Caspases, a family of cysteine proteases, have been recognized as the central executors of programmed cell death. Nonetheless, the information on the caspase family has been limited to mammals, *Drosophila*, and nematodes. To examine the structure and characterization of the *Xenopus* caspase family, we have cloned the cDNAs encoding caspase-2 and -6–10 in addition to caspase-1 and -3, which we characterized previously (Yaoita, Y., and Nakajima, K. (1997) J. Biol. Chem. 272, 5122–5127). First, the existence of these caspases in frog suggests that the caspase cascades clarified in mammals are conserved at least from Amphibia. Interestingly, *Xenopus* caspase-1, -8, and -10 (especially caspase-8) showed a lower degree of identity to human equivalents than the other caspases. Second, mRNAs of many caspases increased during the climax of metamorphosis in regressing organs, tail, and intestine, where programmed cell death occurs, but not in apoptotic tail-derived cultured cells (XLT-15-11) treated with thyroid hormone, showing that new RNA synthesis of caspases is dispensable to programmed cell death. Third, comparison of human and *Xenopus* caspase sequences implies that some proposed regulations of human caspases are not conserved in frog.

Programmed cell death occurs during many developmental processes in a spatially and temporally predetermined way (2, 3). One of the classical examples is resorption of the anuran tail during metamorphosis, which is triggered by a surge of thyroid hormone and coincides with the up-regulation of thyroid hormone receptors (4, 5). The tadpole tail, which is twice as long as the body, disappears completely in several days with characteristic features of apoptosis such as nuclear chromatin condensation and cytoplasmic blebs (6). Apoptosis is also observed when a myoblastic cell line with treatment with thyroid hormone (1). Gills and larval intestinal epithelial cells also degenerate during metamorphosis (7).

A large body of evidence supports the idea that apoptosis is mediated by the sequential and coordinated activation of several caspases (cysteine/aspartic acid-specific proteases), which cleave key substrates after aspartic acid residues (8–10). Over 12 human caspases that share similarities in sequence and activity have been identified. Caspases are synthesized as inactivezymogens composed of a prodomain and large and small subunits.

There are two well characterized pathways of apoptosis: the process triggered by the interaction between the Fas receptor and its ligand (11) and cell death starting from the release of cytochrome *c* from mitochondria into the cytoplasm (9). Upon binding of the Fas ligand to the receptor, oligomers are formed that bring together in close vicinity the intracellular C-terminal death domains, to which the death domain of FADD (Fas-associated protein with death domain) binds through protein-protein interaction. Then, procaspase-8 associates with the receptor-FADD complex by interaction between the death effector domains of FADD and procaspase-8, resulting in the cleavage and activation of caspase-8. Active caspase-8 released from the complex causes the sequential activation of the downstream caspases, including caspase-3, -6, and -7, culminating in apoptotic cell death.

In the second pathway, various stimuli of cell death induce the release of cytochrome *c* from mitochondria; the cytochrome *c* binds to a cytosolic protein, Apaf-1. This Apaf-1-cytochrome *c* complex self-associates through its CED-4 homology domain (12) and binds to procaspase-9 by the interaction of caspase recruitment domains, resulting in transactivation of caspase-9. The activated caspase-9 initiates the caspase cascade (9).

Although the caspase family appears to play a pivotal role in programmed cell death beyond species, the information on caspases has been limited to mammals (human and mouse), *Drosophila*, and *Caenorhabditis elegans* and to few reports on chicken (13) and *Xenopus* (1) caspases. In this paper, we report the comprehensive study of the structure, expression, cytotoxic activity, and activation of the *Xenopus laevis* caspase family members.

**EXPERIMENTAL PROCEDURES**

Cloning of *Xenopus Caspase Genes*—A directionally oriented cDNA library of stage 62 tadpole tail was constructed using the Directional Cloning Toolbox and the Time Saver cDNA synthesis kit (Amersham Pharmacia Biotech) with the ZAP Express vector (Stratagene) according to the manufacturer’s instructions. A 2-μg aliquot DNA from this cDNA library containing ~10^6 plaque-forming units was used as a substrate for polymerase chain reaction (PCR) amplification as described (14) with some modifications. The primary amplification (30 cycles: 94 °C for 40 s, 50 °C for 1 min, and 72 °C for 2 min) was performed with T<sub>1</sub>, vector-specific primer (Stratagene) and a degenera-
Characterization of the Xenopus Caspases

