that of the CASP-3 subfamily transcripts, suggesting that the transcriptional regulation of the CASP members within one subfamily is related, but is quite different between the CASP-1 and the CASP-3 subfamilies. Transient overexpression of each of the seven CASPs induced apoptosis in mammalian cells. Only two, mCASP-1 as well as mCASP-3, were able to process precursor interleukin (IL)-1β to biologically active IL-1β. In addition, mCASP-3 is the predominant PARP-cleaving enzyme in vivo.

Key words: Caspase; Interleukin-1; Family PCR cloning; Tissue expression

1. Introduction

Apoptosis or programmed cell death is an essential process during normal development and homeostasis of a multicellular organism [1–4]. Recently, genes involved in apoptosis of particular cell types in the nematode *Caenorhabditis elegans* have been characterized. Both positive effectors (CED-3 and CED-4) and a negative regulator (CED-9) have been identified [5,6]. Several human homologs of CED-3 and CED-9 have been described, while so far a vertebrate homolog of CED-4 has not been found. The first identified mammalian homolog of CED-3 was interleukin-1β-converting enzyme (ICE [7,8]), a cysteine protease which processes inactive pro-interleukin-1β (pIL-1β) to its biologically active form [9]. Recently, several human (h) and murine (m) ICE or caspase (CASP) homologs have been cloned [10–12]. In the human system, CASP-1 (ICE [13]), CASP-2 (Ich1 [14]), CASP-3 (CPP32, Yama or apopain [15–17]), CASP-4 (TX, Ich2 or ICE_{rel}-II [18–20]), CASP-5 (ICE_{rel}-III or TY [20,21]), CASP-6 (Mch2 [22]), CASP-7 (Mch3, ICE-LAP3 or CMH-1 [23–25]), CASP-8 (MACH, FLICE or Mch5 [26–28]), CASP-9 (ICE-LAP6 [29]) and CASP-10 (Mch4 [26]) were identified. In the mouse, only the molecular cloning of CASP-1 [30,31], CASP-2 (NEDD-2 or Ich1 [32]) and CASP-11 (Ich3 [33]) has been reported to date. These CASP proteins constitute a new class of cysteine proteases, and transient overexpression of each of them caused apoptosis in mammalian or insect cell lines.

Here we report on the molecular cloning and side-by-side comparison of seven mCASPs: mCASP-1, mCASP-2, mCASP-3, mCASP-6, mCASP-7, mCASP-11 (mTX, mCASP-4) and mCASP-12. Transient overexpression experiments in mammalian cells allowed us to assess the role of these cysteine proteases in apoptosis, pro-interleukin-1β processing, and poly(ADP-ribose) polymerase (PARP) cleavage.

2. Materials and methods

2.1. Cloning of mCASP cDNAs

Degenerate oligonucleotides were synthesized with reference to two conserved regions of the CASP family, viz. [L,V][V,A][L,F,I]-[L,M]SHG and [F,I][I,V]QACRG.

The following degenerate sense primers were used:

(A) TGCGAATTC[G,C,T]TIG[C,T]I[C,T,A]TI[T,C]TITC-ICA[T,C]GG;

(B) TGCGAATTC[G,C,T]TIG[C,T]I[C,T,A]TI[T,C]TIAG-[C,T]CA[T,C]GG;

(C) TGCGAATTC[G,C,T]TIG[C,T]I[C,T,A]TIATG[T,A]-[C,G]ICA[C,T]GG;

and the following degenerate antisense primers:

(R) TGCGAATTCICC[A,T,G,C]C[G,T][G,A]CA[G,C,T,A]-GC[T,C]TG;

(N) TCGGGATCCIC[G,T][G,A]CAIGC[T,C]TG[G,C,T,A]A-[T,C][T,G,A]A.

The combinations of primer A and R, primer B and R, and primer C and R were taken for the first PCR amplification. Next, 2 μl aliquots of the resulting products were amplified again with the same sense primer, but with primer N as an antisense primer. 1 μg of murine genomic DNA or 4 ng of a cDNA library derived from L929r2 were used as template. The products were analyzed on a 2% agarose gel. The isolated PCR products were cloned into a pGEM-T vector (Promega Biotec, Madison, WI). 1500 clones were further analyzed by a combination of consecutive hybridizations and cycle sequencing of selected inserts on an ABI373A sequencer (Applied Biosystems, Foster City, CA). PCR clones showing significant amino acid homology with CASP-1 were selected and the inserts were used sequentially as probes to screen 4×10^5 colonies of an L929r2 cDNA library which were transferred to nylon membranes (HybondN; Amersham Life Science, Amersham, UK). Hybridization occurred under stringent conditions.

The L929r2 cells used to construct a cDNA library [34] had been stimulated for 4 h with 1000 IU/ml of human tumor necrosis factor (TNF) and 10 μg/ml cycloheximide.

