The Apoptotic Initiator Caspase-8: Its Functional Ubiquity and Genetic Diversity during Animal Evolution

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Abstract

The caspases, a family of cysteine proteases, play multiple roles in apoptosis, inflammation, and cellular differentiation. Caspase-8 (Casp8), which was first identified in humans, functions as an initiator caspase in the apoptotic signaling mediated by cell-surface death receptors. To understand the evolution of function in the Casp8 protein family, casp8 orthologs were identified from a comprehensive range of vertebrates and invertebrates, including sponges and cnidarians, and characterized at both the gene and protein levels. Some introns have been conserved from cnidarians to mammals, but both losses and gains have also occurred; a new intron arose during teleost evolution, whereas in the ascidian Ciona intestinalis, the casp8 gene is intronless and is organized in an operon with a neighboring gene. Casp8 activities are near ubiquitous throughout the animal kingdom. Exogenous expression of a representative range of nonmammalian Casp8 proteins in cultured mammalian cells induced cell death, implying that these proteins possess proapoptotic activity. The cnidarian Casp8 proteins differ considerably from their bilaterian counterparts in terms of amino acid residues in the catalytic pocket, but display the same substrate specificity as human CASP8, highlighting the complexity of spatial structural interactions involved in enzymatic activity. Finally, it was confirmed that the interaction with an adaptor molecule, Fas-associated death domain protein, is also evolutionarily ancient. Thus, despite structural diversity and cooption to a variety of new functions, the ancient origins and near ubiquitous distribution of this activity across the animal kingdom emphasize the importance and utility of Casp8 as a central component of the metazoan molecular toolkit.

Key words: caspase, FADD, intron insertions, exon shuffling, operon.

Introduction

Caspases (Cysteine-dependent aspartyl-specific protease) belong to a family of cysteine proteases that recognize specific tetrapeptide motifs and cleave after aspartate residues. Caspases are essential for the initiation and execution of apoptosis, as well as for the processing and maturation of the inflammatory cytokines (Nicholson 1999). Twelve caspases (caspases-1 to -10, -12, and -14) have been identified in human (Homo sapiens) (Lamkanfi et al. 2002), and additional caspases, including caspases-15 to -18, are present in some other mammals (Eckhart et al. 2008). The mammalian caspases are classified according to their phylogenic relationships, and these groupings correlate with functional relatedness (Lamkanfi et al. 2002). Caspases are widely distributed across the animal kingdom, and are present in nonmammalian vertebrates (Sakamaki and Satou 2009) as well as many invertebrates, and have been extensively characterized in representative nematodes (e.g., Caenorhabditis elegans).
(Shaham 1998) and arthropods (e.g., fruit fly [Drosophila melanogaster]) (Kumar and Doumanis 2000), in particular. Comparative analyses suggest that many of the invertebrate caspases are likely to be orthologs of specific vertebrate caspase types. For example, a short form of the sponge caspase that is most similar to human caspase-3 (CASP3) also has Casp3-like enzyme activity (Wiens et al. 2003). Therefore, it is likely that the functions of at least some types of caspases have been conserved throughout animal evolution. Despite the apparent deep conservation of some caspase types, both losses and gains have occurred in specific lineages, accounting for the different caspase repertoires observed in extant animals (Shaham 1998; Kumar and Doumanis 2000; Weill et al. 2005; Sakamaki and Satou 2009).

Caspase-8 (Casp8), which is classified as an initiator caspase, contains tandem death effector domain (DED) motifs in its amino-terminal prodomain and an active protease domain (CASCc) at the carboxyl terminus. Casp8 is critically involved in the extrinsic apoptotic signaling pathway of mammals (Ashkenazi 2008), which is triggered by stimulation of cell-surface receptors called death receptors (DRs). Six types of DRs have been identified in mammals; among them, Fas has been most thoroughly characterized (Lavrik and Krammer 2012). Once activated by oligomerization, Fas recruits an adaptor molecule, Fas-associated death domain protein (FADD, also known as MORT1), which contains both DED and death domain (DD) motifs, to the cytoplasmic region of Fas via homophilic interactions mediated by DDs. Casp8 associates in turn with FADD via interactions between DED motifs. Within the Fas–FADD–Casp8 complex, Casp8 undergoes self-cleavage to generate its active form. Activated Casp8 is released to the cytosol, where it cleaves downstream molecules to transmit apoptotic signals. In addition, recent studies have revealed various nonapoptotic functions of Casp8, including roles in cell proliferation, differentiation, suppression of inflammation, tissue homeostasis, and suppression of necrosis, which is a distinct type of cell death (Frisch 2008; Maelfait and Beyaert 2008; Günther et al. 2014; Salvesen and Walsh 2014).

In vertebrates, the Casp8 gene is tightly linked to the paralogous genes, caspase-10 (CASP10) and CASP8- and FADD-like apoptosis regulator (Cflar; gene product, c-FLIP) in the same chromosomal region (Sakamaki and Satou 2009). Furthermore, an additional casp8-like gene caspase-18 (casp18) is located between the casp8 and casp10 loci in chicken (Gallus gallus) and marsupials such as the opossum (Monodelphis domestica), but not in eutherian mammals (Sakata et al. 2007; Eckhart et al. 2008). These four genes diverged from the ancestral casp8 gene by tandem gene duplication early in vertebrate evolution (Sakata et al. 2007). In addition, the fish-specific casp8-like gene, card-casp8 may have been duplicated adjacent to the casp8 gene after chromosomal segregation (Sakamaki and Satou 2009). Within the vertebrate lineage, these molecules have diverged to play distinct physiological roles (Ozturk et al. 2012; Lamy et al. 2013).

casp8 genes are also present in several invertebrates—for example, Dredd was identified as a Casp8 ortholog in both the fruit fly D. melanogaster and the mosquito (Aedes aegypti) (Chen et al. 1998; Cooper et al. 2007). Note that D. melanogaster lacks a functional extrinsic apoptotic pathway, Dredd being involved instead in innate immunity (Falschlehner and Boutros 2012). Although casp8 genes have also been identified in the lancelet (Branchiostoma belcheri tsingtauense), the abalone (Haliotis discus), and the oyster (Crassostrea hongkongensis) (Xu et al. 2011; Lee et al. 2011; Xiang et al. 2013), the functions of Casp8 proteins have not been elucidated in those animals. Therefore, information currently available is insufficient for a comprehensive evaluation of the roles of Casp8 in invertebrates. In an effort to better understand the distribution and diversity of Casp8 and related molecules during animal evolution, we searched for casp8 genes in various animals and found that they arose early in animal evolution. We also investigated the structure and organization of casp8 loci, as well as the structure and function of proteins that they encode. Comparisons of these aspects between animals provide insights into the evolution of casp8, leading to an improved understanding of how the ancestral function(s) of Casp8 have been inherited. In addition, we investigated the ability of Casp8 to interact with FADD in multiple species. Our results clearly demonstrate the conservation of the universal features of Casp8 proteins under evolutionary pressure.

Results and Discussion
Isolation of Casp8 from Various Organisms and Sequence Alignment Analysis
To investigate the distribution of genes orthologous to human CASP8 in animals, the NCBI DNA database was searched using TBLASTN. We thus identified candidate cDNA clones in the stickleback (Gasterosteus aculeatus), skate (Leucoraja erinacea), the ascidians (Ciona intestinalis and Ci. savignyi), the annelid sludge worm (Tubifex tubifex), the mollusc mussel (Mytilus californianus), the planarians (Schmidtea mediterranea and Dugesia japonica), and the cnidian coral (Acropora palmata). In the cases of the lamprey (Lethenteron japonicum), the lancelet (Branchiostoma floridae), and the coral (Acropora millepora), cDNAs encoding casp8 homologs were directly isolated from cDNA libraries by polymerase chain reaction (PCR) amplification based on the expressed sequence tag (EST) sequences. Sequencing of these cDNAs led to the identification of the complete open reading frames, as shown in supplementary table S1, Supplementary Material online. Each of the sequences identified not only exhibited the highest similarity to mammalian Casp8 in reciprocal BLASTP analysis (data not shown) but also shared a common domain architecture (two DED motifs and a CASC protease domain), supporting their assignment as Casp8 orthologs. A novel and so far unique Casp8-like molecule bearing an additional DD in the midportion was also identified in the mussel (see later).