K. Nakajima and Y. Yanoita, unpublished data.
FIG. 1. Amino acid sequence comparisons between *Xenopus* and human caspases. Amino acid sequences of *Xenopus* caspase (xC)-2, -6, -7, -8, -9, and -10 are aligned with those of human caspase (hC)-2, -6, -7, -8, -9, and -10 (14, 54, 62–69). The conserved residues are boxed and...
site. It is possible that Xenopus caspase-8 and -10 might be duplicated genes as a result of doubling chromosomes during evolution since *X. laevis* is allotetraploid. It seems unlikely because we have cloned two different caspase-1 and -2 cDNAs that share 79.5 and 85.6% identities, respectively, whereas *Xenopus* caspase-8 and -10 show only 31% identity. However, we cannot completely exclude the possibility that *Xenopus* caspase-8 and -10 do not correspond to the human counterparts.

**Expression of Xenopus Caspases**—It has been shown that metamorphosis transforms most, if not all, organs and tissues in a tadpole especially after stage 58 (7) and that the stages at which maximal cell death is observed are different in different organs. The degeneration of tail muscle segments proceeds very rapidly after stage 63, and tail is resorbed completely until stage 66 (22), whereas the limbs differentiate and grow. In the intestine, the apoptotic degeneration of larval epithelial cells is most frequently observed at stages 59–61, and the proliferation of adult epithelial cells occurs in a predetermined order (23, 24). Although the central nervous system (brain and spinal cord) undergoes cell death of the mesencephalic fifth nucleus (the proprioceptors of the jaw musculature) at stage 62 (25), no conspicuous change takes place except topographical changes during the climax of metamorphosis (22). Caspase mRNA expression was examined in these organs during metamorphosis and in T3-treated myoblast cell lines derived from tadpole tail (Fig. 3). As has been observed, caspase probes hybridized to transcripts of 2.0 kb (caspase-1), 2.5 kb (caspase-2), 3.7 and 1.6 kb (caspase-3), 2.2 kb (caspase-6), 1.5 kb (caspase-7), 2.5 kb and 3.0 kb (caspase-8), 2.7 kb (caspase-9), and 2.3 kb (caspase-10). Expression of all cloned caspases increased in the intestine. Expression of all caspases except caspase-9 and -10 increased in the tail, whereas caspase expression did not change in the hind limb or the central nervous system. Both XLT-15 and the subline XLT-15-11 expressed constant levels of caspase mRNAs despite T3 treatment, except for caspase-3 mRNA, which increased in T3-treated XLT-15 cells (1). Although the expression of caspase-1 fluctuated during tail regression, in general, it increased somewhat.

**Overexpression of Xenopus Caspases**—Many reports have demonstrated that the elevated expression of a caspase by transfection of the gene into cultured cells induces apoptotic cell death (26, 27). To compare the cytotoxic activity of caspases, *Xenopus* caspase genes in expression vector were transfected into XLT-15-11 cells with a reporter gene (pEGFP-N2), and apoptotic cells were identified by the morphology of GFP-positive cells (Fig. 4). The overexpression of caspases with a long prodomain (caspase-1, -2, -8, -9, and -10) caused greater...
Characterization of the Xenopus Caspases

Numerous lines of evidence have established that caspases play a central role in apoptosis and that this cell death pathway has been conserved throughout the evolution of eukaryotes. Many caspase genes have been characterized, including Ced-3 from C. elegans (8); dcp-1 (30), drICE (31),redd (32), and dronc (33) from Drosophila; 10 mouse caspase genes (34–41); and 12 human caspase genes (42, 43). We have cloned cDNAs of Xenopus caspases corresponding to all human members that are involved in apoptosis. The sequences of Xenopus caspases enable us to compare the corresponding caspases in distant vertebrates and to delineate evolutionarily conserved regions that might be functionally important.

The caspase family members are conserved from frog to human (Figs. 1 and 2), suggesting that the same two caspase cascades triggered by activation of caspase-8 and -9 as apical caspases previously described in mammalian apoptosis are similar in Xenopus. In fact, when Xenopus egg cytosol is incubated with isolated mitochondria, cytochrome c is released from mitochondria, leading to the activation of DEVD-specific caspases and nuclear apoptosis in vitro (44). This might correspond to the cell death pathway caused by the activation of caspase-9 that is mediated through the association of Apaf-1 and cytochrome c. On the other hand, there is so far no report on Xenopus that describes the characterization of the death receptors belonging to the tumor necrosis factor receptor superfamily or that suggests the death receptor signaling pathway. The Xenopus caspase cascade involving caspase-8 activation via its complex with FADD remains to be examined.