2.2. Plasmid construction for eukaryotic and in vitro expression

One clone of each murine homolog was chosen to be subcloned into a pCAGGS eukaryotic expression vector [35] using routine recombinant DNA techniques. The generated vectors are further referred to as pCAGGS-mCASP-*x* ($x=2$, 3, 6, 7, 11 or 12). The human IL-1β

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precursor (pIL-1β) gene was excised as an *Eco*RI fragment from an 88.YH plasmid (a generous gift from Dr. J. DeLamarter, Glaxo Institute for Molecular Biology, Geneva) and subcloned into pCAGGS to generate pCAGGS-pIL-1β. The same insert fragments were cloned into a *Hin*cII- or *Eco*RI-opened pGEM11zf(+) vector (Promega Biotec) to generate pGEM-mCASP-*x* (*x* = 2, 3, 6, 7, 11 or 12) and pGEM-pIL-1β. mCASP-1 cDNA (pCAGGS-mCASP-1) was cloned from an EL4/c cDNA library constructed in pCAGGS. The *Sfi*I/*Not*I insert was blunt/*Not*I subcloned in pGEM11Zf(+) to obtain pGEM-mCASP-1. TRADD cDNA was obtained by PCR from first-strand cDNA derived from human KYM cells and subcloned in pCDNAI (Invitrogen, San Diego, CA). pUT651 contains the gene coding for βGal (Cayla, Toulouse, France).

2.3. In vitro transcription/translation

Coupled transcription/translation was performed using a TNT kit from Promega Biotec according to the manufacturer's recommendations. Plasmids derived from pGEM11zf(+) were used as a template for SP6 or T7 RNA polymerase. The reaction products were analyzed by SDS-PAGE and stored at −70°C until needed. The autoprocessing activity of translated products was tested by adding 2 μl of translation mixture to 18 μl of 'ICE maturation buffer', containing 0.1% CHAPS, 50 mM HEPES pH 7.5 (KOH), 1 mM EDTA, 10 mM DTT, 1 mM PMSF, 50 μM leupeptin and 20 μg/μl aprotinin, for 90 min at 37°C.

2.4. Cell lines and transient transfections

L929s is a TNF-sensitive murine fibrosarcoma. MCF7 is a human breast adenocarcinoma line. COS7 is a replication-defective SV40-immortalized fibrosarcoma line derived from African green monkey kidney cells. HeLa H21 is a human cervix carcinoma. Rat1 is a rat fibrosarcoma. HEK293 is a human embryonal kidney carcinoma. All cell lines were cultured in appropriate media using standard tissue culture procedures. L929s, MCF7, COS7 and HeLa H21 were routinely transfected using the calcium phosphate precipitation method [36]. HeLa H21 and Rat1 were also transfected with lipofectamin (Life Technologies, Paisley, UK) according to the manufacturer's instructions. A lipofectamin:plasmid DNA concentration ratio of 8:1 was used. For both transfection methods cells were seeded the day before at 10^4 cells/96-well, 5×10^4 cells/24-well or 5×10^5/6-well; a total amount of plasmid DNA of 200 ng/96-well, 600 ng/24-well or 6 μg/6-well was added (50% pCAGGS-mCASP-*x* or TRADD, 25% pUT651, 25% pCAGGS-pIL-1β or empty pCAGGS). Cells were transfected for 6–12 h, washed and incubated for another 24 h before the XGal assay, or another 48 h before supernatant was collected and tested in a biological assay for IL-1β.

2.5. βGal assay

βGal activity was visualized by fixing transfected cells with 0.2% glutaraldehyde and 2% formaldehyde in PBS(A) for 5 min at 4°C, followed by extensive washing. Cells were stained in PBS(A) containing 1 mg/ml XGal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.02% NP40 and 0.01% SDS. All blue cells in the well were counted.

2.6. pIL-1β-processing assay

Biologically active IL-1β was determined using growth factor-dependent D10(N4)M cells [37]. Cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin G, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 5 mM β-mercaptoethanol and 10% supernatant of phorbol ester-stimulated EL-4 cells as a source of IL-2, and 10% supernatant of phorbol ester-stimulated P388D1 cells as a source of IL-1. The day before the assay, D10(N4)M cells were washed and transferred to culture containing only 10% EL-4 supernatant. The next day, cells were washed again and added to serial dilutions of IL-1β-containing samples (10^4 cells/96-well), followed by incubation for 24 h at 37°C in a CO_2 incubator; proliferation was quantified by [^{3}H]thymidine incorporation (0.5 μCi/well) for the last 6 h. Cells were harvested and incorporated [^{3}H]thymidine was determined in a microplate scintillation counter (Packard Instrument Co., Meriden, CT). Samples were quantified according to a standard preparation of IL-1β with a specific biological activity of 10^9 IU/mg (obtained from the National Institute for Biological Standards and Control, Potters Bar, UK).

2.7. Northern blot analysis

Multiple tissue Northern blot membranes of murine adult tissues were purchased from Clontech Laboratories (Palo Alto, CA). Each lane contained 2 μg of poly(A)$^+$ RNA. Sequential hybridization and stripping were performed according to the manufacturer's instructions. The human β-actin control probe was also supplied by Clontech Laboratories.

2.8. PARP cleavage assay

5×10^5 HEK293 cells were transfected with 6 μg pCAGGS-mCASP-*x* (*x* = 1, 2, 3, 6, 7, 11 or 12) or empty pCAGGS vector by calcium phosphate coprecipitation. The transfection efficiency was 45% as determined by βGal expression in pUT651-transfected cells. 31 h after transfection, the cells were lysed in lysis buffer, containing 50 mM Tris pH 8.0, 300 mM NaCl, 5 mM EDTA, 15 mM $MgCl_2$, 1% NP40, 50 μg/ml PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin. 90 μg of total protein was loaded on 12.5% SDS-PAGE and Western blot analysis performed using a CI-10 anti-PARP antibody (a kind gift from Dr. W. Earnshaw, Johns Hopkins School of Medicine, Baltimore), which detects the 85 kDa cleavage product of PARP.