The amino acid sequence alignment of Casp8 proteins illustrates the degree of similarity across this protein family. In particular, amino acid residues in the CASCc are highly conserved (fig. 1A). With few exceptions, the glutamine residue at Q61 in the human CASP8 sequence, which immediately
FIG. 1. Analysis of the protein structures of Casp8 proteins. (A) Multiple alignment of amino acid sequences corresponding to a protease domain (CASc) of Casp8 proteins from human (H. sapiens), chicken (G. gallus), Xenopus (X. laevis), zebrafish (D. rerio), lamprey (L. japonicum), lancelet (B. floridae), ascidian (Ci. intestinalis), sea urchin (P. lividus), Drosophila (D. melanogaster), sludge worm (T. tubifex), mussel (M. californianus), planarian (Sc. mediterranea), and coral (Ac. millepora). Identical and similar amino acids in all family members are indicated by red and blue, respectively. Identical amino acids with one exception are also indicated by pink. The number of amino acids in brackets indicates hidden residues. The assignments of /α/1-helix and /α/2-sheet secondary structure elements are based on a previous study (Watt et al. 1999). Specifically conserved amino acid residues representing typical characteristics of Casp8 proteins are indicated by [1], [2], and [3]. (B) Front and back views of active human CASP8. Active CASP8, consisting of a dimer of two large and two small subunits, is shown as a ribbon diagram. Large and small subunits are indicated in dark blue and yellow, respectively. The peptide inhibitor, colored in red, is shown as a stick model, except for one unit in the left panel. The left panel highlights the one active site of the CASP8 dimer. The essential amino acid residues H317 and C360 in the active site are indicated by colored balls. The CASP8-specific amino acids Q361 and Y412 are also indicated by a spherical ball. The right panel highlights the spatial area against for the active site. The CASP8-specific amino acid D438 are localized face-to-face.
follows the active site cysteine residue (C360 in human), is conserved from cnidarians to mammals (fig. 1A and table 1). This high degree of evolutionary conservation implies functional significance. In a previous study, it was reported that the side chain of Q361 is required for hydrogen-bond interactions with neighboring residues, suggesting that this residue is involved in orienting single large and small subunits in relation to the second unit within the caspase dimer (Blanchard et al. 1999). This mode of interaction distinguishes Casp8 proteins from other caspases, in which an arginine (R) residue is present at the corresponding position in primary sequence comparison (supplementary fig. S1, Supplementary Material online). On the other hand, the tryptophan (W) residue located in the S2/4 pocket of many other caspases (Chereau et al. 2003), is typically not present in Casp8 orthologs (table 1 and supplementary fig. S1, Supplementary Material online). The S2 pocket is the region of caspase proteins responsible for binding the substrate. Instead, either a tyrosine (Y412 in human) or a phenylalanine (F) residue occurs at this position, except in the putative Casp8 molecules of coral and sea anemone (table 1). The S4 pocket of CASP8 differs from that of CASP3: the hydrogen-bond participants have moved away to accommodate a nonpolar residue (Watt et al. 1999), and the face of Y412 seems to form a part of the hydrophobic S4 pocket. The Casp8-paralogs Casp10, Casp18, and CARD-casp8 proteins retain these characteristic features of Casp8 (Q rather than R, and a Y or F rather than W in the S2/4 pocket; data not shown), which, given the positions of these residues in the structures of the proteins (fig. 1B), may be involved in substrate recognition and cleavage. We addressed this issue as described further later. Interestingly, the aspartic acid residue at D438 in the human sequence also appears away to accommodate a nonpolar residue (Watt et al. 1999), that of CASP3: the hydrogen-bond participants have moved (supplementary fig. S1, Supplementary Material online). The genomic sequence covering the casp8 locus of the coral Ac. millepora was assembled for this study (GenBank accession number: KJ639818), and the locus was sequenced in the case of the sludge worm T. tubifex (GenBank accession number: FJ890021). With the sole exception of the third splicing acceptor site of the West African clawed frog Xenopus tropicalis gene, the exon–intron boundaries of the casp8 genes conformed to the GT-AG rule (Breathnach and Chambon 1981). On the basis of these data, we found that the splice junction sites of the casp8 genes are basically

### Table 1. Summary of the Amino Acids Conserved in Casp8 Proteins.

<table>
<thead>
<tr>
<th>Species</th>
<th>[1]</th>
<th>[2]</th>
<th>[3]</th>
</tr>
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<tbody>
<tr>
<td>H. sapiens</td>
<td>CGQ</td>
<td>SYR</td>
<td>DDI</td>
</tr>
<tr>
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<td>SYR</td>
<td>DDI</td>
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<td>SYR</td>
<td>NDI</td>
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<td>SYR</td>
<td>EDI</td>
</tr>
<tr>
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<td>CGQ</td>
<td>SYR</td>
<td>EDI</td>
</tr>
<tr>
<td>Me. gallopus</td>
<td>CGQ</td>
<td>SYR</td>
<td>EDI</td>
</tr>
<tr>
<td>X. laevis</td>
<td>CGQ</td>
<td>SFR</td>
<td>NDI</td>
</tr>
<tr>
<td>X. tropicalis</td>
<td>CGQ</td>
<td>SFR</td>
<td>DDI</td>
</tr>
<tr>
<td>Da. rerio</td>
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<td>SYR</td>
<td>EDI</td>
</tr>
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<td>SFR</td>
<td>DDI</td>
</tr>
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<td>SFR</td>
<td>DDI</td>
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<td>SFR</td>
<td>EDI</td>
</tr>
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<td>SFR</td>
<td>NDI</td>
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<td>NDI</td>
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<tr>
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<td>NDV</td>
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<tr>
<td>Sc. mediterranea</td>
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</tr>
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<td>KSL</td>
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<td>Ac. millepora</td>
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<td>YDV</td>
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<td>FDV</td>
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<td>H. sapiens</td>
<td>CRG</td>
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<td>LEF</td>
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</table>

Both the glutamine residue (Q) and aspartic acid residue (D) are conserved in positions [1] and [3], as shown in fig. 1A. The amino acid residue in position [2] is unlikely to be tryptophan (W), except in coral and sea anemone Casp8 proteins. The superior sharp indicates a molecule bearing the DD motif. The species name are as follows: A. aegypti (mosquito); Ac. digitifera (coral); Ac. millepora (coral); Ac. palmita (coral); B. florisae (lancelet); B. lanceolatum (lancelet); Cr. hongkongensis (oyster); Ci. intestinalis (ascidian); Ci. savignyi (ascidian); Ca. teleta (polychaete worm); Da. japonica (planarian); D. melanogaster (fruit fly); Da. rerio (zebrafish); Gas. aculeatus (stickleback); Ge. cylindronium (sponge); G. gallus (chicken); Ha. discus (abalone); Hy. magnipapillata (hydra); H. sapiens (human); Leu. erinaceus (skate); Lo. gigantea (owl limpet); L. japonicum (lamprey); M. californianus (mussel); Mon. domestica (opossum); Mo. tectiformis (turkey); Mu. musculus (mouse); Mo. tectiformis (ascidian); N. vectensis (sea anemone); O. latipes (medaka); P. lividus (sea urchin); Pe. marinus (lamprey); Sa. kowalevskii (acorn worm); Sc. mediterranea (planarian); S. purpuratus (sea urchin); Su. scrofa (pig); T. tubifex (sludge worm); X. laevis (African clawed frog); X. tropicalis (West African clawed frog).
Fig. 2. Organization of the casp8 genes. (A) Splice junction sites of the casp8 genes are presented. Vertical lines indicate the splice junction sites, defined by comparisons between the genomic and cDNA sequences of *H. sapiens* (human), *M. musculus* (mouse), *G. gallus* (chicken), *X. tropicalis* (West African clawed frog), *D. rerio* (zebrafish), *O. latipes* (medaka), *T. rubripes* (pufferfish), and *D. melanogaster* (fruit fly). The evolutionary tree of listed animals is presented on the left side. (B) The alignment of amino acid sequences corresponding to DED1 (a) and CASc/CASc* (b) of Casp8, Casp10 and c-Flip from *O. latipes* (medaka), *G. aculeatus* (stickleback), *T. rubripes* (pufferfish), *D. rerio* (zebrafish), and *P. marinus* (lamprey). Splice junction sites are indicated as boxes where they fall between two codons, and as circles (phase 1) where they interrupt codons. Asterisks indicate inactive CASc domains. (C) A molecular phylogenetic tree of Casp8 proteins and paralogs in medaka, stickleback, pufferfish, and zebrafish. The arrow indicates the position at which the additional introns were acquired in
Fig. 2. Continued

