The pyrin domain was identified recently in multiple proteins that are associated with apoptosis and/or inflammation, but the physiological and molecular function of these proteins remain poorly understood. We have identified Caspy and Caspy2, two zebrafish caspases containing N-terminal pyrin domains. Expression of Caspy and Caspy2 induced apoptosis in mammalian cells that were inhibited by general caspase inhibitors. Biochemical analysis revealed that both Caspy and Caspy2 are active caspases, but they exhibit different substrate specificities. Caspy, but not Caspy2, interacted with the zebrafish orthologue of ASC (zAsc), a pyrin- and caspase recruitment domain-containing protein identified previously in mammals. The pyrin domains of both Caspy and zAsc were required for their interaction. Furthermore, zAsc and Caspy co-localized to the “speck” when co-transfected into mammalian cells. Enforced oligomerization of zAsc, but not simple interaction with zAsc, induced specific proteolytic activation of Caspy and enhanced Caspy-dependent apoptosis. Injection of zebrafish embryos with a morpholino antisense oligonucleotide corresponding to caspy resulted in an “open mouth” phenotype associated with defective formation of the cartilaginous pharyngeal skeleton. These studies suggest that zAsc mediates the activation of Caspy, a caspase that plays an important role in the morphogenesis of the jaw and gill-bearing arches.

Apoptosis, a morphologically distinguished form of programmed cell death, is critical during development and tissue homeostasis and plays a role in the pathogenesis of a variety of diseases. The morphological features of apoptosis and demise of the cell are because of the cleavage of structural and non-structural proteins by a family of proteases called caspases (for review, see Ref. 1). Upon stimulation by apoptotic stimuli, proximal caspases are activated and cleave downstream effector caspases leading to an amplification of proteolytic activity within the dying cells. Activation of proximal caspases requires upstream regulators that have domains homologous to those present in the N-terminal prodomains of proximal caspases, namely death effector domains (DEDs) and caspase recruitment domains (CARDs). Homophilic interactions between the CARDs or DEDs of upstream regulators and proximal caspases mediate caspase activation (1). For example, homophilic interactions between the DEDs of Fas-associated death domain protein and procaspase-8 and between the CARDs of Apaf-1 and procaspase-9 are essential for caspase activation (2). Activation of proximal caspases is thought to be induced via proximity of their catalytic subunits, a process that is mediated through oligomerization of their upstream regulators. For example, Fas-associated death domain protein and Apaf-1 contain a death domain (DD) and nucleotide-binding domain, respectively, that mediate their oligomerization in a ligand-dependent manner (1).

Bioinformatic and protein structure analyses have revealed that the CARD, DED, and DD share similar structural and functional features (3–6). These domains are found not only in apoptosis regulators but also in proteins that are associated with inflammation, cell cycle regulation, and cytoskeletal organization (4, 7). Thus, these α-helical-rich domains mediate homophilic interactions between signaling components of diverse signaling pathways, most notably those involved in apoptosis and inflammation (8–11).

The pyrin domain (also called DAPIN, PYRIN, and PAAD) was originally found in pyrin, the product of the familial Mediterranean fever-associated gene (12), and in ASC, a component of the “speck,” a granular structure induced in the cytosol of certain apoptotic cells (13). Subsequently, pyrin domains were also identified in multiple proteins whose molecular functions are largely unknown (14–19). Computer modeling has predicted that the pyrin domain is α-helix-rich and structurally related to the CARD, DED, and DD. Furthermore, the pyrin domain mediates homophilic interactions between pyrin domain-containing proteins (16, 20). However, the functional significance of these pyrin-pyrin homophilic interactions is presently poorly understood.
The vertebrate zebrafish (Danio rerio) is a model organism whose genome encodes the great majority of the components that mediate apoptosis and inflammation in mammals including humans (10, 21). Thus, analysis of zebrafish components should provide insight into the mechanisms that mediate apoptosis and inflammation in mammalian systems. Here we report the identification and initial characterization of the zebrafish orthologue of ASC (zAsc) and two zebrafish pyrin-containing caspases, Caspy and Caspy2. We provide evidence that zAsc associates with and activates Caspy through a homophilic pyrin-pyrin domain interaction. In addition, we show that Caspy is required for normal development of the jaw and pharyngeal arches.

