Cloning and Characterization of a Novel Nuclear Bcl-2 Family Protein, zfMcl-1a, in Zebrafish Embryo

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The importance of the Bcl-2 family proteins in normal vertebrate embryogenesis is being recognized; however, their regulatory mechanism is poorly understood. To elucidate the embryonic roles of Bcl-2 family proteins, we cloned and characterized the first zebrafish Bcl-2 family protein, zfMcl-1a. Zebrafish Mcl-1a shows the highest homology to rat Mcl-1 and contains several conserved BH domains of the Bcl-2 family proteins. It also contains a nuclear localization signal (NLS). Using EGFP reporter analysis, we verified the nuclear localization of zfMcl-1a. Deletion of the NLS resulted in distribution of the fusion protein in the cytoplasm. Northern blot analysis indicated that zfMcl-1a mRNA is 1.5 kb and was expressed in oocytes and throughout embryonic development. Notably, the expression of zfMcl-1a transcript was significantly downregulated during gastrulation. These results suggest that zfMcl-1a is a novel nuclear Bcl-2 family protein and is likely to play an important role in zebrafish oogenesis and embryogenesis.© 2000 Academic Press

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Apoptosis is essential to normal embryonic development. In vertebrates, apoptosis functions in sculpturing organs, deleting unwanted structures, adjusting cell numbers and eliminating nonfunctional, harmful, abnormal, or misplaced cells (1, 2). Studies aiming to uncover the underlying molecular basis of apoptosis have identified many apoptotic genes from diverse organisms. Sequence comparison analysis and functional studies indicated that the molecules and regulation modes of apoptosis have been well preserved during evolution (3–5). Among these apoptotic regulators, the Bcl-2 family proteins are the best characterized. Recent gene-targeting studies in mice demonstrated that the Bcl-2 family proteins are key regulators in embryonic apoptosis, and they are essential to normal embryonic development (2, 6–10).

Bcl-2 family proteins occupy a key position in apoptotic signaling pathways linking various upstream cell death signals to a group of common downstream effectors (11, 12). This still growing protein family contains members of opposing activities, and is characterized structurally by the possession of at least one of the four conserved motifs (the Bcl-2 homology domains, BH1-4) (3, 13). The location of BH domains corresponds to α-helical segments of the molecule (3). Many Bcl-2 family proteins also contain a putative transmembrane domain (TM) at their C-termini, and at least to Bcl-2, the TM domain is critical for both its subcellular localization and apoptotic activity (3, 14, 15).

Members of the Bcl-2 family have been divided into three subfamilies: the Bcl-2 subfamily, the Bax subfamily and the BH3-only subfamily (3). Proteins of the Bcl-2 subfamily are all anti-apoptotic. Their BH domains share higher homology to Bcl-2 protein than members in the other two subfamilies. Both the Bax and the BH3-only subfamilies are composed of pro-apoptotic proteins. As implied by its name, proteins of the BH3-only subfamily only contain a recognizable domain that shares significant sequence homology to the BH3 domain of Bcl-2 protein.

High degree of homology in the BH domains foretells their importance. Mutagenesis analyses established a requirement for BH domains in protein-protein interaction both within and out of the Bcl-2 protein family and this BH-mediated interaction is critical for proteins of the Bcl-2 family to exert their function. For instance, BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax (16). BH 3 domain was shown to be essential for
proteins of the BH 3-only subfamily to both heterodimerize with anti-apoptotic Bcl-2 family members and exert their killing activity (17). BH 4 domain is essential to the heterodimerization between Bcl-2 and Bax, and disruption of this interaction abolishes the inhibitory effect of Bcl-2 on the killing activity of Bax (18). Finally, BH4 domain was speculated to play a regulatory role in apoptosis since it has been shown to serve as a docking site for several signaling molecules (19–21).

The expression of Bcl-2 family proteins is subject to multiple regulations. In various model systems, cytokines induce transcriptionally the expression of several antiapoptotic Bcl-2 family proteins (23–26). Posttranslational levels of regulation such as phosphorylation, degradation, and proteolytic cleavage have been shown to influence the activity of several Bcl-2 family proteins (27–31). Finally, subcellular localization serves another level of regulation on the activity of Bcl-2 family proteins. The proapoptotic protein Bim is sequestered to microtubules and kept inactive through the binding of LC8 dynein light chain (32), and Bax translocates from cytosol to mitochondria after cells received apoptotic stimuli (33). Members of the Bcl-2 family have also been shown to distribute to other subcellular compartments, such as endoplasmic reticulum (ER), nuclear membrane, and cytosol (28, 33, 34), but the significance of such localization is so far unclear.