3. Results and discussion

3.1. Cloning of seven mCASPs

In an attempt to identify all mCASPs, we have used family PCR both on genomic DNA and on cDNA. Two largely conserved domains in the sequence of CED-3 and the murine and human counterparts of CASP-1 and CASP-2 were selected to construct degenerate primers: [L,V][V,A][L,F,I][L,M]SHG and [F,I][V,I]QACRG (Fig. 1A). These two domains are located within one exon in the murine *casp-1* gene [38], the human *casp-1* gene [39] and the *ced-3* gene [7]. We used both murine genomic DNA as well as murine cDNA derived from L929r2 cells as PCR template: the former in order to isolate CASPs independent of cell-specific expression patterns, the latter with the intention not to miss homologs with an exon-intron boundary between the SHG and QACRG boxes of homology. The nucleotide sequence of the cloned PCR products revealed seven different murine members of the CASP family, including the already reported mCASP-1 [30,31], mCASP-2 [32] and mCASP-11 [33]. Remarkably, PCR both on genomic DNA as well as on L929r2 cDNA revealed the same seven CASPs. Obviously, the murine counterparts of CASP family members in which the regions of the degenerate primers are not completely conserved, were missed using this cloning procedure, e.g. CASP-8, CASP-9 and CASP-10, in which the QACRG box is not conserved [26,27,29]. The inserts of the seven identified PCR clones, except for the mCASP-1 fragment, were used as a probe to isolate the corresponding full-length cDNAs from an L929r2 cDNA library. mCASP-1 had been isolated previ-

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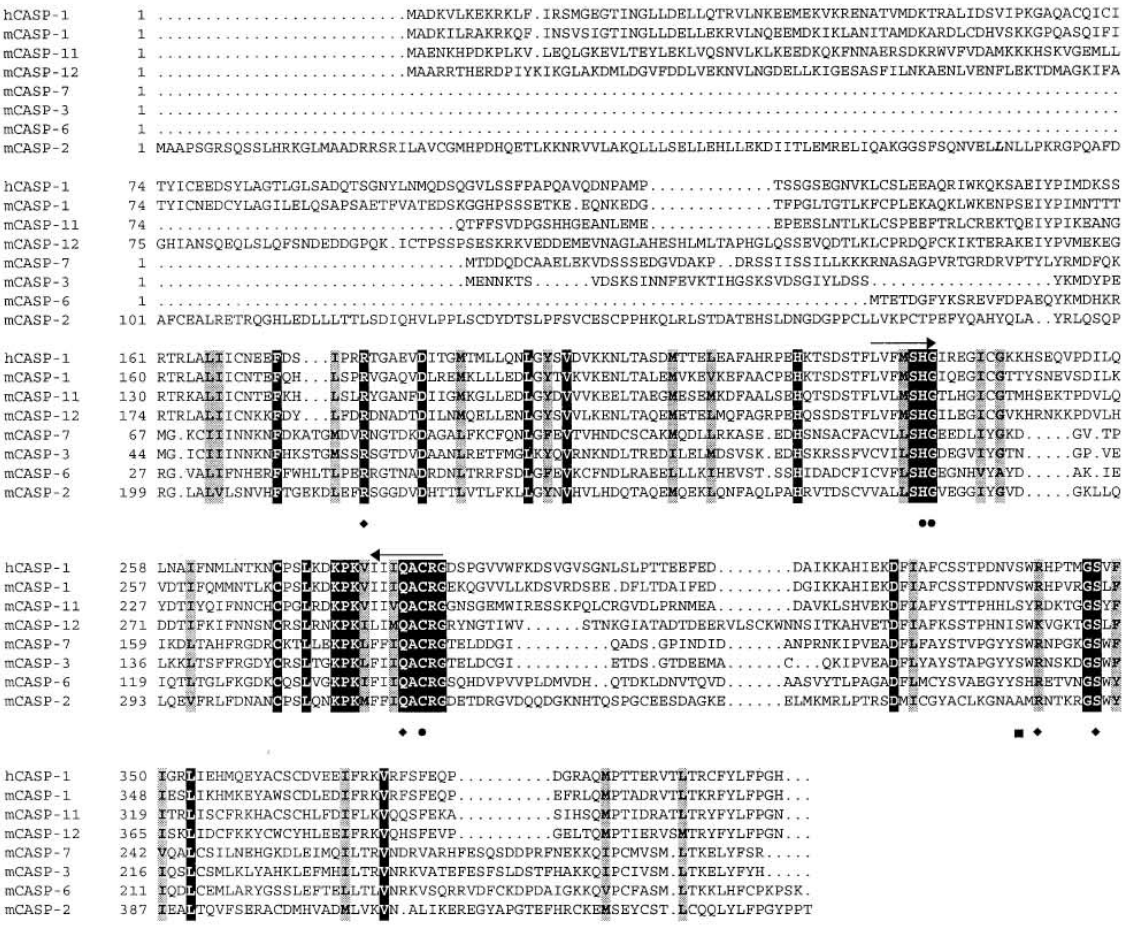
Fig. 1. Alignment of amino acid sequences of murine members of the CASP family and their relationships. (A) Deduced amino acid sequences for hCASP-1 [13] and mCASP-1, mCASP-11, mCASP-12, mCASP-7, mCASP-3, mCASP-6 and mCASP-2. The sequences were aligned using the Genetics Computer Group (Madison, WI) PILEUP algorithm (gap weight = 3.0; gap length weight = 0.1). Identical and similar amino acids are boxed in black and grey, respectively. Conserved QACRG and SHG boxes used for designing degenerate primers are shown by arrows. Leu_{88} of mCASP-2 is italicized. Amino acids that align with the residues of hCASP-1 forming the binding pocket for P1 Asp, are indicated by ♦ (Arg_{179}, Gln_{283}, Arg_{341} and Ser_{347}); amino acids involved in catalysis are indicated by ● (His_{237}, Gly_{238} and Cys_{285}). A putative protein kinase C phosphorylation site is marked by ■. (B) Phylogenetic comparison of hCASPs and mCASPs originating from a CED-3-like ancestor. The alignment was achieved as described above and was used to generate the dendrogram by the CLUSTAL W program [50]. The CASP-3 subfamily (red) and hCASP-9 (purple) are weakly related to each other. The CASP-2 subfamily is shown in green, the CASP-1 subfamily in blue.