fish species. (D) Physical map of the region including the *Ci. intestinalis* casp8 gene. The bold arrows indicate the coding region and orientation of the gene. The casp8 and abca1 genes align without a gap. Abbreviated genes: ap1m1, AP-1 complex subunit mu-1; abca1, ATP-binding cassette subfamily A member 1. (E) Separation of casp8 and abca1 mRNAs from a polycistronic pre-mRNA by spliced-leader (SL) trans-splicing. The casp8 cDNA clones terminate at the SL trans-splicing acceptor site “AG”, and the abca1 cDNA clones start after “AG”. (F) The genomic structure of the *S. purpuratus* casp8 gene, C8A/B. The exon–intron composition of the *S. purpuratus* C8A/B gene was determined by referring to the sequence of the related *P. lividus* casp8 gene. Exons of the C8A/B gene are depicted as closed boxes under the physical map. (G) Assessment of the splicing pattern of transcripts from the C8A/B gene. The upper panel shows an alignment of the nucleotide sequences of Exon 10a and 10b. A forward primer that hybridizes to the sequence in the Exon 9 and a reverse primer that hybridizes to the identical regions in Exon 10a and 10b are indicated by arrows. The lower panel shows the result of clone analysis. Twenty-one cDNA clones amplified from a blastula cDNA library were analyzed by sequencing. All clones were transcribed from Exon 10a.
conserved in the DED motifs and protease-like domain, whereas their positions fluctuate in the nonfunctional hinge region (fig. 2A).

Although the number of exons comprising the casp8 transcript varied form 1 (Ciona) to 11 (medaka) across the range of animals studied, 5 intron positions are conserved from coral to human (fig. 2A). Against this background, additional losses and gains of introns have occurred in some lineages. Among the vertebrates, the casp8 exon–intron structure is remarkably similar (seven introns in the coding sequence), although comparison with zebrafish (Danio rerio) and other vertebrates implied the presence of two novel introns in the medaka casp8 locus, one in the first DED domain and the second in the CASc domain (fig. 2A, shown by arrowheads). To further investigate the origins of these novel introns, we compared the splice junction sites of casp8 genes and their paralogs casp10 and cflar genes from medaka (Oryzias latipes), stickleback, pufferfish (Takifugu rubripes), zebrafish, and lamprey (Petromyzon marinus) (fig. 2B). Splice junction sites corresponding to the novel introns were detected in the casp8 genes of stickleback and pufferfish, as well as in medaka, but not in those of zebrafish and lamprey. No additional sites were observed in the paralogous genes. As shown in figure 2C, these data imply that new intron insertions in the casp8 gene postdate the divergence of the acantho-morph fishes (~300 million years ago [Ma]), but predate the medaka divergence (100–150 Ma).

As shown in figure 2A, the coding sequence of the C. intestinalis casp8 gene consists of a single exon, and this is also the case in C. savignyi (data not shown). Analysis of the C. intestinalis genome indicates that the casp8 gene is located between the ap1m1 and abca1 (ATP-binding cassette subfamily A member 1) loci (fig. 2D), and that the casp8 and abca1 genes constitute an operon (fig. 2D). Based on our previous demonstration (Satou et al. 2006), it is likely that both casp8 and abca1 are transcribed as a polycistronic pre-mRNA, which is subsequently processed into monocistronic mRNAs by spliced leader (SL) trans-splicing (fig. 2E). In support of this hypothesis 1) distinct casp8 and abca1 cDNAs were identified, and the 5’-end of abca1 directly abutted on the 3’-end of casp8, indicating a typical Ciona operon structure with no intergenic region, and 2) the splice-acceptor site indicated by “AG” was detected at the junction between these two genes. Thus, during evolution of the ascidian lineage, the casp8 gene lost all introns, and Casp8 protein is now translated from mature mRNA that is generated by processing of a polycistronic transcript.

We identified the casp8 cDNA clone from the cDNA library of the European purple sea urchin (Paracentrotus lividus) and predicted the amino acid sequence (GenBank accession number: ABW34712). On the basis of the sequence of P. lividus Casp8, we searched for the most similar protein in another purple sea urchin (Strongylocentrotus purpuratus) by analyzing the data published in the NCBI genomic database using TBLASTN. We found one candidate gene (LOC585496) whose product had 71% identity to P. lividus Casp8 at the amino acid level (fig. 2F). In this case, a putative additional exon, tentatively numbered as 10b, was identified approximately 8.9 kb downstream from Exon 10a (fig. 2F).

As summarized in supplementary fig. S2A, Supplementary Material online, the possibility that alternatively spliced products are transcribed is of particular interest because, in the case of human CASp8-paralog CASP10, alternate splicing has been shown to generate transcripts in which either one of two last exons are present (Ng et al. 1999). For convenience, these putative products are referred to here as C8A and C8B. In order to determine which exon is used for transcription termination, we examined S. purpuratus cDNA libraries generated using mRNAs isolated from four-cell, blastula, or gastrula embryos or pluteus larvae. We performed PCR amplification from these libraries using a forward primer coming from the Exon 9, and a reverse primer designed against sequence present in both Exon 10a and 10b (fig. 2G, upper panel). The PCR products were isolated from a blastula cDNA library, and the resultant DNA fragments were cloned and sequenced. This analysis revealed that Exon 10a, but not Exon 10b, was present in most transcripts (fig. 2G, lower panel). Consequently, Exon 10a may be more often used for transcriptional termination from the C8A/B gene.

Although arthropods and annelids diverged over 500 Ma (Ayala et al. 1998), D. melanogaster and T. tubifex share a novel intron in the CASc domain that is not present in the range of other animals initially studied (fig. 2A, shown by open and black arrowheads). Further investigation revealed that this intron is also present in another fruit fly (Drosophila virilis) and another annelid, the polychaete worm (Capitella teleta) (supplementary fig. S2B, Supplementary Material online). Although these data support an early origin within the arthropod lineage for this intron, it has apparently been lost in the mosquitos (A. aegypti and Anopheles gambia). However, both mosquito species have retained the lineage-restricted DED1 intron present in fruit flies (gray arrowhead in fig. 2A; supplementary fig. S2C, Supplementary Material online). As shown figure 2A, no casp8 gene is present in the genome of C. elegans, even though other caspase genes such as ced3 are present in this species (Shaham 1998), suggesting that the casp8 gene was lost from this lineage during evolution. Taken together, our data indicate that evolutionary divergence of the casp8 gene has occurred.