To further our understanding of the function and regulation of apoptosis in normal vertebrate embryonic development, we have chosen to study embryonic apoptosis in the zebrafish, Danio rerio, because it has high fecundity, large and transparent embryos, characters that will facilitate embryonic studies. Furthermore, recent observations indicate that zebrafish expresses a variety of apoptotic genes (35), and is capable of launching apoptotic response upon genetic insults (36) and cytotoxic treatments (37, 38).

As the first step toward the above stated goal, here we report the cloning and characterization of the first zebrafish Bcl-2 family protein, zfMcl-1a.

MATERIALS AND METHODS

Cells and fish. NIH3T3 cells were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin sulfate (100 µg/ml) at 37°C. ZFL cells were maintained in L15 supplemented with 10% FBS, penicillin (100 U/ml), streptomycin sulfate (100 µg/ml) at 28°C. The maintenance of adult zebrafish, generation of embryos, and determination of developmental stages of embryos are based on Westerfield (39).

Cloning of zebrafish Mcl-1a cDNA and sequence analysis. To clone cDNAs for zebrafish Bcl-2 family proteins, degenerate RT-PCR (reverse transcription-polymerase chain reaction) was first conducted to generate short cDNA fragments coding for conserved protein regions among known Bcl-2 family proteins. Two pairs of degenerate primers were designed for the amplification based on Kiefer et al. (40) with modifications. The sense primers (NWG-1, AAC/ TGGGGGIA/C/GIA/GT/GI/GC and NWG-2, AAC/TGGGGGIA/C/GIA/GTA/G/GTA/T/G/GCC) were designed against the conserved BH1 domain (NWRGR/V/MVA). The antisense primers (GGW-1, TCA/ GAAI/C/GITC/CC/CACI/CICCCG and GGW-2, TCA/GAAI/C/GITC/CC/A /CT/G/GCA/T/G/GCC) were designed against the conserved BH2 domain (GGW/ID/EAX/3FE). Total RNA was extracted from dechorionated zebrafish embryos using Trizol reagent (Life Technologies) and then reverse transcribed by Superscript II reverse transcriptase (Life Technologies). Two micrograms of total RNA was used as template for first strand cDNA synthesis in a 20- µl RT reaction primed with oligo(dT)12–18 primer (Promega). The RT reaction was allowed to proceed for 2 h at 42°C, and then stopped by heating at 70°C for 15 min. One tenth of the RT products was used as template for PCR amplification of the target sequence. The PCR reaction was composed of 2 µl RT products, 5 µl 10× buffer (Clontech), 25 pmol of each sense and antisense primers, 200 µM dNTPs (Promega), and 0.5 µl Advantage cDNA polymerase mix (Clontech) in a 50- µl reaction volume. A “step down” thermal cycling strategy was used. Each thermal cycle was composed of a 20-s denaturation step at 94°C, a 30-s annealing step at decreasing temperature starting from 67 to 55°C at the speed of 3 degree every two cycles, and a 30-s extension step at 72°C. After an initial 2-min denaturation, PCR was allowed to proceed as programmed above. Once the annealing temperature has dropped to 55°C, the PCR was allowed to proceed for another 32 cycles with the same cycling parameters and a constant 55°C annealing temperature. The PCR was controlled by a final extension step. Five microliters of each PCR reaction was analyzed by electrophoresis, and agarose plugs containing products with size close to 180 bp were excised from the gel and directly used as template for another round of PCR amplification. The PCR products were eluted from agarose using QIAquick gel extraction system (Qiagen), blunted by Pfu DNA polymerase (Stratagene) at 72°C for 30 min, and subcloned using Zero Blunt TOPO PCR cloning system (Invitrogen). The cloned PCR products were sequenced by dye termination method using ABI PRISM 377 DNA sequencer (PE Biosystems), and scanned against the GenBank database by use of the BLAST program. One of them, denoted as ZM5-1, whose sequence closely resembled human Mcl-1 (human myeloid cell leukemia-1 (40), was used to clone longer cDNAs. A 24-h zebrafish embryo cDNA library (Stratagene) was screened using 32P-labeled ZM5-1 (rediprime II, Amersham Pharmacia Biotech) following standard procedures (41). Insert cDNAs were prepared by in vivo excision procedure according to the instruction of the manufacturer (Stratagene) and sequenced on both strands. The primary sequences of the cDNAs were analyzed using various computer programs, including BLAST (42), PESTfind (43), PSORT II (44), while its secondary structure was analyzed by using PHD program (45–46). Multiple alignments were performed with CLUSTAL W version 1.6 (47). The graphic presentations were prepared with GenDoc (48).