A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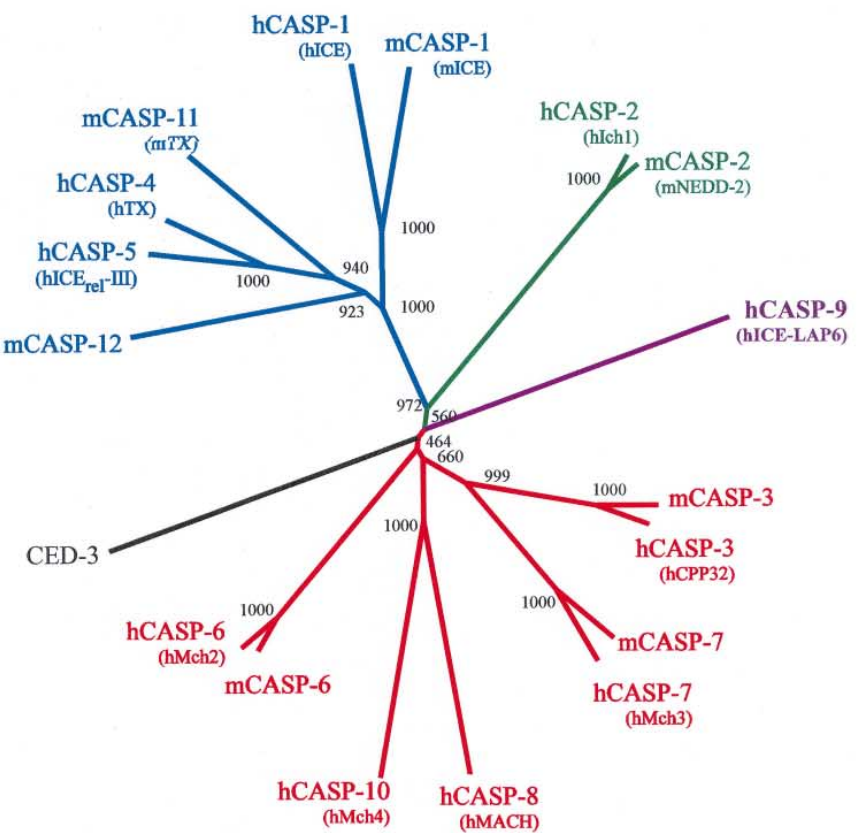
B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(hICE$_{rel}$-III)
mCASP-12
hCASP-2
(hIch1)
mCASP-2
(mNEDD-2)
hCASP-9
(hICE-LAP6)
CED-3
mCASP-3
hCASP-3
(hCPP32)
mCASP-7
hCASP-7
(hMch3)
hCASP-6
(hMch2)
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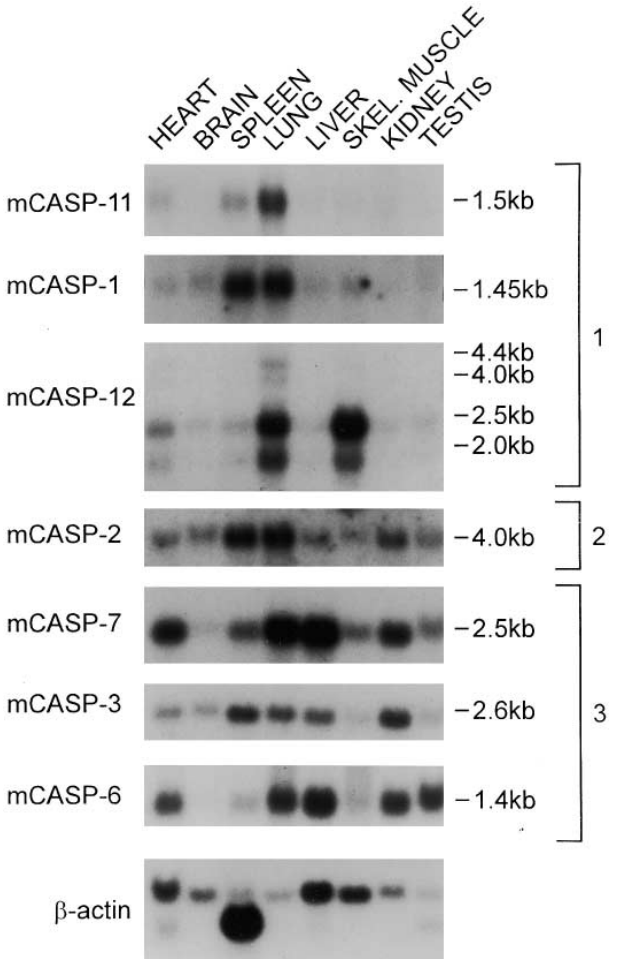