EXPERIMENTAL PROCEDURES

Identification of Caspy, Caspy2, and zAsc cDNAs and Preparation of Expression Plasmids—Zebrafish genes with homology to the pyrin domain of human ASC were searched in public data bases of ESTs, using the program TBLASTN (National Center for Biotechnology Information). The nucleotide sequences of the EST clones AW174631, AI384922 (Caspy), and BF156256 (Caspy2) were determined by dideoxy sequencing. The entire open reading frames of zebrafish Caspy, Caspy2, and zAsc were amplified by polymerase chain reaction from a dideoxy sequencing. The EST clones AW174631 and BF156256, respectively, were cloned into pEGFP-C2, pDsRed-N1 (Clontech), pFLAG-CMV-4 (Sigma), pcDNA3, pcDNA3-Myc, pcDNA3-HA (22), and pcDNA3-Fpk3-Myc (23) to generate pEGFP-Caspy, pcDNA3-Caspy-Myc, pcDNA3-Caspy2-Myc, pcDNA3-zAsc-Myc, and pcDNA3-zAsc-Fpk3-Myc. pcDNA3-Caspy-(1–100)-Myc and pcDNA3-Caspy2-(1–100)-Myc were generated from the DNA fragments corresponding to the regions between residue 1 and 100 and between residue 101 and 383, respectively; in Caspy subcloned in pcDNA3-Myc, pcDNA3-zAsc-(1–103)-Myc and pcDNA3-zAsc-(104–203)-Myc were generated from the DNA fragments corresponding to the regions between residue 1 and 103 and between 104 and 203, respectively, in zAsc subcloned in pFLAG-CMV4. The plasmids pCDNA3-Caspase-9-FLAG, pCDNA3-Apaf-1XL-Myc, and pEFBOS-β-gal have been described (9, 24).

Genetic Mapping and in Situ Hybridization—Genetic mapping was performed on the heat shock doubled haploid meiotic mapping panel by single strand conformation polymorphism as described (25), using the following mapping primers: caspy, 5’-GAATGAAAAATGGCGCACAGT-TACCTA-3’ and 5’-AGGGTCCTCCAATTCGCTAAA-3’; caspy2 Set1, 5’-TCAAGGGGAAACAGACAGG-3’ and 5’-TAAAGCCCAAGCAATAC-AATAAA-3’; Set2, 5’-CAGCAGGCAGCTCCAATCT’ and 5’-GACCGGAGACCTCAGGATCAGTCCAATCT-3’; Set3, 5’-CAGCAGGCAGCTCCAATCT’ and 5’-TCCGGGTTCTCCGTCTGCTG-3’.

For in situ hybridization, digoxigenin-labeled antisense RNA probes were synthesized from full-length cDNAs using an in vitro transcription kit (Promega). As a control, sense RNA-labeled probes were synthesized and used for hybridization as above. In situ hybridization and development of whole-mount zebrafish embryos were performed as described (26).

Transfection, Expression, Immunoprecipitation, and Immunodetection of Tagged Proteins—Cells were transfected, and immunoprecipitation assay was performed as described with slight modifications (27). The total amount of transfected plasmid DNA was adjusted with pcDNA3 to always be the same within individual experiments. 1 × 10⁶ COS-7 cells were transfected with 2.4 μg of expression plasmids using LipofectAMINE-PLUS reagent (Invitrogen) according to the manufacturer’s instructions. The supernatant was immunoprecipitated with 20 μl of protein G-Sepharose 4B (Amersham Biosciences) conjugated with anti-Myc polyclonal antibody (Santa Cruz Biotechnology, Inc.). Immunoprecipitated proteins were subjected to 15% SDS-polyacrylamide electrophoresis and detected by Western blotting using anti-FLAG monoclonal antibody M2 (Sigma). For cellular localization assay, COS-7 cells were transiently cotransfected with pEGFP-zAsc and pEGFP-Caspy. For corexpression assay, COS-7 cells were transiently cotransfected with pDsRed-zAsc and pEGFP-Caspy. After 24 h, cells were fixed
with 70% ethanol. Signals were detected by immunofluorescence microscopy.

Cell Death Assays and Caspase Enzymatic Assay—Cell death assays with 293T cells were performed as described (28). For Fig. 4B, transfected cells were incubated with 20 \( \mu \)M zVAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-(OMe)-fluoromethylketone) or left untreated. FLAG-Caspy and Caspy2-Myc were purified by immunoprecipitation from 293T cells transfected by pcDNA3-FLAG-Caspy and pcDNA3-Caspy2-Myc, respectively, as described above, and caspase enzymatic assays were performed as described (29) using caspase-type-specific substrates (Calbiochem, La Jolla, CA) indicated in the figure legends. The concentration of the cleavage products was estimated from comparison with the fluorescence of AMC (7-amino-4-methyl-coumarin) or AFC (7-amino-4-(trimethyl-fluoromethyl) coumarin) standards.

Microinjection of Morpholino Oligonucleotides and Phenotypic Analyses—Each morpholino (Gene Tools, LLC) was resuspended in sterile water to a concentration of 1 mM. For injections, the stock solution was diluted to 250 nM in Danieau buffer. A 3-ml volume of the solution was injected into one to four cell-stage embryos at the yolk and cytoplasm interface. The sequences of caspy and control morpholino oligonucleotides were 5'-GCCATGTTAGCTCAGGGCGCTGAC-3' and 5'-CCTCTTACCTCAGTTACATTATA-3', respectively. For the morphological assay, larvae were euthanized in tricane, fixed in 4% paraformaldehyde, and washed for 3 min in acid-alcohol (0.37% HCl, 70% ethanol). The fixed larvae were stained for 1 h with 0.1% Alcian blue. After rinsing in ethanol, animals were suspended in 50% glycerol, 0.25% KOH overnight. Stained preparations were clarified with 1% H2O2 and mounted in glycerol-KOH.