Caspase-1, -8, and -10 are more divergent between frog and human than the other caspases (Table I). However, it does not mean that the latter caspases are more essential to normal development than the former because caspase-2 knockout mice reach adulthood without any gross abnormalities (45), and homozygous targeted disruption of the mouse caspase-8 gene results in death in utero (46). Caspase-2 is important for survival after birth since apoptosis mediated by granzyme B and perforin is defective in caspase-2-deficient B lymphocytes (45). A lower degree of identity of caspase-8 below 40% (Table I and Fig. 2) between human and frog might reflect a developmental change in the immune system during evolution in vertebrates (47).

In regard to caspase-10, only a human gene other than Xenopus has been cloned. The human caspase-10 gene is located between the CASH (a proteolytically inactive homolog of caspase-8 and -10) and caspase-8 genes on the chromosome, whereas the mouse caspase-8 gene is located 30 kb downstream of the CASH gene in a genomic clone (46), implying a possibility included a small amount of apoptotic floating cells, in which the overexpressed caspase was activated, modified, and detected by affinity labeling. On the other hand, the extracts from cells introduced with the caspase-2, -8, -9, or -10 gene showed no additional signal as compared with the vector-transfected cell extract (Fig. 6A). In each case, the affinity labeling reagent Z-EK(bio)D-aomk might not bind to the active caspase efficiently, yet cell death might be induced before sufficient amounts of endogenous caspase-3, -6, and -7 for detection are processed.

To estimate which caspases are activated in T3-treated XLT-15 and XLT-15-11 cells, their cell extracts were reacted with the affinity labeling reagent. The T3 treatment augmented the intensity of several signals, including two polypeptides that comigrated with processed caspase-6 and -7 (Fig. 6B). The intensity of a signal (17.1 kDa) that might correspond to processed caspase-3 increased in the T3-treated XLT-15 cell extract, but not in dying XLT-15-11 cells.

DISCUSSION

Affinity Labeling of Active Caspases—To identify an activated caspase in the caspase-overexpressing cells, we used an affinity labeling method that enabled us to detect the large subunits of processed caspases without preparing antibodies against Xenopus caspases. If a caspase overexpressed by transfection of its gene is activated during the cell death process (including spontaneous cell death) and the active form of this caspase has an affinity for Z-EK(bio)D-aomk, the caspase should be modified covalently by the reagent. The extracts from cells transfected with the caspase-1, -3, -6, or -7 gene showed signals by labeled active caspases (Fig. 6A). We interpreted these signals as follows based on the predicted cleavage sites by comparison of human and Xenopus caspase sequences (Fig. 1) and the molecular masses of the large subunits from the affinity labeling experiment. The single bands at 17.7 and 19.0 kDa (Fig. 6A, lanes 6 and 7) observed in the extracts of cells transfected with the caspase-6 or -7 gene correspond to the large subunits of transfected gene products processed by cutting at Asp16, Asp36, Asp104, and Asp296 of caspase-1 generated two polypeptides, 29.8 kDa (a prodomain plus a large subunit) and 21.4 kDa (a large subunit). The other signal (19.0 kDa) might represent cleavage of an endogenous caspase-7 (Fig. 6A, lane 3).

The extracts from adherent cells overexpressing caspase-1, -3, -6, or -7 contained much less labeled active caspase than the extracts from adherent plus floating cells, which indicates that most of the signal was derived from floating dead cells (29). Cells transfected with the caspase-3, -6, or -7 gene expressed a small amount of apoptotic floating cells, in which the overexpressed caspase was activated, modified, and detected by affinity labeling. On the other hand, the extracts from cells introduced with the caspase-2, -8, -9, or -10 gene showed no additional signal as compared with the vector-transfected cell extract (Fig. 6A). In each case, the affinity labeling reagent Z-EK(bio)D-aomk might not bind to the active caspase efficiently, yet cell death might be induced before sufficient amounts of endogenous caspase-3, -6, and -7 for detection are processed.

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that there is no caspase-10 gene in mouse chromosomal DNA. The existence of Xenopus and human caspase-10, however, suggests that a vertebral animal at least from Amphibia basically possesses the caspase-10 gene.