Fig. 2. mRNA expression pattern of mCASPs. Murine, multiple, adult tissue Northern blots were hybridized sequentially with mCASP-11, mCASP-1, mCASP-12, mCASP-2, mCASP-7, mCASP-3, mCASP-6 and human β-actin probes. The size of the hybridizing bands is indicated in kb. (1) mCASP-1 subfamily, (2) mCASP-2 subfamily, (3) mCASP-3 subfamily.

ously from an EL4/c cDNA library [40]. For each murine homolog one clone, containing the largest open reading frame, was retained for further characterization. These cDNA clones were identified as coding for the presumed murine homologs of hCASP-2, hCASP-3, hCASP-4 (mCASP-11), hCASP-6 and hCASP-7 (Fig. 1A). The translated open reading frames are 91.5, 92.5, 73.5, 96 and 90.5% similar, and 86, 86.5, 60, 90.5 and 82% identical, respectively, to their human counterparts. It should be noted that the homology between hCASP-4 (TX) and mCASP-11 (Ich3) is as significant as between human and murine CASP-1, which have 75% similarity and 62% identity [30,31]. In addition, amino acid alignment between hCASP-4 and mCASP-11 shows only a 4 amino acid gap located in the prodomain (data not shown). Moreover, we were also able to show functional homology, since both hCASP-4 and mCASP-11 cleave CASP-1 ([18]; Van de Craen et al., in preparation). Consequently, we conclude that mCASP-11 is the counterpart of hCASP-4, but the recommendation is to refer to this sequence as mCASP-11 (Drs. J. Yuan and N.A. Thornberry, personal communication on behalf of the nomenclature committee). On the other hand, mCASP-12 has only 62% similarity and 44.5% identity to hCASP-5; amino acid alignment of these two homologs reveals several gaps (data not shown). Hence, it seems that mCASP-12 is not the murine counterpart of hCASP-5, but constitutes a new CASP-1 subfamily member. Our cloned mCASP-2 is identical to mNEDD-2 [32], except for the insert of an extra Leu at position 88 (Fig. 1A).

3.2. Sequence comparison of CASPs

The multiple sequence alignment for hCASP-1, mCASP-1 and the newly cloned mCASPs is shown in Fig. 1A. Residues Arg_{179}, Gln_{283}, Arg_{341} and Ser_{347} constitute the Asp-binding pocket in hCASP-1 [41,42] and are conserved in all cloned members of the CASP family, except for mCASP-12 in which Arg_{341} is conservatively replaced by Lys. This makes mCASP-12 the only known CASP family member, including the related hCASP-5, with an amino acid substitution at this important position and argues again in favor of mCASP-12 being a new CASP-1 subfamily member. The residues involved in catalysis, His_{237}, Gly_{238} and Cys_{285} of hCASP-1, are conserved in all family members. The dendrogram in Fig. 1B shows that the CASP protein family comprises three subfamilies. The CASP-3 subfamily consists of h/mCASP-3, h/mCASP-6, h/mCASP-7, hCASP-8 and hCASP-10. This subfamily, especially h/mCASP-6, displays the highest homology with CED-3, which agrees with the presumed central role of CASP-3-like proteases in different pathways of apoptosis [17,43]. Presumably, also hCASP-9 belongs to the CASP-3 subfamily, but, depending on the type of phylogenetic analysis, it can also be related to the second subfamily, the CASP-2 subfamily. The latter consists of human and murine CASP-2. A characteristic feature is the absence of a putative protein kinase C phosphorylation site SXR (Ser_{339} of hCASP-1), which is conserved in all other CASPs. The third subfamily, viz. CASP-1, consists of h/mCASP-1, hCASP-4/mCASP-11, hCASP-5 and mCASP-12. This subfamily is characterized by a wider amino acid sequence divergence between the human and murine homologs ($< 75\%$ similarity as compared to $> 90\%$ similarity for the other two subfamilies). This might indicate that CASP-1 subfamily members do not fulfill such a central role as the CASP-3 subfamily. In this respect it may be noted that CASP-1-deficient mice are quite healthy, and their cells, except for Fas-induced apoptosis of thymocytes, are not affected in their capacity to undergo cell death [44,45].

3.3. Tissue distribution of mCASP cDNAs

The expression of the different murine *casp* gene family members was examined by Northern blot analysis under stringent hybridization conditions. The full-length cDNA clones were used as a probe for sequential hybridization of a multiple tissue Northern blot (Fig. 2). The sizes of the predominantly hybridizing transcripts were consistent with the length of the cloned cDNAs for mCASP-1 (1322 bp), mCASP-2 (3463 bp), mCASP-6 (1262 bp), mCASP-7 (2371 bp), mCASP-11 (1340 bp) and mCASP-12 (2262 bp), except for the mCASP-3 transcript which was approx. 1300 bp larger than the cDNA clone (1297 bp); presumably, the cDNA clone isolated lacked most of the 3′-UTR sequences. The weakly hybridizing, larger transcripts could correspond to incompletely or alternatively processed mRNAs, while some smaller species could be alternatively spliced isoforms or degradation products (results not shown). Cross-hybridization under the conditions used is unlikely, but cannot be completely excluded.