RESULTS

Identification of a Novel Class of Caspases with Pyrin Domain—To identify novel pyrin domain-containing proteins in zebrafish, we screened the zebrafish GenBank™ EST data base for cDNAs encoding amino acid sequences with homology to the pyrin domain (amino acids 1–91) of human ASC by the program TBLASTN. We identified the zebrafish orthologue of human ASC (referred to here as zAsc) and two zebrafish caspases (designated here as Caspy and Caspy2). Sequence analysis of zAsc cDNA revealed that it encodes a protein of 203 amino acid residues composed of a N-terminal pyrin domain linked to a C-terminal CARD as reported for its mammalian orthologues (GenBankTM accession numbers were as follows: human ASC, AB023416; mouse ASC, AB033249; bovine ASC, AB050006; rat ASC, AB053165). Caspy and Caspy2 contained N-terminal pyrin domains and C-terminal caspase catalytic.
domains (Fig. 1A). The N-terminal pyrin domain of Caspy was most homologous to that present in zAsc (87% similarity) whereas that of Caspy2 was most homologous to that of human Cryopyrin/PYPAF1 (46% similarity), a pyrin-domain containing Nod-family protein (30, 31). Comparison of these zebrafish and human caspases showed that the catalytic domains of Caspy and Caspy2 share highest homology with those of human caspase-1 and caspase-5, respectively (54 and 57% similarity, respectively). Caspy and Caspy2 contained conserved His and Cys amino acid residues, which are essential for proteolytic catalysis (depicted by arrows in Fig. 1A), and also conserved residues, which are essential for interaction with critical core residues of substrates, suggesting that both Caspy and Caspy2 are enzymatically active. Notably, Caspy, like human caspase-1, has His at position 318, corresponding to the residue at position 342, which binds the specific P3 residue of the substrate (Ala of YVAD) in human caspase-1 (32), whereas Caspy2 and human caspase-5, which can not digest peptides carrying Ala residue at P3 (33), have Asn and Asp residues, respectively, at the corresponding positions (arrowhead; see Fig. 1A).

Genomic Mapping of asc, caspy, and caspy2—Genomic mapping experiments using the heat shock doubled haploid meiotic mapping panel revealed that caspy and caspy2 are located on linkage groups 16 and 1, respectively (Fig. 2). Human CASP1 and CASP5, which are homologues of caspy and caspy2, are located as tandem genes on human chromosome 11. We did not find any conclusive evidence supporting conserved syntenies between the zebrafish and human regions containing the zebrafish and human caspase genes (Fig. 2). Notably, caspy was adjacent to asc on linkage group 16 (z066003336 at www.ensembl.org/Danio_rerio/blastview), whereas the human orthologue of asc is located on chromosome 16. Sequence analysis also showed that the pyrin domain of Caspy2 exhibited significant homology not only to pyrin domains but also to the CARDs of *Xenopus* caspase-1 (49% similarity) and bovine caspase-13 (45% similarity, previously known as human caspase-13) (Fig. 1B). Thus, both the pyrin domain and CARD are predicted to be α-helix rich and to share a similar organization in their predicted secondary structure (Fig. 1B).

*zAsc*, *Caspy*, and *Caspy2* mRNA Exhibit a Similar Pattern of Expression in the Zebrafish Embryo—We performed *in situ* hybridization to assess the expression of zAsc, Caspy, and Caspy2 mRNA in zebrafish embryos. At embryonic development of 48 h post-fertilization, zAsc mRNA was observed (Fig. 3, A–C), Caspy (Fig. 3, E–G), and Caspy2 (Fig. 3, I–K) mRNA exhibited a similar labeling pattern with expression being detected primarily in epidermis (arrows), mouth (single arrowhead), and pharyngeal arches (white arrowheads) shown in Fig. 3. Significantly, the expression of asc and caspy2 appeared stronger than that observed for caspy. Similar results were observed in embryos at 36 h post-fertilization. The expression pattern of caspy (Fig. 3, A and B) is consistent with that of human ASC, which was reported to be expressed in epithelial cells including squamous epithelium of skin and mouth (34).

**Caspy and Caspy2 Induce Apoptosis in Mammalian Cells**—To begin to assess the molecular function of Caspy and Caspy2, we constructed expression plasmids of tagged and non-tagged Caspy and Caspy2 and transiently transfected the plasmids into 293T cells, respectively. A significant percentage of Caspy- and Caspy2-transfected cells displayed morphological features of adherent cells undergoing apoptosis such as rounding, membrane blebbing, and detachment from the dish (Fig. 4A). At 24 h post-transfection, about 20–40 and 95% of the cells transfected with the highest dose of both tagged and non-tagged Caspy and Caspy2 constructs, respectively, displayed morphological features of apoptosis compared with less than 1% of the cells transfected with control plasmid (Fig. 4A).