EGFP reporter analysis of zfMcl-1a subcellular distribution. The expression construct encoding N-terminal EGFP-tagged zfMcl-1a was constructed in two steps. First, the coding sequence of zfMcl-1a was PCR-amplified from zfMcl-1a (zfMcl-1a cDNA in pBluescript) using the sense primer, CGGGAATCTTATGCTTTGGTATT (EcoRI site is underlined, the start codon of zfMcl-1a was in bolded letter) and the universal T7 primer (Promega). Then, after restriction digestion with EcoRI and KpnI, the PCR product was ligated with identically predigested pEGFP-C1 vector (Clontech) to create pEGFP-zfMcl-1a. The expression construct encoding fusion protein between EGFP and the zfMcl-1a variant with a deletion from aa 43 to 49 was constructed by a whole plasmid amplification strategy. First, the entire sequence of pEGFP-zfMcl-1a except for the region intended for deletion was PCR-amplified using the sense primer, CCGGGTTAGACAGGCCCTGAAG and the antisense primer, CGC- CTTCCCTTCCTCTAGTGA. Then the linear PCR product was circularized by self-ligation to generate pEGFP-zfMcl-1a-D143-49. Sequencing was performed to verify the intended deletion and the
absence of any secondary mutations in the coding sequence of zfMcl-1a for both constructs. For cell transfection, both newly confluent NIH3T3 and ZFL cells were seeded at 2 x 10^5 cells to each well of a 6-well culture plate one day before transfection was conducted. Five micrograms of either pEGFP, pEGFP-zfMcl-1a, or pEGFP-zfMcl-1aD43-49 were introduced into these cells using Lipofectamine 2000 (Life Technologies). Fluorescence microscopy (Olympus) using FITC filter was employed to visualize the expression of the EGFP fusion proteins.

Northern blot analysis of embryonic expression of the zfMcl-1a mRNA. Total RNA was isolated from embryos as described above. Total oocyte RNA was extracted from oocytes collected from one sacrificed mature female. Stages of embryonic development were determined based on the criteria of Westerfield (39). Ten micrograms of total RNA were analyzed for each selected developmental stages. Northern blotting was performed based on Sambrook et al. (41) except that the RNA samples were fractionated in 1% native agarose gel. Prehybridization was done in a blocking solution (6x SSC, 5x Denhardt’s solution, 100 μg/ml sheared and denatured calf thymus DNA, 0.5% SDS) at 68°C for 1 h. After prehybridization, hybridization was initiated by addition of denatured, 32P-labeled zfMcl-1a probe prepared with rediprime II. Hybridization was allowed to proceed for more than 16 h at 68°C, after which the membrane was washed three times at 60°C for a total of 2 h with wash solution (0.1x SSC, 0.1% SDS). The blot was autoradiographed at -20°C against Kodak film to visualize the expression of zfMcl-1a mRNA.

RESULTS AND DISCUSSION

To isolate zebrafish Bcl-2 family members, we designed degenerate PCR primers to amplify the conserved polypeptide region between the BH1 and BH2 domains of the Bcl-2 family proteins. We used cDNA derived from zebrafish total embryonic RNA as PCR template to increase chances of identifying Bcl-2 family proteins involved in zebrafish embryonic apoptosis. Sequence analysis of the PCR-amplified cDNA fragments revealed significant sequence homology to known Bcl-2 family proteins. One of the cDNA fragments, ZM5-1 was most closely related to human Mcl-1. To isolate cognate full-length cDNA for ZM5-1, a commercial zebrafish embryonic cDNA library was probed with isotope-labeled ZM5-1. A total of twelve ZM5-1-positive plaques were isolated from one million of plaque formation units after three round of plaque purification. Insert cDNAs were obtained from purified phage particles by in vivo excision procedure and sequenced on both strands. The longest cDNA was 1253 bp and contained a single open reading frame of 768 bp (bases 9 to 776) encoding a polypeptide of 255 residues, an 8-bp 5’ untranslated region (UTR) and a 477-bp 3’ UTR (Fig. 1). The first start codon was located in a favored location (49) preceded by an upstream in-frame stop codon. Initial BLASTP search of GenBank database identified rat Mcl-1 as its closest relative, and therefore this protein was denoted as zebrafish Mcl-1 like protein 1 (zfMLP1). Later during the preparation of this report, BLASTP search showed that zfMLP1 was identical to a recently deposited protein sequence, zebrafish Mcl-1a (35, 40). We therefore renamed zfMLP1 as zebrafish Mcl-1a (zfMcl-1a) to comply with this recent report. Primary sequence analyses of zfMcl-1a identified two PEST sequences (43), one putative nuclear localization signal (NLS) and one transmembrane sequence (TM) (44), and two consensus polyadenylation signals (nt: 952–957 and 1205–1210) are double-underlined.