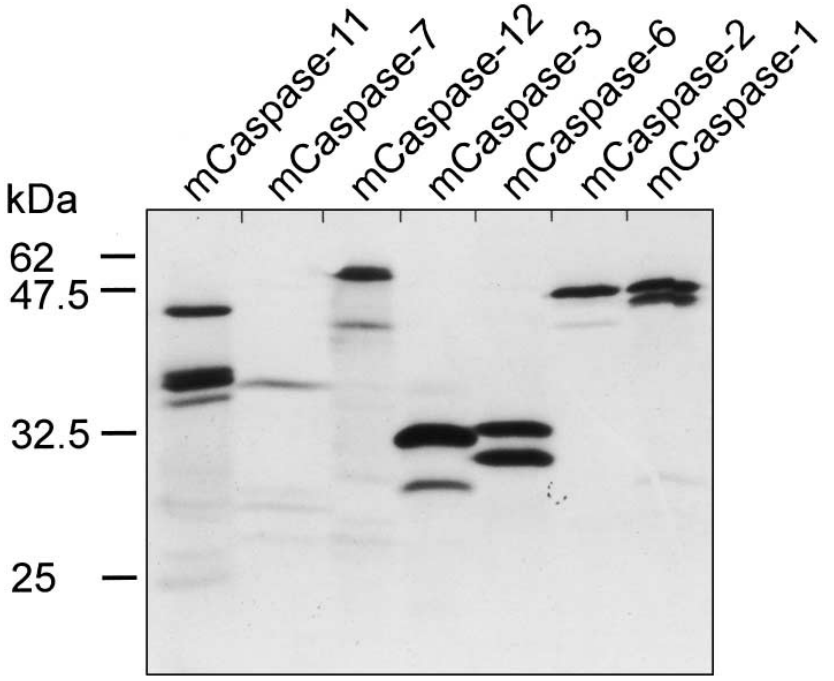

Fig. 3. In vitro transcription and translation of mCASPs (pGEM11zf(+)-based vectors) in reticulocyte lysate in the presence of [^{35}S]methionine for 90 min at 30°C. 2 μl of this mixture was loaded on an SDS-polyacrylamide gel. Molecular mass markers are indicated on the left.

As regards the mRNA tissue distribution (Fig. 2 and Table 1), mCASP-11 mRNA was most expressed in lung, weaker in spleen and heart, and little in liver, skeletal muscle, kidney and testis. No message could be detected in the brain. Transcripts of mCASP-7 were prominent in most tissues, but especially in liver, lung, kidney and heart. The 2.5 kb mCASP-12 transcript was most abundant and was mainly expressed in skeletal muscle and lung, and moderately expressed in the other tissues analyzed. It may be noted that the potential mCASP-12 counterpart, hCASP-5, is only clearly expressed in lung, liver, placenta and pancreas [20]. Moreover, hCASP-4 (mCASP-11 counterpart) has a comparable expression pattern to mCASP-11 and hCASP-8 (hICE$_{rel}$-III) [20], while the tissue distribution of mCASP-11 and mCASP-12 expression is not related (Table 1). This suggests once more that mCASP-12 is not the murine homolog of hCASP-5, while mCASP-11 is presumably the murine counterpart of hCASP-4. mCASP-3 mRNA (2.5 kb) is mostly expressed in spleen, kidney, lung and liver, but also the other tissues reveal relatively high levels. mCASP-6 mRNA is clearly present in all tissues and the expression is especially high in liver, lung, kidney, testis and heart. mCASP-2 (4 kb) is expressed in all

Table 1
Distribution of CASP mRNA in murine and human tissues

	Heart	Brain	Spleen	Lung	Liver	Skeletal muscle	Kidney	Testis
mCASP-11 (1.5 kb)	++	–	+++	++++	+	+	+	+
hCASP-4 (1.5 kb) [19]	++	–	++++	+++	++	+	+	+
mCASP-1 (1.45 kb)	+	+	+++	+++	+	+	+	+
hCASP-1 (1.5 kb) [19]	++	–	++++	++++	++	+	+	+
mCASP-12 (2.5 kb)	+	+	+	++++	+	++++	+	+
hCASP-5 (1.5 kb) [20]	+	–	ND	+++	++	+	+	ND
mCASP-2 (4 kb)	++	++	++++	++++	++	+	+++	++
hCASP-2 (4 kb) [14]	++	++	ND	+++	+++	++	+++	ND
mCASP-7 (2.5 kb)	+++	+	++	++++	++++	++	+++	++
hCASP-7 (2.4 kb) [23,24]	+++	+	+++	++++	+++	++++	+++	++
mCASP-3 (2.6 kb)	+++	++	++++	++++	++++	++	++++	+
hCASP-3 [15,24]	+++	++	+++	++++	+++	++++	+++	++
mCASP-6 (1.4 kb)	+++	+	++	++++	++++	+	+++	+++
hCASP-6 (1.4 kb)	ND	ND	ND	ND	ND	ND	ND	ND

++++, very strong; +++, strong; ++, moderate; +, low; –, not detectable expression; ND, not determined. Data for the murine system are from results shown in Fig. 2; data for the human system are from the references mentioned in the first column.