Evolutionary Conserved Ability of Casp8 Proteins to Induce Apoptosis

We next investigated the propoptotic potential of Casp8 proteins in cultured mammalian cells. First, the expression of chicken, Xenopus, skate, ascidian, sea urchin, slug worm, mussel, planarian, and coral Casp8 proteins in transfected HEK293T cells was confirmed by immunoblot analysis. To detect these proteins in transfected cells, Casp8 was tagged with a FLAG epitope. In each case, we detected proteins with the correct predicted size (supplementary fig. S3A, Supplementary Material online). Previous studies have demonstrated that overexpression of human or mouse Casp8

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proteins induces cell death without exogenous apoptotic stimulation (Boldin et al. 1996; Muzio et al. 1996; Sakamaki et al. 1998). Therefore, this killing assay was used to examine whether nonmammalian Casp8 proteins could also induce cell death when expressed in mammalian cell lines. Human HeLa cells were used for these experiments, because this cell line is sensitive to extrinsic apoptotic signals in association with CASP8 activation. After transfecting into HeLa cells, the killing activity of nonmammalian Casp8 proteins was assessed by monitoring Venus (a variant of yellow fluorescent protein)-positive cells in the presence or absence of cytokine response modifier A (CrmA) (fig. 3A), a viral product that specifically blocks the protease activity of Casp8 (Zhou et al. 1997). The number of Venus-positive HeLa cells did not change in the presence or absence of CrmA when Casp8 was not exogenously expressed (top left in fig. 3A). By contrast, the number of Venus-positive HeLa cells that coexisted with nonmammalian Casp8 derived from various animals decreased compared with the number of control cells, suggesting that many transfected cells died and disappeared. When the inhibitor CrmA was additively expressed, the number of Venus-positive cells was recovered, indicating that Casp8-induced cell death was prevented. To demonstrate that the cell death occurring in these experiments was caspase-dependent apoptosis, we examined the protease activity of CASP3 in cells expressing sea urchin or mussel Casp8. For this experiment, we generated plasmid constructs in which the DED motifs of Casp8 were replaced with an FK506-binding protein 12 variant (Fv) domain (fig. 3B). Dimerization of an Fv-human CASP8 fusion protein by the synthetic dimerizer AP20187 converts Casp8 to an active form, leading to, in turn, CASP3 activation and apoptotic cell death of transfected cells (Chen et al. 2002; Kominami et al. 2012). The expression of fusion proteins in transfected cells was confirmed by immunoblot analysis (supplementary fig. S3B, Supplementary Material online). In the presence of AP20187, CASP3 activation was observed in both Fv/P.lividus/Casp8- and Fv/M.californianus/ Casp8-expressing transfected cells (fig. 3C, shown by arrows). This suggests that P. lividus and M. californianus Casp8 fusion proteins are converted to the corresponding active forms by dimerization and carry out the processing of endogenous CASP3 that is necessary for its activation. In summary, these data demonstrate that Casp8 proteins from a range of nonmammalian species are able to induce apoptosis in human cells, and that, as in the case of mammalian Casp8, these protease activities are inhibited by CrmA.

Phylogenetically Restricted Casp8-Related Molecules

As mentioned earlier, in addition to a conventional Casp8 ortholog, a clearly related but previously undescribed molecule was identified in the mussel M. californianus. This protein, known here as “Casp8-like” (C8L), has an additional DD between the N-terminal DEDs and C-terminal CASc (fig. 4A). Scanning of the owl limpet (Lottia gigantea) database at Joint Genome Institute (JGI) revealed a presumed ortholog of Mytilus C8L (fgensh2_pg.C_sca_30000086; see fig. 4A), suggesting that related proteins may be distributed more generally throughout the Mollusca. Further analysis showed that the DD of Mytilus C8L is most similar to the DD of FADD (fig. 4B), whereas the DD of Lottia C8L is most similar to the DD of PIDD (p53-induced protein with a DD) (supplementary fig. S4A, Supplementary Material online). FADD is an adaptor protein that binds to a DR, FAS, via DD–DD interactions following apoptotic stimulation (Boldin et al. 1996; Muzio et al. 1996). PIDD was originally identified as a modulator that contains leucine-rich repeat domains at the N-terminus and a DD at the C-terminus (Bock et al. 2012). To define the function of a DD of Mytilus C8L, C8L_DD, we investigated whether its characteristic is similar to that of the DD of FADD. We constructed a fusion protein, C8L(DD)-Venus, consisting of C8L_DD and Venus (fig. 4C) and examined its homotypic interactions with DD-bearing proteins by immunoprecipitation. To investigate whether C8L_DD can associate with the DD of FAS (FAS-DD), we constructed a fusion protein, FLAG-HsFAS(DD)-mCherry, consisting of FAS-DD sandwiched between a FLAG-tag and a red fluorescent protein, mCherry (fig. 4C). We observed C8L_DD in the complex communoprecipitated from transfected cells along with FLAG-HsFAS(DD)-mCherry, indicating that C8L_DD associates with human FAS_DD (fig. 4D). A truncated form of C8L_DD, C8L_tDD, which lacks 22 amino acids in DD corresponding to α5 and α6 helices, also associated with FAS_DD (fig. 4D). In a previous study, we generated a truncated X. laevis FADD, FADD-XIFADD-DD, containing the DD motif as the only functional domain (fig. 4C) (Sakamaki et al. 2004). In the complex immunoprecipitated with FADD-XIFADD-DD, we detected C8L_DD, but not C8L_tDD (supplementary fig. S4B, Supplementary Material online). Thus, by communoprecipitation and immunoblot analyses, we could detect interactions of C8L_DD and DD-containing molecules, and also found differences in binding strength that may be arisen from distinct interaction types of the DD-DD interface as shown in the previous study (Wang et al. 2010). These results suggest that Mytilus C8L molecule has an additional motif that functions as a protein–protein interaction domain (Kersse et al. 2011). In addition, we postulated that C8L diverged from Casp8 over the course of evolution of the phylum Mollusca. To test this idea, we further examined the organization of the casp8-like gene by analyzing its exon–intron structure. We sequenced DNA fragments produced by genomic PCR amplification, and determined the locations of exons in the M. californianus casp8-like gene (fig. 4E). The data indicated that the region containing the DD motif is composed of a single exon, suggesting the strong possibility that a new exon was inserted into an intron of a preexisting casp8 gene. According to the exon shuffling theory, shuffled exon(s) create functional diversity in preexistent proteins, resulting in greater adaptive potential during evolution (Liu and Grigoriev 2004). The modification of Casp8 by exon shuffling might lead to the evolutionary plasticity of Casp8 specificity. Thus, a divergent form of an ancient caspase has appeared over the course of evolution.
FIG. 3. Assessment of the proapoptotic activity of the members of the Casp8 subfamily. (A) Cytotoxicity assays of human HeLa cells expressing Casp8 derived from various animal species. Plasmid constructs encoding FLAG-Casp8 and the pCAG-Venus plasmid were cotransfected into HeLa cells with or without pCAG-CrmA, which encodes the Casp8-specific inhibitor CrmA. After 3 days of culture, cells were washed to remove floating cells, and then fixed; viability was determined by monitoring Venus-positive cells. Both phase-contrast and fluorescence images were captured for each field under the microscope. The species names are as follows: *G. gallus* (chicken); *X. laevis* (African clawed frog); *Leu. erinacea* (skate); *Mo. tectiformis* (ascidian); *P. lividus* (sea urchin); *T. tubifex* (sludge worm); *M. californianus* (mussel); *Sc. mediterranea* (planarian); *S. purpuratus* (sea urchin); and *Ac. millepora* (coral). Scale bars represent 100 μm. (B) Structure of fusion proteins. An Fv domain tagged with HA was fused with the CASc domain of either *P. lividus* or *M. californianus* Casp8. The numbers indicate positions in the amino acid sequence of Casp8 proteins. (C) Detection of CASP3 activation in transfected cells. Transfected cells exogenously expressing both Fv-Casp8 fusion protein and mCherry were incubated with the detection reagent for CASP3 activation after 4 h of treatment with or without AP20187, and green fluorescence in mCherry-positive transfected cells was monitored by microscopy. Images of transfected cells were captured as bright-field (upper panels), red fluorescence for mCherry (middle panels), and green fluorescence for the reagent (lower panels) under the microscope. CASP3-active cells were shown by arrows. Scale bars indicate 10 μm.
FIG. 4. Characterization of Casp8-like molecules. (A) A schematic diagram of *M. californianus* and *L. gigantea* Casp8-like (C8L) proteins. Two DED motifs and a CASc are indicated by boxes, respectively. Both molecules contain an extra DD motif in their middle regions. (B) The alignments of amino acid sequences of DD motifs. A DD of *Mytilus* C8L protein and both DDs of human and mouse FADD are aligned. Identical and similar amino acids in three DD motifs are shown by bold letters, respectively. (C) Schematic structure of fusion proteins. C8L(DD)-Venus was generated by connecting a DD motif of *Mytilus* C8L with the fluorescent protein Venus. C8L(tDD)-Venus carries a truncated DD from which 22 amino acids have been deleted. FLAG-HsFAS(DD)-mCherry bears a DD motif from the cytoplasmic region of human FAS, sandwiched between the FLAG epitope tag and mCherry. FLAG-XIFADD(DD) is a truncated mutant lacking the amino acid region 2–58 of the DED motif of *X. laevis* FADD, fused with a FLAG-tag. (D) Coimmunoprecipitation and immunoblot analysis of physical interactions between *Mytilus* C8L-DD and human FAS. Human HEK293T cells were cotransfected with either pCMV-FLAG/hFAS(DD)/mCherry in conjunction with pCS2 empty vector, pCMV-McC8L(DD)/Venus, or pCMV-McC8L(tDD)/Venus. After 2 days of cultivation, transfected cells were harvested and lysed in a lysis buffer. The cell lysates were immunoprecipitated with an anti-FLAG M2 affinity gel. Immunoblotting of cell lysates was performed by immunoblotting analysis with anti-FLAG, anti-GFP, and anti-actin antibodies. Abbreviations: MWM, molecular weight marker; IP, immunoprecipitation. (E) The exon–intron structure of the casp8-like gene. Exons spanning the coding region are shown as closed boxes. The numbers on boxes show the positions in the coding sequence of the casp8-like cDNA. The vertical lines on the schematic protein structure indicate splice junction sites.
The Coral Casp8 Protein Has Typical Casp8-Type Specificity despite Casp3-Like Residues