To determine whether the proapoptotic activity of Caspy and Caspy2 is because of the their enzymatic activities, the ability of these proteins to induce apoptosis was tested in the presence of the caspase inhibitors, p35, CrmA, and zVAD-fmk. Expression of baculoviral p35 attenuated apoptosis induced by both Caspy and Caspy2 whereas incubation with zVAD-fmk greatly inhibited apoptosis (Fig. 4B). Interestingly, CrmA, an inhibitor of human caspase-1 and certain caspases (35), inhibited apoptosis induced by Caspy but not by Caspy2 (Fig. 4B). These data were also similar compared with the results using non-tagged Caspy and non-tagged Caspy2 (Fig. 4B). These results suggest that apoptosis induced by Caspy and Caspy2 is dependent on caspase activity and that the proapoptotic activity of Caspy and Caspy2 can be dissociated based on the inhibition with CrmA.

**Caspy and Caspy2 Are Active Caspases with Different Substrate Specificity**—To test more directly whether Caspy and Caspy2 are active enzymes, a panel of fluorogenic peptide substrates of mammalian caspases were incubated with Caspy or Caspy2 immunoprecipitated from extracts of cells transfected with plasmids producing the relevant caspases. Both caspases cleaved several caspase peptide substrates, demonstrating that Caspy and Caspy2 are active caspases (Fig. 4, C and D). Nota-

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*W. Zhou and J. Y. Kuwada, unpublished data.*
bly, Caspy preferentially cleaved AcYVAD-AMC, a caspase-1 substrate, whereas Caspy2 was more active on AcWEHD-AFC, a preferred substrate of caspase-5, and it did not cleave AcY-VAD-AMC (Fig. 4, C and D). These results suggest that Caspy and Caspy2 are enzymatically active caspases that exhibit different substrate specificity.

Zebrafish ASC Interacts with Caspy but Not with Caspy2—Because Caspy and Caspy2 contain a pyrin domain highly homologous to that of zAsc, the ability of both caspases to interact with zAsc was tested using a co-immunoprecipitation assay. zAsc was co-immunoprecipitated with Caspy but not with Caspy2 (Fig. 5). Mutational analysis revealed that a zAsc mutant containing the pyrin domain but lacking the CARD was co-immunoprecipitated with Caspy (Fig. 5, FLAG-zAsc-PD), suggesting that the pyrin domain of zAsc mediates the binding to Caspy. Furthermore, a zAsc mutant-lacking pyrin domain did not co-immunoprecipitate with Caspy (Fig. 5, FLAG-zAsc-PD), indicating the pyrin domain of zAsc is necessary and sufficient for the interaction with Caspy. In addition, a Caspy mutant lacking the pyrin domain (Fig. 5, Myc-Caspy-ΔPD) failed to interact with a zAsc mutant containing a pyrin domain (Fig. 5, FLAG-zAsc-PD) suggesting that zAsc and Caspy associate through a homophilic pyrin-pyrin domain interaction. To further assess the association between zAsc and Caspy, we constructed plasmids to express GFP-Caspy, GFP-zAsc, and DsRed-zAsc fusion proteins to facilitate the analysis of their subcellular localization. Expression of GFP-Caspy and GFP-

Fig. 4. Both Caspy and Caspy2 induce apoptosis in mammalian cells and exhibit differential substrate specificity. A, dose-dependent induction of apoptosis by Caspy and Caspy2 in 293T cells. 5 × 10⁴ 293T cells were cotransfected with the indicated amount of empty vector (−), pcDNA3-FLAG-Caspy (Caspy, tagged), pcDNA3-Caspy2-Myc (Caspy2, tagged), pcDNA3-Caspy (Caspy, non-tagged), or pcDNA3-Caspy2 (Caspy2, non-tagged) and 73 ng of pcDNA3-β-gal. 24 h post-transfection, the percent of apoptotic cells ± S.D. was calculated in triplicate cultures. B, suppression of Caspy- and Caspy2-induced apoptosis by caspase inhibitors. 5 × 10⁴ 293T cells were co-transfected with 667 ng of pcDNA3-FLAG-Caspy (Caspy, tagged), 333 ng of pcDNA3-Caspy2-Myc (Caspy2, tagged), 667 ng of pcDNA3-Caspy (Caspy, non-tagged), 333 ng of pcDNA3-Caspy2 (Caspy2, non-tagged), and 167 ng of empty vector (−), pcDNA3-β-gal (p35), or pcDNA3-CrmA (CrmA) in the presence of pcDNA3-β-gal. 8 h post-transfection, transfected cell were incubated with 20 μM zVAD-fmk (zVAD-fmk) or medium alone (other lanes). 24 h post-transfection, the percent of apoptotic cells ± S.D. was calculated in triplicate cultures. C and D, Caspy and Caspy2 exhibit differential substrate specificity. 3 × 10⁴ 293T cells were co-transfected with 10 μg of pcDNA3-FLAG-Caspy (A) or pcDNA3-Caspy2-Myc (B). 24 h post-transfection, the caspases were immunopurified with anti-Myc and anti-FLAG Abs, and ½ of the aliquot was incubated with the indicated fluorogenic peptide substrates for 60 min.