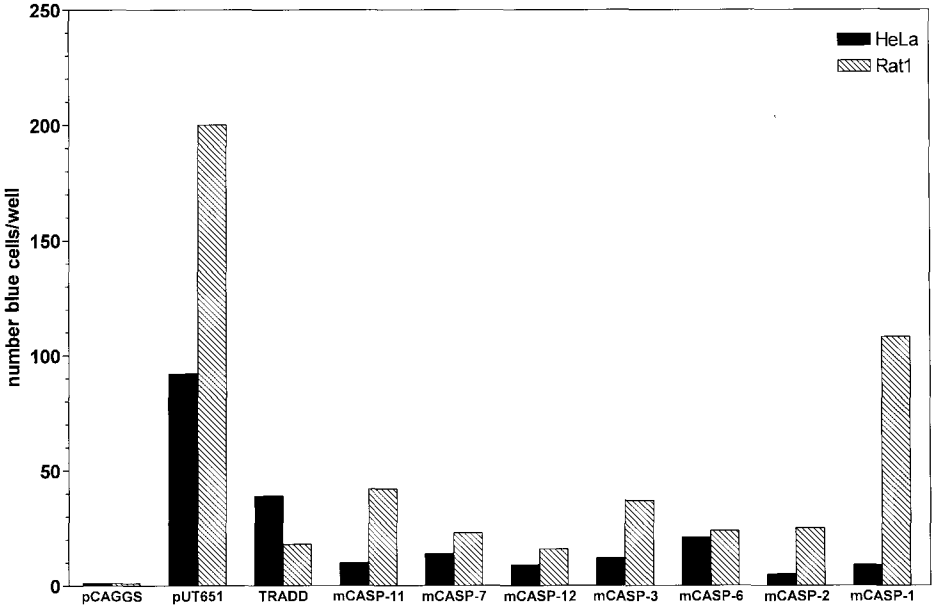

Fig. 4. mCASP-mediated induction of apoptosis by transient overexpression in mammalian cell lines. HeLa and Rat1 cells were cotransfected in 24-well plates with pUT651, containing βGal, and pCAGGS-mCASP-x ($x = 1$, 2, 3, 6, 7, 11 or 12), TRADD or empty pCAGGS as control. 24–36 h after transfection, the cells were stained with XGal and counted. The ordinate indicates the number of blue cells counted in each well for HeLa and Rat1. The data are representative of three independent experiments. The relative counting variation was $< 10\%$.

tissues, but mainly in spleen and lung. mCASP-1 hybridization revealed a band of 1.45 kb, mainly in spleen and lung.

An important conclusion which can be drawn from Table 1, is that, except for mCASP-12 and hCASP-5, discussed above, and for h/mCASP-6 (where no data are available for the human system), there is a remarkable agreement in mRNA abundance of the various homologs in different human and murine tissues; this suggests a similar function and regulation. Furthermore, it may be noted that the tissue distribution of the CASP-1 subfamily transcripts, on the one hand, and the CASP-3 subfamily transcripts, on the other hand, are quite different. In general, the former have a restricted expression pattern, mainly confined to spleen and lung, tissues with important immune functions, and are only moderately expressed in the other tissues examined. Members of the latter subfamily have a much broader mRNA expression pattern. This could indicate that the transcriptional regulation of CASPs within one subfamily is related, but is quite different between the CASP-1 and CASP-3 subfamilies. This suggests that transcription of CASP-1 subfamily members is mainly controlled by other transcription factors than in the case of the CASP-3 subfamily members. In this respect, it has been reported that

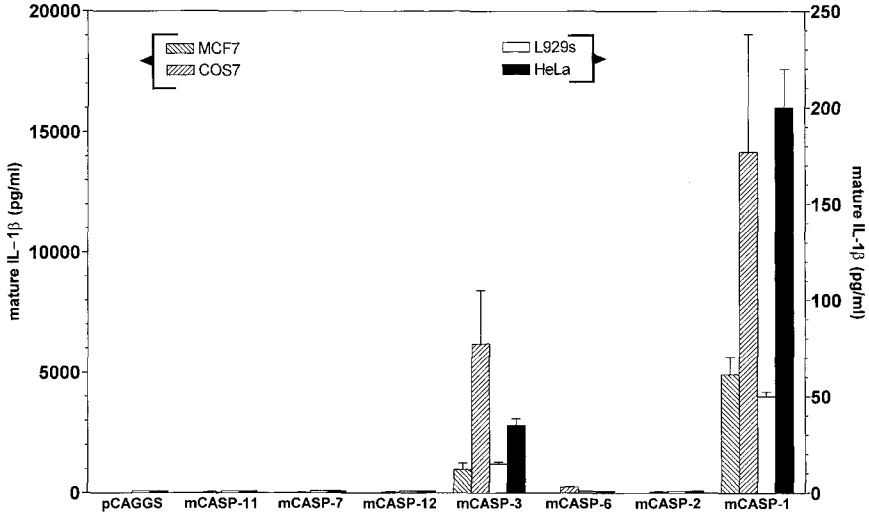

Fig. 5. In vivo cleavage of human pIL-1β by cotransfection with mCASPs. The plasmids pCAGGS-mCASP-x ($x = 1$, 2, 3, 6, 7, 11 or 12) or empty pCAGGS were cotransfected with pCAGGS-pIL-1β in MCF7, COS7, L929sA or HeLa cells in 96-well microtiter plates. 48 h after transfection, the concentration of secreted IL-1β was determined in a D10 bioassay. The left Y-axis indicates IL-1 processing in MCF7 and COS7 cells (transfection efficiencies of 26 and 17%, respectively), the right Y-axis in L929s and HeLa cells (transfection efficiencies $< 2\%$). The experiments were performed in triplicate.

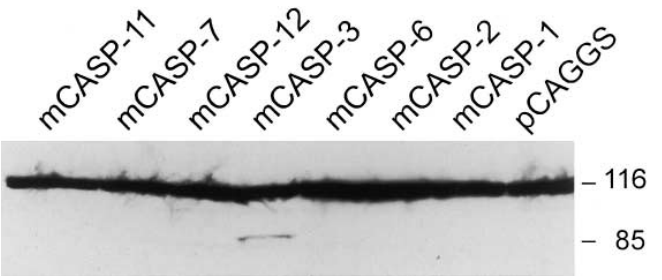

Fig. 6. In vivo cleavage of PARP by mCASPs. HEK293 cells were transfected with pCAGGS-mCASP-x (x=1, 2, 3, 6, 7, 11 or 12) or empty pCAGGS vector (transfection efficiency of 45%). 31 h later, cells were lysed and proteins analyzed by SDS-PAGE (90 μg total protein per lane). After Western blotting, cleavage of endogenous PARP was analyzed by means of anti-PARP antibody (CI-10), which revealed an 85 kDa-specific degradation product.