As shown in figure 1, one of the distinguishing characteristics of Casp8 proteins is the presence of a glutamine residue (Q361 in human) immediately following the active site cysteine. However, the Casp8 proteins from each of three species of the coral Acropora (Ac. millepora, Ac. digitifera, and Ac. palmata) have arginine (R) residues (figs. 1B and 5A) at the corresponding position, as in other caspase types (supplementary fig. SSA, Supplementary Material online).

Although a tyrosine (Y412 in human) or phenylalanine residue is typically present in the P4 pocket of Casp8 orthologs, the coral sequences have a tryptophan (W) residue at the corresponding position, characteristic of other caspases such as Casp3 (fig. 5A and supplementary fig. SSA, Supplementary Material online). Additionally, in the genomic database of the sea anemone (Nematostella vectensis), a gene analogous to the coral casp8 gene was identified (fig. 5A and supplementary table S2, Supplementary Material online), indicating that these Casp8 sequence characteristics are likely to apply throughout the Hexacorallia (and perhaps to the Anthozoa in general). On the basis of these observations, we hypothesized that the protease activity of the putative coral Casp8 might be Casp3-like, rather than Casp8-like. To test this hypothesis, we generated a recombinant Ac. millepora Casp8 protein in a cell-free protein synthesis system (fig. 5B), and subjected the resultant protein to in vitro cleavage assays. We also generated a mutant version of the coral Casp8, Casp8(C414S), exchanging the cysteine (C414) residue in the active site with serine (S). Recombinant hIL21R (human interleukin-21 receptor) was prepared as a CASP8-recognizing substrate (Akagi et al. 2011). Although hIL21R contains two caspase recognition sites, “VESD344G” and “DTFD491S” (supplementary fig. S5A, Supplementary Material online), active human CASP8 has a strong preference for the first of these, whereas active Casp3A can cleave both sites (supplementary fig. S5C, Supplementary Material online). A recombinant protein derived from wild-type coral Casp8, Casp8-His, promoted autoprocessing and was able to cleave the FLAG-hIL21R substrate in vitro (fig. 5C, lane 1 in both lower and upper panels). In a mixture containing Casp8-His, a FLAG-hIL21R(D491A) mutant protein was cleaved, but neither FLAG-hIL21R(D344A) nor FLAG-hIL21R(DD/AA) mutant was cleaved (fig. 5C, upper panel). Thus, mutation of the VESD site in the substrate, but not the DTFD site, blocks cleavage by the coral caspase, implying that like human CASP8, coral Casp8-His specifically recognizes and cleaves the first of these sites, but not the second, in hIL21R. A protease-deficient mutant, Casp8(C414S)-His, could not cleave the FLAG-hIL21R substrate (fig. 5C, lane 5 in the upper panel). Contrary to expectations, these results showed that the protease activity of this protein resembles human CASP8. This suggests the involvement of additional amino acid residue(s) in the substrate specificity of Casp8 proteins. To understand the substrate-recognition properties of coral Casp8, we constructed a three-dimensional model and compared this with the known structures of human CASP8 and CASP3 (fig. 5D).

By searching for structural resemblance, we found that the arginine residue R312 of coral Casp8 is protruded on the pocket at a similar position to the arginine residue R258 in human CASP8 (fig. 5D, shown by arrow). A previous study demonstrated that this arginine residue of human CASP8 interacts (by hydrogen bonding) with the side chains of the P3 glutamic acid residue of the z-EVD peptide inhibitor (Blanchard et al. 1999). Thus, this structural feature, which may contribute to the stringent specificity of human CASP8, is shared by coral Casp8. In coral Casp8, R312 may therefore enable the specificity observed in the recognition of hIL21R substrate. Taken together, these results indicate that coral Casp8 has a specific protease activity similar to that of human CASP8.

Evolutionary Conservation of the Interaction between Casp8 and FADD Proteins

FADD is an adapter molecule responsible for specifically recruiting Casp8 into the DR complex through homotypic DED–DED interactions (Boldin et al. 1996; Muzio et al. 1996). To investigate the binding ability of DED of Ac. millepora Casp8, we isolated a cDNA encoding a clear homolog of human FADD from the same organism. The Ac. millepora fadd cDNA encodes a 222 amino acid residue protein containing both DED and DD motifs (fig. 6A). In addition to sharing a similar domain structure, the amino acid sequence of the coral FADD protein was 22% identical and 44% similar to that of human FADD (fig. 6B). To examine interactions of coral Casp8 with coral or human FADD without the complication of inducing cell death, we employed protease-deficient Casp8CS mutants and coexpressed p35 in the cells. As the baculovirus p35 protein is known to play a role as a pan-caspase inhibitor (Zhou et al. 1998), this protein was used to retain transfected cells by inhibiting cell death. Coimmunoprecipitation assays clearly indicated that coral Casp8CS (fig. 6C, lane 8). Parallel coimmunoprecipitation experiments demonstrated that coral FADD, as well as human FADD, interacts with zebrafish Casp8CS (fig. 6C, lanes 9 and 12). Although interaction between coral Casp8 and human FADD could not be demonstrated (fig. 6C, lane 11), these experiments not only confirm that the Casp8–FADD interaction is evolutionarily ancient but also suggest a high degree of conservation of the conformation of the DED domain.