Fig. 5. Interaction between zAsc and Caspy, zAsc interacts with Caspy but not with Caspy2. 1 × 10⁶ COS-7 cells were transfected with 2.4 μg of pcDNA3-Myc (vector), pcDNA3-Caspy-Myc (Caspy-WT), pcDNA3-Caspy-(101–383)-Myc (Caspy-ΔPD), or pcDNA3-Caspy2-Myc and pFLAG-zAsc (zAsc-WT), pFLAG-zAsc-(1–103) (zAsc-ΔPD), or pFLAG-zAsc-(104–203) (zAsc-ΔPD). 24 h post-transfection cells were lysed, and proteins were immunoprecipitated by anti-Myc polyclonal Ab. Co-immunoprecipitated proteins were detected by anti-FLAG M2 Ab (upper panel). As controls, co-immunoprecipitated proteins were detected by anti-Myc monoclonal Ab (middle panel). Proteins in total lysate were detected by anti-FLAG Ab (bottom panels).
zAsc induced the formation of specks (Fig. 6B, **GFP-Caspy** and **GFP-zAsc**, respectively), and double labeling analysis of the same cells revealed that both GFP-Caspy (Fig. 6A, **Green** and **DeRed-zAsc** (Fig. 6A, **Red**)) co-localized to the same speck visualized in **yellow** (Fig. 6A, **Merge**). These observation demonstrates that zAsc interacts with Caspy in the same speck.

**Enforced Oligomerization of zAsc Induces Caspy-dependent Apoptosis**—To determine the functional relevance of the interaction between Caspy and zAsc, we first tested whether co-expression of zAsc enhances apoptosis induced by Caspy and *Fig. 7.* The induced proximity of zAsc enhanced apoptosis induced by Caspy and processing of Caspy. *5 × 10^4* 293T cells were co-transfected with *17 ng* of pcDNA3, pcDNA3-Caspase-9-FLAG, pcDNA3-FLAG-Caspy, pcDNA3-Caspy2-Myc, pcDNA3-Caspy, or pcDNA3-Caspy2 and *33 ng* of pcDNA3-zAsc-Myc, pcDNA3-zAsc-Fpk3-Myc, or pcDNA3-Apaf-1XL-Myc in the presence of pEFBOS-β-gal. 8 h post-transfection the cells were stained by DAPI (4’,6-diamidino-2-phenylindole dihydrochloride) (**Blue**). Signals were detected by immunofluorescence microscopy. Green fluorescence, red fluorescence, and blue fluorescence were visualized in the same field (**Merge**). GFP-Caspy signal (**Green**) and DeRed-zAsc signal (**Red**) accumulate in the same speck (**arrowheads**). A, as controls, cells were transiently transfected with pEGFP or pDsRed as a vector control. Diffuse green or red florescent signals were detected in the cells (**GFP** and **DsRed**, respectively). Cells were transiently transfected with pEGFP-zAsc alone or pEGFP-Caspy alone (**GFP-zAsc** and **GFP-Caspy**, respectively). Green fluorescence signals were detected in the cells (**arrowheads**).
Caspy2. In these experiments we expressed low amounts of plasmid so that we could assess whether zAsc could enhance tagged and non-tagged Caspy- and Caspy2-induced apoptosis. Under these experimental conditions, expression of zAsc did not augment apoptosis induced by either Caspy or Caspy2 (Fig. 7, A and B). This result suggested that the interaction between Caspy and zAsc was not sufficient to induce caspase activation. Enforced oligomerization of the CARD of Apaf-1 and that of the DD of Fas, which are protein modules structurally related to the pyrin domain, is known to induce the activation of target caspases (36, 37). Therefore, we hypothesized that zAsc might similarly promote Caspy activation through zAsc oligomerization. To test this hypothesis, we constructed a plasmid to express a zAsc-Fpk3 fusion protein that can be oligomerized with the ligand AP1510 (14). Co-expression of zAsc-Fpk3, like that of zAsc, did not enhance apoptosis induced by Caspy in the absence of AP1510 (Fig. 7, A and B). In contrast, greater than 50% of the cells underwent apoptosis when both zAsc-Fpk3 and Caspy were co-expressed in the presence of AP1510 (Fig. 7, A and B). In control experiments, co-expression of zAsc-Fpk3 did not enhance the ability of Caspy2 or caspase-9 to induce apoptosis in the presence of AP1510 (Fig. 7B). In reciprocal experiments, co-expression of Apaf-1, a regulator of caspase-9, enhanced apoptosis induced by caspase-9, but it did not augment Caspy- or Caspy2-mediated apoptosis (Fig. 7B). These results suggest that oligomerization of zAsc is required for enhancement of Caspy-dependent apoptosis. Oligomerization of zAsc is presumably induced through an interaction between the C-terminal CARD of zAsc and a putative upstream regulator that needs to be identified. Because Caspy2 did not interact with zAsc, we suggest that Caspy2, which possesses caspase-5 like enzymatic activity, mediates apoptosis and/or inflammation in a zAsc-independent manner.