IRF-1, an IFN- and TNF-inducible transcription factor, controls the expression of CASP-1 and might in this way play a role in inflammation [46].

3.4. *In vitro transcription/translation*

Fig. 3 shows the [^{35}S]methionine-labeled in vitro transcription/translation products of the murine cDNA clones coding for the CASP precursors. Translation of mRNA from mCASP-1 (45.6 kDa), mCASP-11 (42.7 kDa), mCASP-7 (35.5 kDa), mCASP-12 (47.8 kDa), mCASP-3 (31.5 kDa), mCASP-6 (31.5 kDa) and mCASP-2 (47 kDa) gave rise to products of a size expected on the basis of the cDNA sequences (mentioned in brackets). Lower molecular weight bands were presumably due to internal initiation and/or premature termination. No degradation was observed during the in vitro synthesis and no obvious changes in molecular weight patterns were detectable after further incubation for 1.5 h in 'ICE maturation buffer' (results not shown). Hence, we could conclude that, at the enzyme concentration examined, no autoprocessing occurred. It may be noted that in vitro transcribed and translated CED-3 did show autoprocessing activity under similar assay conditions [47].

3.5. *All mCASPs induce apoptosis in transfected cells*

In order to determine the apoptotic capacity of mCASPs, different cell lines were transiently transfected. To this end, the aforementioned CASP cDNAs were cloned in a pCAGGS expression vector [35]. The extent of apoptosis in the transfected cells was quantified by means of a cotransfected plasmid containing a βGal reporter gene. The extent of cell death in the transfected population was measured by the decrease in number of surviving βGal-expressing cells, using XGal as an indicator substrate. As a positive control for the induction of apoptosis, an expression vector coding for the TNF p55 receptor-associated death domain (TRADD) [48] was cotransfected. As a negative control, the empty pCAGGS vector was used. In the cell lines tested, transient overexpression of the TRADD-containing plasmid caused a profound reduction in the number of βGal-expressing cells as compared to the negative controls. Transient overexpression of mCASP-1 and all murine homologs in HeLa and Rat1 cells resulted in a clear reduction in the number of XGal-positive cells (Fig. 4 and unpublished results), although the extent of apoptosis varied from experiment to experiment. Since precursor CASP genes were used for these experiments, these results mean either that the full-length product was active, or if not, that the transfected cells were capable to generate processed, active forms. Remarkably, and in contrast to TRADD overexpression, transient transfection of CASPs in L929s cells induced little or no apoptosis (data not shown).

3.6. *pIL-1β is mainly processed by mCASP-1, but also by mCASP-3*

CASP-1 was discovered originally as the protease responsible for processing of pIL-1β [9,13]. We examined, by transient cotransfection of the CASP-containing expression plasmids with a human pIL-1β-coding vector, whether other CASPs in addition to CASP-1 were able to cleave inactive pIL-1β into mature IL-1β. An IL-1β-processing activity by transient cotransfection may be due to direct processing by the cotransfected CASP or can be explained by CASP-mediated activation of endogenous CASP-1. Four cell lines with different transfection efficiency were used, viz. MCF7, COS7, HeLa and L929s. Secreted mature IL-1β was quantified in a bioassay using D10 cells [37] (Fig. 5). As expected, cotransfection with mCASP-1 expression vector resulted in a high level of biologically active mature IL-1β. Except for mCASP-3 (see below), none of the other mCASPs were able to induce pIL-1β processing. This also means that neither of the other CASPs could activate sufficient levels of endogenous CASP-1 or CASP-3 to result in detectable amounts of IL-1β. Cells transfected with an mCASP-3 expression vector clearly resulted in mature IL-1β levels in the four cell lines examined, at a level of 10–30% of the amounts obtained with mCASP-1. In agreement with these findings, we could show in vitro that only bacterially expressed mCASP-1 and, to a lesser extent, mCASP-3, were able to process pIL-1β to the expected 28 and 17 kDa fragments; the other bacterial mCASP preparations did not exhibit this activity (Van de Craen et al., in preparation). As hCASP-3-mediated cleavage of pIL-1β has not been demonstrated [16], this indicates that the substrate specificity between human and murine CASP counterparts might be different. Kamens et al. [19] reported in vitro cleavage of pIL-1β by hCASP-4; however, two other groups could not confirm this result [18,20].

3.7. *mCASP-3 is the predominant PARP-cleaving CASP in vivo*

Cleavage of PARP has frequently been reported to correlate with apoptosis [16,17]. In some cell lines, inactivation of this repair enzyme may even directly contribute to cell death [49], although mice lacking PARP develop normally and no effects on death pathways are in evidence [14]. mCASP-x expression plasmids (x=1, 2, 3, 6, 7, 11 or 12) were transiently trans-

fected in HEK293 and endogenous PARP cleavage was examined by Western blotting. Fig. 6 shows that especially in cells transfected with mCASP-3, PARP was clearly cleaved releasing the characteristic 85 kDa fragment, suggesting that mCASP-3 is the main PARP-cleaving enzyme.

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