The Evolutionary Distribution of Casp8 Proteins in Animals

Finally, to understand the derivation of caspase-8, we used computational analysis to search for the casp8 gene in simpler metazoans. Although searching the JGI database failed to identify caspases in the unicellular choanoflagellate (Monosiga brevicollis), single caspases (presumed orthologs) were detected in two ctenophores, the sea walnut (Mnemiopsis leidyi) and the sea gooseberry (Pleurobrachia pileus) (ML154125a National Human Genome Research Institute.)
Fig. 5. Evolutionary transition in protease activity of coral Casp8. (A) Amino acid sequence alignment corresponding to a protease domain of *Ac. millepora* and *N. vectensis* Casp8 and human and mouse CASP8 and CASP3 proteins. The Casp8-specific and Casp3-specific amino acid residues are indicated by red and blue, respectively. Asterisks indicate the critical histidine and cysteine residues in the active site, an arrowhead is placed on the focused amino acid residue, and an arrow points to the arginine residues (human R258 and *Ac. millepora* R312). The right side numbers of *N. vectensis* Casp8 show the positions in the genomic sequence. (B) Structure of coral Casp8 recombinants. A CASc domain of *Ac. millepora* Casp8 fused with a His- tag, Casp8-His, was produced in a cell-free protein synthesis system. Casp8(C414S)-His is a mutant generated by replacing the cysteine residue in the active site of Casp8-His with serine. (C) Examination of the enzymatic properties of coral Casp8 and its mutant recombinants. After incubation of coral Casp8 and hIL21R recombinants in the mixture, autoprocessing of coral Casp8 and cleavage of hIL21R were detected by SDS-PAGE, followed by immunoblotting with anti-His and anti-FLAG antibodies, respectively. (D) Prediction of the three-dimensional structure of *Ac. millepora* Casp8. Molecular surface representations overlaid on ribbon diagrams of the predicted *Ac. millepora* Casp8 3D model (c), with Casp3 (PDB ID code 2C20) (a) and human CASP8 (PDB ID code 1F9E) (b) are shown. Hydrophobic, positive, and negative patches on the surfaces are indicated in green, blue, and red, respectively. Peptide inhibitors are shown in cyan. The active site residues (H and C) are colored in pale pink. The figure was generated using the Molecular Operating Environment (MOE) software package (Chemical Computing Group, MOE, version 2012.10). The arrow indicates the amino acid residue of human R258 and coral R312.
Acropora Casp8-like (Wiens et al. 2003) and Ac. millepora Casp8 proteins were used as bait. Four distinct caspases (gw1.7.775.1, e_gw1.7.784.1, e_gw1.13.62.1, gw1.13.169.1) were also reported in the Superfamily database for another simple multicellular animal, Trichoplax adhaerens, belonging to the phylum Placozoa. However, each of these five caspases has higher similarity to Casp3 than to Casp8 and do not contain DED motifs (data not shown). We further examined animals belonging to the phylum Porifera. In the freshwater sponge (Ephydatia fluviatilis), we found the casp8 gene (DNA Data Bank of Japan [DDBJ] accession number AB898058) by transcription data analysis. The predicted amino acid sequence indicates that this molecule contains two DED motifs and a CASc in the amino- and carboxyl-terminal regions, respectively (supplementary table S1, Supplementary Material online). The alignment of the CASc with human CASP8 indicated that amino acid residues conserved in other animals are also conserved in sponge Casp8 (fig. 7A). Searching the NCBI genomic database for the sea sponge (Amphimedon queenslandica) using the E. fluviatilis Casp8 protein sequence resulted in identification of a putative ortholog (AMPQUscaffold_13479: 285,603–290,623) (fig. 7A and supplementary table S2, Supplementary Material online). Thus, casp8 genes are present in a number of diploblastic Phyla and appear to predate eumetazoan origins.

Furthermore, to confirm the distribution of Casp8 proteins in animals, we conducted molecular phylogenetic analyses on Casp8 and its paralogous proteins, Casp10, Casp18, c-Flip and CARD (caspase-recruitment domain)-casp8. In previous studies, we demonstrated that these paralogous molecules diverged from Casp8 by gene duplication during...
Fig. 7. The evolutionary analyses of the distribution of Casp8 proteins in animals. (A) Alignment of amino acid sequences corresponding to a CASc of two species of sponge (E. fluviatilis and Am. queenslandica) and human Casp8 proteins. Identical and similar amino acids in all three proteins are shown by bold letters, respectively. Asterisks indicate the critical histidine and cysteine residues in the active site. The right side numbers of the Am. queenslandica Casp8 sequence show the positions in the genomic sequence. (B) Molecular phylogenetic analysis of Casp8 and its paralogous proteins. The molecular phylogenetic tree of Casp8 and its paralogs, Casp10, Casp18, c-Flip, and CARD-casp8, was constructed by RAxML based on an alignment of the protease and protease-like domains. The Casp8 proteins and paralogs in the tree are listed in supplementary tables S1 and S2, Supplementary Material online. Casp3 and Casp3-like proteins, are also listed in supplementary table S2, Supplementary Material online, were used as outgroups to root the tree. The number by a node indicates the percentage of support for that branching pattern based on a bootstrap method with 1,000 replicates and
vertebrate evolution (Sakata et al. 2007; Sakamaki and Satou 2009). In addition to known amino acid sequences, we searched for deposited homologs in a set of sequence databases to identify new members in this family. As listed in supplementary table S2, Supplementary Material online, several Casp8 and related proteins were identified in those databases. We generated a molecular phylogenetic tree of these Casp8 and related proteins using the Randomized Accelerated Maximum Likelihood (RAxML) program (Stamatakis 2006) based on an alignment of Casc domains. The tree clearly indicated that Casp8 proteins and their paralogous proteins form a large subfamily (fig. 7B). According to amino acid sequence characteristics, members of this subfamily could be further divided into several subgroups, i.e., vertebrate Casp8s, Casp10s, Casp18s, c-Flips, fish CARD-casp8s, and invertebrate Casp8s. However, the constructed phylogenetic tree and the taxonomic tree do not completely correspond with each other. In particular, a subclade consisting of c-Flip proteins derived from vertebrates diverged from other subclades (including vertebrate Casp8 and its paralogous proteins) earlier than expected. This result may be explained by a higher rate of evolution of cflar genes as a consequence of their loss of protease function (manuscript in preparation). Thus, based on a phylogenetic tree of the Casp8 subfamily, Casp8 proteins are widely distributed in various animals.

The Ubiquity and Diversity of Casp8

Although first discovered in mammals, the presence of Casp8 in sponges and cnidarians indicates a early origin for this class of caspase and its near ubiquitous distribution throughout the animal kingdom implies that it has critical functions. In the mouse, which has lost the paralogs casp10 and casp18, defects in casp8 cause embryonic lethality (Varfolomeev et al. 1998; Sakamaki et al. 2002), which is consistent with Casp8 activity being essential for mammalian embryonic development. To every rule, there is an exception, however, and the absence of casp8 from C. elegans is a case in point. It has been shown that gene loss and sequence divergence are particularly extensive in the model ecdysozoans, C. elegans and D. melanogaster (Kortschak et al. 2003). As well as losing casp8, C. elegans has highly derived and dispersed HOX genes (Abboobaker and Blaxter 2003), highlighting the atypical nature of this animal. Therefore, we suggest that Casp8 is utilized as a central component of the metazoan molecular toolkit.

One of the key interactions of mammalian Casp8 is with FADD, and it is interesting to observe that the distributions of these two genes across the animal kingdom correspond perfectly. In every animal in which Casp8 was present, including sponges and cnidarians, we were able to identify a fadd gene (data not shown). Intriguingly, as well as the casp8 gene, the nematode C. elegans has lost fadd (data not shown). These lines of evidence suggest that the primary role of FADD is its involvement in conserved Casp8 functions. In support of this idea, we were able to demonstrate Casp8–FADD interactions mediated through their DED folds (fig. 6), even between heterotypic proteins separated by at least 500 Ma of evolution. In mammals, both Casp8 and FADD are indispensable for apoptosis mediated through DRs (Lavrik and Krammer 2012). A recent report documented the presence of DRs in corals and the induction of apoptosis in coral cells by a human death ligand, TNFα, implying that the extrinsic apoptosis machinery is present in Cnidaria (Quistad et al. 2014). This report is also consistent with the hypothesis that both coral Casp8 and FADD function in the transmission of apoptotic signals received via these DRs. Taken together, our and these studies suggest that the programmed cell death mechanism was already in place in the eu metazoan ancestor more than 500 Ma and predates the divergence of the bilaterian and cnidian lineages.

The apparent paradox of Casp8-like specificity despite the presence of Casp3-like “diagnostic” amino acid residues observed in the case of coral Casp8 protein (table 1 and fig. 5) highlights the need to analyze three-dimensional structural characteristics of Casp8 proteins from a more comprehensive range of animals in order to better understand structure-function relationships in this family. Moreover, investigating the broader functional context of Casp8 proteins in nonbilaterian animals may also shed some light not only on the evolution of function in this protein family but also the evolution of apoptotic cell death.

Materials and Methods

Reagents

Antibodies were obtained from the indicated suppliers: anti-FLAG (M2, Sigma-Aldrich, St. Louis, MO), anti-HA (HA124, Nacalai Tesque, Kyoto, Japan), anti-GFP (598, MBL, Nagoya, Japan), and anti-HA (Neova, Tokyo, Japan). Reagents and procedures for the preparation of neuronal cell cultures, including the generation of lentiviral vectors harboring human Casp8 (Yang et al. 2008), are described elsewhere. For many experiments, we utilized HEK 293T cells and HEK 293T cells expressing human FADD or human c-FLIP. Immunohistochemical staining for the detection of Casp8 and FADD was performed as described previously (Yang et al. 2008).
Japan), anti-His (Wako Pure Chemical Industries, Ltd., Osaka, Japan), anti-actin (MAB1501R, Chemicon International Inc., Temecula, CA), and HRP-conjugated anti-mouse IgG antibody (Cell Signaling Technology, Danvers, MA). The synthetic FK506 analog AP20187 was a gift from ARIAD Pharmaceuticals (Cambridge, MA).