Human caspase-9 is inactivated by Akt phosphorylation (48) and contains sites that conform to the consensus Akt phosphorylation motif R\(\text{X}\)RXX(S/T) (49) at Ser\(^{183}\) (RTRTGS) and Ser\(^{196}\) (RRRFSS). The latter site is recognized and phosphorylated by Akt. The corresponding sequences of Xenopus caspase-9 (STRTGS and ANRMRS) do not, however, fulfill the requirements of the consensus sequence (Fig. 1). There are two possibilities to explain this. First, the regulation of caspase-9 by Akt phosphorylation may not be conserved in Xenopus. Second, Xenopus Akt may have a different recognition sequence compared with the human protein. This seems unlikely because strong amino acid sequence conservation is observed from Dicyostelium to human (50), and bovine Akt can rescue Drosophila Akt mutants (51). On the other hand, since RGD-containing peptides induce procaspase-3 autoprocessing and activation directly in a cell-free system without any requirement for integrin-mediated cell clustering or signals and since human procaspase-3 contains an RGD-binding motif (DDX), it has been proposed that RGD peptides exert their effects by the interaction with a potential RGD-binding site of procaspase-3, leading to the conformational changes that promote its autoprocessing and activation (52). However, the region of Xenopus caspase-3 corresponding to an RGD-binding motif is not conserved (1). Moreover, Tyr\(^{277}\) and Ser\(^{401}\) of Xenopus caspase-10 correspond to Leu\(^{242}\) and Val\(^{367}\) of human caspase-10, respectively, missense mutations of which are found in two kindreds with autoimmune lymphoproliferative syndrome type II, decrease caspase activity, and interfere with death receptor-induced apoptosis (53). Leu\(^{242}\) is substituted by Phe in the one family, and Val\(^{367}\) is changed to Ile in the other. Although Xenopus caspase-10 contains substitutions by quite different amino acids, its overexpression showed a strong cytotoxic activity as compared with other caspases (Fig. 5). The regulation of caspases might be different between human and frog in some details.

Our results clearly show that the enforced expression of
XLT-15-11 is derived from XLT-15 and maintains T₃ sensitivity, although the expression profile of caspase genes was a little different from the pattern of its parental cell line (Fig. 3). After T₃ treatment, caspase-3 mRNA was up-regulated in XLT-15 cells, but not in XLT-15-11 cells, whereas the expression of the other caspase genes did not increase in XLT-15 or XLT-15-11 cells. This result indicates that the increase in expression of any caspase gene is not necessary for T₃-induced cell death of XLT-15-11 cells. On the other hand, the up-regulation of caspase expression was observed in a regressing and a remodeling organ during metamorphosis, but not in an organ that grows or undergoes minimal changes (Fig. 3). In addition, the ced-3 mRNA is most abundant during nematode embryogenesis, when most programmed cell deaths occur (8). In late third instar larvae of *Drosophila*, dronc mRNA is dramatically up-regulated in salivary glands and midgut before histolysis of these tissues (33). The expression of the caspase-1 gene is induced in post-lactational involution of mouse mammary gland (57). Since T₃ treatment causes apoptosis of XLT-15-11 cells without an increase in any caspase mRNAs, it is reasonable that the increase in caspase expression promotes the apoptotic process efficiently, but is not essential to cell death. However, we cannot rule out the possibility that caspase expression is up-regulated in the cultured cell lines treated with T₃ by translational regulation.

Signals of several polypeptides in the affinity labeling assay were increased in the extracts of T₃-treated XLT-15 and XLT-15-11 cells (Fig. 6B). Two main molecules among them comigrated with large subunits of caspase-6 and -7, although it has been reported that caspase-3 and -6 are the major active caspases in apoptotic cells in many cases (58, 59). It is possible that the apical caspase (caspase-8 or -9) that is supposed to process procaspase-3 (60) can afford to activate procaspase-7 in T₃-treated XLT-15 and XLT-15-11 cells (61) because these cells express only a small amount of caspase-3. In T₃-treated XLT-15 cells, a faint signal that might correspond to a large subunit of caspase-3 appeared in the affinity labeling assay (Fig. 6B, lane 2) as caspase-3 expression was induced. The polypeptides other than caspase-3, -6, and -7 observed in the extract of T₃-treated cells remain to be identified.

Our data show that the caspase family is conserved at least from Amphibia to mammals in terms of amino acid sequence and function and that programmed cell death in regressing organs does not depend on the transcription of caspase genes. The sequence information on *Xenopus* caspases might enable us to clone caspase genes of other animals, including fishes and ascidians, by utilizing the sequences of the conserved regions between frog and human caspases and to elucidate the duplication and diversification process of caspase genes during evolution.

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Structure, Expression, and Function of the *Xenopus laevis* Caspase Family
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