**Enforced Oligomerization of zAsc Induces Proteolytic Processing of Caspy**—To examine whether the activation of Caspy is enhanced by zAsc, we determined whether oligomerization of zAsc induces the processing of Caspy. Analysis of cell extracts by immunoblotting revealed that a processed form of Caspy was immunodetected in extracts from cells co-expressing zAsc-Fpk3 and Caspy in the presence but not in the absence of dimerizer AP1510 (Fig. 7C). When Caspy was immunoprecipitated with antibody, processing of Caspy was observed in extracts from cells co-expressing Caspy and zAsc even in the absence of the dimerizer (Fig. 7C). A possible explanation for the latter finding is that Caspy activation can be induced through antibody-mediated aggregation of zAsc-Caspy complexes in vitro resulting from the immunoprecipitation procedure. Significantly, zAsc was still required for processing of Caspy in vitro, suggesting that the interaction of zAsc with Caspy is important for caspase processing and activation.

**Morpholino Antisense Oligonucleotide-based Knocked-down of Caspy Affects Development of the Jaw and Posterior Pharyngeal Arches**—To assess whether Caspy was required for normal development of zebrafish, we designed a morpholino antisense oligonucleotide to interfere with caspy translation. Microinjection of the blocking antisense oligonucleotide into one- to four-cell stage embryos resulted in an open mouth phenotype as early cleavage embryos and stained the head cartilage of the resulting larvae with Alcian blue. The analysis revealed that 12/19 larvae injected with the caspy antisense morpholino exhibited several abnormalities in the development of the cartilaginous pharyngeal skeleton. The abnormal phenotype included deformed and thinned Meckel’s and palatoquadrate cartilages, which form the jaw, deformed ceratohyal cartilages, which are point posteriorly, and disorganized branchial cartilages (Fig. 8). The larvae were still alive approximately 1 week post-fertilization but then started to die as yolk disappeared. Of the seven remaining larvae derived from early embryos injected with the caspy antisense oligonucleotide, 5/19 showed a similar but less marked phenotype, whereas 2/19 larvae appeared normal. The phenotype is consistent with the expression of caspy in the pharyngeal arches (Fig. 3). No other abnormalities were identified in any other part of the body including the brain, eyes, or pectoral fins of the larvae injected with the caspy antisense oligonucleotide. The latter is consistent with the observation that caspy is not expressed in the brain, eyes, or pectoral fins during embryonic development (Fig. 3). None of the embryos injected with control morpholino oligonucleotide showed any alteration in the cartilaginous structures of the jaw or gills.

**DISCUSSION**

In this report we provide evidence that the zebrafish ASC mediates the activation of Caspy, a pyrin domain-containing caspase. Although mammalian orthologues of zebrafish Caspy have not been yet identified, ASC is highly conserved in vertebrates. It is possible that mammals have Caspy-like molecules...
with typical pyrin domains, but they need to be identified. Because the CARD is highly related to the pyrin domain, it is also possible that one of the mammalian CARD-containing caspases is the Caspy orthologue. Caspy exhibits the highest homology to human caspase-1 and preferentially cleaved caspase-1 substrates whereas Caspy2 showed the highest homology to human caspase-5 and preference for caspase-5-like substrates. Apoptosis induced by Caspy was blocked by CrmA, a caspase inhibitor that inhibits human caspase-1 at low concentrations (35) (Fig. 4B). Both caspase-1 and caspase-5 belong to the same subfamily of CARD-containing caspases (33), but their activator(s) are unknown or are poorly understood. Thus, it will be important to test for interactions between these known CARD-containing caspases and mammalian ASC.

zAsc, like its mammalian orthologues, is composed of an N-terminal pyrin domain linked to a C-terminal CARD. Recent studies of the three-dimensional structure of several apoptosis regulators have suggested that the stoichiometry of the homophilic interaction between the CARD and related domains is one by one (38, 39). Therefore, it is unlikely that the pyrin domain of zAsc binds to other pyrin or CARD-containing factors in the Caspy/zAsc signaling complex. Enforced oligomerization of zAsc enhanced Caspy-induced apoptosis. These findings suggest that the C-terminal CARD of zAsc and that of its mammalian orthologues might be important for oligomerization. In this model, zAsc acts as an adaptor molecule linking a putative upstream factor to caspases such as Caspy. Potential upstream factors include Defcap/NAC/CARD/NALP1 and Ipaf-1/upstream factor to caspases such as Caspy. Potential upstream factors include Defcap/NAC/CARD/NALP1 and Ipaf-1/upstream factor to caspases such as Caspy.