**Database Search**


**Construction of cDNA Libraries**

cDNA libraries of the sea urchin *S. purpuratus* were prepared as described previously (Yajima et al. 2012). Embryos of each developmental stage were collected and subjected to total RNA extraction using the RNeasy Mini kit (Qiagen, Valencia, CA). The RNA was reverse-transcribed into cDNA using the TaqMan RT-PCR kit (Roche, Indianapolis, IN).

**Isolation of *casp8* cDNAs**

c*asp8* cDNAs were isolated from cDNA libraries of the lancelet *B. floridae* and lamprey *L. japonicum* by PCR amplification using appropriate primer sets.

**Preparation of Genomic DNAs and Isolation of DNA Fragments by PCR Amplification**

Genomic DNAs were isolated from the stages 15–18 embryos of *T. tubifex* and the gill of *M. californianus* using the GenomicPrep Cells and Tissue DNA Isolation Kit (Amersham Pharmacia Biotech, Piscataway, NJ) or the Tissue NucleoSpin kit (Macherey-Nagel, Bethlehem, PA). Genomic DNA was also prepared from *Ac. millepora* sperm essentially as previously described (McMillan and Miller 1989). Genomic DNA fragments were amplified by PCR using appropriate primer sets based on the corresponding *casp8* cDNA sequences and the resultant products were cloned into the cloning vector pCR2.1 (Life technologies, Carlsbad, CA) or pTA2 (TOYOBO, Tokyo, Japan).

**DNA Sequencing**

The nucleotide sequence of EST cDNA clones, including *S. mediterranea* (Zayas et al. 2005), isolated cDNAs, and genomic DNA fragments, was confirmed using Taq DyeDeoxy Terminator Cycle sequencing (Applied Biosystems Inc., Foster City, CA) on the automated DNA sequencer (3130XL Genetic Analyzer, Applied Biosystems Inc.).

**Homology Modeling of Acropora Casp8**

A three-dimensional (3D) model of a CASc domain of *Ac. millepora* Casp8 protein was built based on alignments calculated by FORTE, a profile–profile comparison method for protein structure prediction (Tomii and Akiyama 2004). Several 3D models of *Ac. millepora* Casp8 were constructed using MODELLER9.8 (Eswar et al. 2006), based on 4j8 (Vickers et al. 2013) and 4jr1 (Thomsen et al. 2013) with the highest Z-scores, and side-chain conformations of the model were optimized using SCWRL4.0 (Krivov et al. 2009). The model of *Ac. millepora* Casp8 was selected and validated using VERIFY3D (Eisenberg et al. 1997).

**Alignment and Tree Building**

Amino acid sequences were aligned using Clustal W with BLOSUM alignment matrices. To construct a molecular phylogenetic tree, we collected 72 published or predicted amino acid sequences of CASc and CASc-like domains from Casp8 and its paralogous proteins, Casp10, Casp18, c-flip and CARD (caspase-recruitment domain)-casp8 from several databases for vertebrates and invertebrates (supplementary tables S1 and S2, Supplementary Material online). For phylogenetic reconstruction the amino acid sequences were multiply aligned using Prin (Gotto 1996), which optimizes a weighted sum-of-pairs score by an iterative procedure. We removed poorly aligned regions from multiple sequence alignments using trimAl (Capella-Gutierrez et al. 2009). The phylogenetic tree was constructed from the entire tailored alignment (236 aa) by RAxML (Stamatakis 2011). For molecular phylogenetic reconstruction the amino acid sequences were multiply aligned using Prin (Gotto 1996), which optimizes a weighted sum-of-pairs score by an iterative procedure. We removed poorly aligned regions from multiple sequence alignments using trimAl (Capella-Gutierrez et al. 2009). The phylogenetic tree was constructed from the entire tailored alignment (236 aa) by RAxML (Stamatakis 2011) using the CAT-GTR amino acid substitution model and visualized using MEGA 5 (Tamura et al. 2011).

**Generation of Plasmid Constructs**

To express Casp8 protein derived from several animals in cultured human cells, plasmid constructs were generated. Plasmids, pCS2-FLAG/GgCasp8, pCMV-FLAG/XICasp8, pCMV-FLAG/LeCasp8, pCMV-FLAG/MtCasp8, pCMV-FLAG/PIcasp8, pCMV-FLAG/TrCasp8, pCS2-FLAG/McCasp8, pCMV-FLAG/SmCasp8, and pCMV-FLAG/AmCasp8, were generated by inserting the FLAG-tagged cDNA sequences, obtained by PCR amplification, from *G. gallus*, *X. laevis* (African clawed frog), *Leu. erinacea*, *Molgula tectiformis* (ascidian), *P. lividus*, *T. tubifex*, *M. californianus*, *S. mediterranea*, and *Ac. millepora*, respectively.
into expression vector pCS2, pcDNA1 (Invitrogen, Carlsbad, CA), or pCMV-SPORT6 (Invitrogen). Plasmids pCAG-p35 and pCAG-CrmA, for the inhibition of protease activity of caspases and the specific inhibition of Casp8 activation, respectively, were generated previously (Sakamaki et al. 2004; Sakata et al. 2007). Plasmids, pCAG-Venus and pCAG-mCherry, for detection of transfected cells, were generated previously (Sakamaki et al. 2005; Sakamaki et al. 2012).

To express Fv-Casp8 fusion proteins consisting of the FK506-binding protein 12 variant (Fv) and the Casp8 CASc protease domain from either the sea urchin P. lividus or the mussel M. californianus, plasmids pCS2-Fv/P.lividus_Casp8 and pCS2-Fv/M.californianus_Casp8 were generated by fusing a ha-Fv DNA fragment from pSH1/HA-Fvls-caspase8 (Chen et al. 2002), a gift from Dr. J. Wang, with the coding sequence for the protease domain amplified by PCR, and cloning the resultant sequence into the pCS2 expression vector.

To examine interactions between Casp8 and FADD proteins, plasmid pCMV-FLAG/AmCasp8CS was generated. Briefly, Ac. millepora casp8 cDNA encoding a protease-deficient CS mutant was generated by replacing the cytotoxic residue in the protease active site with serine; mutagenesis was performed by exchanging a PCR-amplified DNA fragment containing the mutant sequence. This cDNA was inserted along with the sequence encoding the FLAG-tag into the expression vector pCMV-Tag2 (Agilent Technologies, Santa Clara, CA). Plasmid pME-FLAG/DrCasp8CS, encoding both FLAG-tag and protease-deficient Da. rerio Casp8 was generated previously (Sakata et al. 2007). Both pME-HA/AmFADD and pME-HA/HsFADD were generated by inserting the Ac. millepora fadd cDNA or the human FADD cDNA fused with the sequence encoding the HA tag into the pME18S expression vector.

To assess the interactions between M. californianus Casp8-like protein (CBL) and vertebrate DD-bearing molecules mediating their DD domains, plasmid pCMV-McC8L/DD)/Venus was generated by fusing a PCR-amplified DNA fragment covering a region containing a DD motif (413–498) of CBL, with the cDNA for Venus (Nagai et al. 2002); the resulting sequence was inserted into the pCMV expression vector. Plasmid pCMV-McC8L(tDD)/Venus was generated by replacing the original sequence with a DNA fragment corresponding to a truncated DD motif (413–476). Plasmid pCMV-FLAG/AmHsFAS/DD)/mCherry was generated by fusing a PCR-amplified DNA fragment, containing a DD motif (217–315) from human FAS, with sequences encoding the FLAG-tag and mCherry (Shaner et al. 2004), and inserting the resultant sequence into pCMV. The pCS2-FLAG/XfFADD/DD) plasmid, which encodes an X. laevis FADD mutant deleting a DEVD motif fused with a FLAG-tag, was generated previously (Sakamaki et al. 2004).

Cell Culture and Transfection

Human cervical carcinoma HeLa cells and embryonal kidney HEK293T cells were cultured in Dulbecco’s Modified Eagle’s medium with 10% fetal calf serum. Transfection of plasmid DNAs into cells was performed using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions.

Cell Death Assays

For detection of proapoptotic activity of Casp8 proteins, HeLa cells were transiently transfected with plasmids encoding FLAG-tagged Casp8 proteins, with or without pCAG-CrmA. Plasmid pCAG-Venus was also cotransfected to permit distinction between untransfected and transfected cells. At 3 days after transfection, fragile and vulnerable cells were washed out with phosphate buffered saline (PBS) and the remaining cells were fixed in PBS containing 3% formaldehyde. Phase-contrast and fluorescent images were acquired using a fluorescent microscope (DMI2E, Leica Microsystems, Wetzlar, Germany).

Detection of CASP3 Activation

HeLa cells exogenously coexpressing Fv-Casp8 protein and mCherry were treated with 100 nM AP20187 for 4 h by addition of this compound into the growth medium after 4 days’ cultivation. To detect Casp3 activation in transfected cells, cells were further incubated in the medium containing 5 μM CellEvent Caspase-3/7 Green Detection Reagent (Invitrogen) for 30 min; bright-field and red and yellow fluorescence images were captured by microscopy.

Immunoblot and Immunoprecipitation Analyses

To detect ectopic expression of Casp8 proteins in cultured mammalian cells, plasmids containing cDNAs encoding FLAG-tagged Casp8 were transiently transfected into HEK293T cells with pCAG-p35. After 48 h of cultivation, transfected cells were lysed in the lysis buffer (50 mM Tris-HCl [pH 7.5], 20 mM MgCl₂, 0.5% Nonidet P-40, 150 mM NaCl and the protease inhibitor cocktails [Nacalai Tesque]). Similarly, to detect Fv-Casp8 fusion proteins in transfected cells, cells were lysed in a lysis buffer 48 h after transfection with plasmid constructs. After cell debris was removed by centrifugation, all cell lysates were denatured in the Laemmli sample buffer, resolved by SDS-PAGE, and analyzed by immunoblotting with anti-FLAG, anti-HA, and anti-actin antibodies. After incubation with an HRP-conjugated antimouse IgG antibody, immune complexes were visualized with Immobilon Western (Millipore Corporation, Billerica, MA) using a luminescent image analyzer (LAS-3000, Fujifilm, Tokyo, Japan).

To detect the physical association of Casp8 proteins with Ac. millepora FADD or human FADD, pME-FLAG/Ac. millepora FADD or human FADD, pME-HE/Ac. millepora FADD, pME-HE/HsFADD, and pME-HE/HsFADD were generated by inserting the Ac. millepora fadd cDNA or the human FADD cDNA fused with the sequence encoding the HA tag into the pME18S expression vector.

To detect the interactions between M. californianus Casp8-like protein (CBL) and vertebrate DD-bearing molecules mediating their DD domains, plasmid pCMV-McC8L/DD)/Venus was generated by fusing a PCR-amplified DNA fragment covering a region containing a DD motif (413–498) of CBL, with the cDNA for Venus (Nagai et al. 2002); the resulting sequence was inserted into the pCMV expression vector. Plasmid pCMV-McC8L(tDD)/Venus was generated by replacing the original sequence with a DNA fragment corresponding to a truncated DD motif (413–476). Plasmid pCMV-FLAG/AmHsFAS/DD)/mCherry was generated by fusing a PCR-amplified DNA fragment, containing a DD motif (217–315) from human FAS, with sequences encoding the FLAG-tag and mCherry (Shaner et al. 2004), and inserting the resultant sequence into pCMV. The pCS2-FLAG/XfFADD/DD) plasmid, which encodes an X. laevis FADD mutant deleting a DEVD motif fused with a FLAG-tag, was generated previously (Sakamaki et al. 2004).
Preparation of Recombinant Proteins with a Cell-Free System

To prepare recombinant Ac. millepora Casp8 and its mutant proteins with His-tag at both ends, a DNA fragment encoding the CASc domain of Ac. millepora Casp8, amplified by PCR, was cloned into the expression vector pEU as previously described (Sawasaki et al. 2002). For the preparation of mutant protein, the DNA fragment encoding Casp8(C41S) mutant was generated by amplifying the region corresponding to a CASc domain in pCMV-Flag/AmCasp8CS, and then cloned with a His-tag into pEU. To synthesize recombinant human interleukin-21 receptor (hIL21R) protein, the transcription template with the sequence encoding a FLAG-tag was prepared by split-primer PCR method (Sawasaki et al. 2002) using the pcDNA3.2-hIL21R plasmid (Akagi et al. 2011). Caspase cleavage-deficient hIL21R mutants were also generated using the PrimeSTAR Mutagenesis Basal kit (Takara Bio, Otsu, Japan). To produce recombinant proteins in a cell-free protein synthesis system, plasmid constructs were transcribed from the SP6 promoter, and the resulting transcripts were translated in wheat embryo extracts (WEPRO-1240, CellFree Sciences, Matsuyama, Japan). Recovery of reactive products was confirmed by SDS-PAGE and followed by immunoblot analysis with appropriate antibodies.

In Vitro Cleavage Assays with Recombinant Proteins

Mixtures of each cell-free-synthesized FLAG-hIL21R and each His-purified Casp8 were incubated in the caspase cleavage buffer (20 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid [HEPES] [pH 7.8]), 100 mM NaCl, 10 mM dithiothreitol [DTT], 1 mM ethylene-diamine-tetra-acetic acid [EDTA], 10% sucrose) at 30 °C for 2 h. These reaction mixtures were boiled in the Laemmli sample buffer for 5 min, resolved by SDS-PAGE, and immunoblotted with the appropriate antibodies.

Acknowledgments

We are greatly thankful to M. Kato (RIKEN, Japan), N. Ueno, and K. Naruse (National Institute for Basic Biology, Japan), D. W. Towle (Mount Desert Island Biological Laboratory, USA), B. Freeman (Harvard Medical School, USA), P. Newmark (University of Illinois, USA), M. Medina (University of California, Merced, USA) for providing cDNA clones, D. Barnes (Mount Desert Island Biological Laboratory, USA), S. Kuratani (RIKEN, Japan), H. Takahashi (National Institute for Basic Biology, Japan), and T. Fujisawa (NIG, Japan) for providing cDNA libraries. J. Wang (Baylor College of Medicine, USA) for the gift of a plasmid construct, and ARIAD Pharmaceuticals for dimerizer AP20187. We also thank N. Iwabe and H. Kato (Kyoto University, Japan) for discussion, T. Todo and T. Ishikawa (Osaka University, Japan) for technical supports, and J. H. Postlethwait (University of Oregon, USA) for critical reading.

The complete sequences of the Gas. aculeatus, Lei. erinacea, L. japonicum, B. floridae, C. intestinalis, C. savignyi, T. tubifex, M. californianus, Sc. mediterranea, A. palma, and Ac. millepora casp8 cDNAs (GenBank accession numbers: HQ285317, EU719070, GQ405966, FJ937786, JP920434, JQ920435, EU719069, FJ937785, KF644848, GU808361, KJ639816), and Du. japonica casp8 and E. fluviatilis casp8 transcript (DDBJ accession numbers: AB897787 and AB898058), the M. californianus casp8-like cDNA (GenBank accession number: FJ937784), the Gas. aculeatus casp10 cDNA (GenBank accession number: DQ010055), G. gallus, L. gallus, A. mellifera, and O. latipes eflar/cflp cDNAs (GenBank accession numbers: HQ687225, AY519261, HQ285319, FJ937783), the Ac. millepora fadd cDNA (GenBank accession number: KJ639817), the T. tubifex and Ac. millepora casp8 genes (GenBank accession numbers: FJ980021, KJ639818), and the M. californianus casp8-like gene (DDBJ accession number: AB924644) have been deposited. This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas, 20017016 (K. Sa.) and by Platform for Drug Discovery, Informatics, and Structural Life Science (K. T.) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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