Activation of Caspy by Zebrafish ASC

Analysis of animals in which caspy activity was knocked-down and analysis of animals in which caspy activity was knocked-down and expressed by zebrafish ASC revealed a large number of loci required for pharyngeal arch development (49, 50). Some of the mutant fish exhibit phenotypes that are similar but not identical to that observed in caspase morpholo knocked-down animals. Thus, it is possible that Caspy is a component of a signaling pathway that includes some of the genes identified in the genetic screens. Additional studies are needed to understand the precise role of Caspy in the genetic program involved in the development of the jaw and branchial arch cartilages.

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References

1. Núñez, G., Benediet, M. A., Hu, Y., and Inohara, N. (1998) Oncogene 17, 3257–3264.
2. Salvesen, G. S., and Dixit, V. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10964–10967.
3. Huang, B., Eberstäd, M., Olejniczak, E. T., Meadows, H. P., and Fesik, S. W. (1996) Nature 384, 638–641.
4. Hofmann, K., Bucher, P., and Tschopp, J. (1997) Trends Biochem. Sci. 22, 155–156.
5. Eberstäd, M., Huang, B., Chen, Z., Meadows, R. P., Ng, S. C., Zheng, L., Lenardo, M. J., and Fesik, S. W. (1998) Nature 392, 941–945.
6. Qin, H., Srinivasula, S. M., Wu, G., Fernandes-Alnemri, T., Alnemri, E. S., and Shi, Y. (1999) Nature 399, 549–557.
7. Hofmann, K., and Tschopp, J. (1995) FEBS Lett. 371, 321–323.
8. Hu, S., Huang, J., Shu, H. B., Baiwäl, V., and Goedel, D. V. (1996) Immunity 4, 387–396.
9. Inohara, N., Koseki, T., del Peso, L., Hu, Y., Yee, C., Chen, S., Carrio, R., Merino, Liu, D., Ni, J., and Núñez, G. (1999) J. Biol. Chem. 274, 14560–14568.
10. Inohara, N., and Núñez, G. (2000) Cell Death Differ. 7, 509–510.
11. Poyet, J. L., Srinivasula, S. M., Lin, J. H., Fernandes-Alnemri, T., Yamaoka, S., Toschil, P. N., and Alnemri, E. S. (2000) J. Biol. Chem. 275, 37966–37977.
12. International IMMIF Consortium (1997) Cell 90, 797–807.
13. Masamoto, J., Taniguchi, S., Ayukawa, K., Sarvationh, H., Kohino, T., Nukawa, N., Hitaka, E., Katsuyama, T., Higuchi, T., and Sagara, J. (1999) J. Biol. Chem. 274, 33835–33838.
14. Inohara, N., Koseki, T., Lin, J., del Peso, L., Lucas, P. C., Chen, F. F., Ogura, Y., and Núñez, G. (2000) J. Biol. Chem. 275, 27583–27591.
15. Bertin, J., and DiStefano, P. S. (2000) Cell Death Differ. 7, 1273–1274.
16. Staub, E., Dahl, E., and Rosenthal, A. (2001) Trends Biochem. Sci. 26, 83–85.
17. Okewski, K., Poo, P., Chu, Z., Reed, J. C., and Gedik, A. (2001) Trends Biochem. Sci. 26, 85–87.
18. Martinon, F., Hofmann, K., and Tschopp, J. (2001) Curr. Biol. 2001, 851–818–R120.
19. Fairbrother, W. J., Gordon, N. C., Humke, E. W., O’ourke, K. M., Starosvarnik, M. A., Yin, J. P., and Dixit, V. M. (2001) Protein Sci. 10, 1911–1918.
20. Masamoto, J., Taniguchi, S., and Sagara, J. (2001) Biochem. Biophys. Res. Commun. 280, 652–655.
21. Arvidin, L., Dixit, V. M., and Komin, E. V. (2001) Science 291, 1279–1284.
22. Inohara, N., Koseki, T., Chen, S., Wu, X., and Núñez, G. (1998) EMBO J. 17, 2526–2533.
23. Hu, Y., Ding, L., Spencer, D. M., and Núñez, G. (1998) J. Biol. Chem. 273, 8461–8468.
24. Benediet, M. A., Hu, Y., Inohara, N., and Núñez, G. (1998) J. Biol. Chem. 275, 8461–8468.
25. Wang, I. G., Kelly, P. D., Chu, F., Ngo-Hazelett, P., Yan, L. Y., Huang, H., Postlethwait, J. W., and Talbott, W. S. (2000) Genome Res. 10, 1903–1914.
26. Yee, C. S., Chandrasekhar, A., Halloran, M. C., Shoji, W., Warren, J. T., and Kowada, Y. (1999) Brain Res. Bull. 48, 581–593.
27. Masamoto, J., Taniguchi, S., Nakayama, K., Ayukawa, K., and Sagara, J. (2001) Exp. Cell Res. 2001, 126–128–133.
28. Inohara, N., Koseki, T., Hu, Y., Chen, S., and Núñez, G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 17,977–17,982.
29. Wu, D., Wallen, H. D., Inohara, N., and Núñez, G. (1997) J. Biol. Chem. 272, 21449–21454.
30. Hoffmann, H. M., Mueller, J. L., Brodie, D. H., Wanderer, A. A., and Kolodner, R. D. (2001) Nat. Genet. 29, 301–305.
31. Manji, G. A., Wang, L., Geddes, B. J., Brown, M., Merriam, S., Al-Garawi, A., Ltd. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 27,177–27,182.
32. Wilson, K. P., Black, J. F., Thomson, J. A., Kim, E. E., Griffith, J. P., Navia, M. A., Murcko, M. A., Chambers, S. P., Ailpae, R. A., Rayback, S. A., and Livingston, D. J. (1994) Nature 370, 270–275.
33. Thornberry, N. A., Chapman, K. T., and Nicholson, D. W. (2000) Methods Enzymol. 322, 100–110.
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34. Masumoto, J., Taniguchi, S., Nakayama, J., Shichara, M., Hisaka, K., Katsuyama, T., Murase, S., and Sagara, J. (2001) J. Histochem. Cytochem. 49, 1269–1275
35. Garcia-Calvo, M., Peterson, E. P., Leiting, B., Ruel, R., Nicholson, D. W., and Thornberry, N. A. (1998) J. Biol. Chem. 273, 32608–32613
36. Hu, Y., Benedict, M. A., Ding, L., and Núñez, G. (1999) EMBO J. 18, 3586–3596
37. Muzio, M., Stockwell, B. R., Stennicke, H. R., Salvesen, G. S., and Dixit, V. M. (1998) J. Biol. Chem. 273, 2926–2930
38. Chou, J. J., Matsuo, H., Duan, H., and Wagner, G. (1998) Cell 94, 171–180
39. Zhou, P., Chou, J., Olea, R. S., Yuan, J., and Wagner, G. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11265–11270
40. Hlaing, T., Guo, R. F., Dilley, K. A., Loessia, J. M., Morrise, T. A., Shi, M. M., Vincenz, C., and Ward, F. A. (2001) J. Biol. Chem. 276, 9230–9238
41. Chu, Z. L., Pio, F., Xie, Z., Walsh, K., Krajewksa, M., Krajewski, S., Godzik, A., and Reed, J. C. (2001) J. Biol. Chem. 276, 9239–9245
42. Poyet, J. L., Srinivaula, S. M., Tnani, M., Razmaria, M., Fernandes-Alnemri, T., and Alnemri, E. S. (2001) J. Biol. Chem. 276, 28309–28313
43. Geddes, B. J., Wang, L., Huang, W. J., Lavelle, M., Manji, G. A., Brown, M., Jurman, M., Cao, J., Morgenstern, J., Merriam, S., Glucksmann, M. A., DiStefano, P. S., and Bertin, J. (2001) Biochem. Biophys. Res. Commun. 284, 77–82
44. Damiano, J. S., Stelblik, C., Pio, F., Godzik, A., and Reed, J. C. (2001) Genomics 75, 77–83
45. Ogura, Y., Inohara, N., Benito, A., Chen, F. F., Yamaoka, S., and Núñez, G. (2001) J. Biol. Chem. 276, 4812–4818
46. Le Deaurain, N. M., Dupin, E., and Ziller, C. (1994) Curr. Opin. Genet. Dev. 4, 685–695
47. Yanagisawa, H., Yanagisawa, M., Kapur, R. P., Richardson, J. A., Williams, S. C., Clouthier, D. E., de Wit, D., Emoto, N., and Hammer, R. E. (1998) Development 125, 825–836
48. Miller, C. T., Schilling, T. F., Lee, K., Parker, J., and Kimmel, C. B. (2000) Development 127, 3815–3828
49. Schilling, T. F., Poizyowski, T., Grandel, H., Brand, M., Heisenberg, C. P., Jiang, Y. J., Beuchle, D., Hammerschmidt, M., Kane, D. A., Mulline, M. C., van Eeden, F. J., Kelsh, R. N., Furutani-Seiki, M., Granato, M., Haffter, P., Odenthal, J., Warga, R. M., Trowe, T., and Nusslein-Volhard, C. (1996) Development 123, 329–344
50. Poizyowski, T., Schilling, T. F., Brand, M., Jiang, Y. J., Heisenberg, C. P., Beuchle, D., Grandel, H., van Eeden, F. J., Furutani-Seiki, M., Granato, M., Haffter, P., Hammerschmidt, M., Kane, D. A., Kelsh, R. N., Mulline, M. C., Odenthal, J., Warga, R. M., and Nusslein-Volhard, C. (1996) Development 123, 345–356