FIG. 1. The nucleotide and deduced amino acid sequences of zfMcl-1a. The amino acid sequence is shown below the nucleotide sequence in single letter code. The translation and stop codons are marked as bolded M and asterisk (*), respectively. The putative nuclear localization signal (aa 43–49) is boxed. Two PEST sequences (aa 26–44 and 61–86) are underlined. The putative transmembrane sequence is labeled with dashed line. Two polyadenylation signals (nt: 952–957 and 1205–1210) are double-underlined.
zfMcl-1a mRNA was expressed at high level as expected (Fig. 3, middle panel), and both 18 and 28s rRNAs were intact as indicated by the sharp RNA bands (Fig. 3, bottom panel).

The low expression of zfMcl-1a at gastrula stage suggests that it may be a candidate of negative regulator of apoptosis. Ikegami et al. showed that embryonic apoptosis abruptly becomes activated at gastrula stage in zebrafish (38). The authors suggested, among other possibilities, that a maternally supplied negative regulator might inhibit apoptosis induction during early embryonic development (38). It was further proposed that down-regulation of this negative regulator, for instance through RNA degradation and/or transcription suppression of the embryonic gene, would then allow the embryo to activate apoptosis pathways during the gastrula stage to remove damaged cells (38). Our observation that zebrafish Mcl-1a mRNA expression experienced a gradual down-regulation after fertilization through the gastrula stage, which correlates with the time course of the embryonic apoptosis, provides a possible basis for the molecular mechanism underlying this developmentally programmed embryonic apoptosis. More experiments are needed to determine the consequences of low zebrafish Mcl-1a mRNA levels on normal zebrafish embryonic development, and whether or not zfMcl-1a plays a role in the developmentally programmed embryonic apoptosis described above.
To obtain clues on how zfMcl-1a functions during zebrafish embryogenesis, we set out to determine the subcellular localization pattern for zfMcl-1a. As described earlier, the PSORT II program has predicted a putative NLS (aa 43 through 49) in zfMcl-1a protein sequence (Fig. 1). Since an intranuclear localization has not previously been described for any Bcl-2 family proteins, we were interested to find out whether zfMcl-1a is indeed a nuclear protein. To do so, an expression plasmid, pGFP-zfMcl-1a expressing EGFP-zfMLP1 fusion protein was constructed and then introduced into both mammalian (NIH3T3) and zebrafish (ZFL) cultured cells by liposome-mediated transfection. The intracellular location of the fusion protein in living cells was then directly monitored by fluorescence microscopy. As shown in Figs. 4B and 4B', ZFL cells transfected with pEGFP-zfMcl-1a displayed a clear nuclear EGFP fluorescence with minor cytosolic EGFP fluorescence. However, ZFL cells transfected with pEGFP-C1 displayed a ubiquitous intracellular EGFP fluorescence (Figs. 4A and 4A'). Similar observations were also made in NIH3T3 cells (unpublished results). Therefore, zfMcl-1a is indeed a nuclear protein. We then tested whether the PSORT-predicted NLS (aa 43–49) is a prerequisite of zfMcl-1a nuclear localization by introducing an aa 43–49 deleted EGFP-zfMcl-1a variant into cultured cells, and then determine the intracellular location of the EGFP-zfMcl-1a variant by monitoring EGFP fluorescence. As shown in Figs. 4C and 4C', ZFL cells transfected with pEGFP-zfMcl-1Δ43–49 expressed predominantly cytosolic EGFP fluorescence with very faint nuclear EGFP fluorescence. Clearly, aa 43–49 were essential to the nuclear translocation of zebrafish Mcl-1a. As far as we are aware, zfMcl-1a is the first Bcl-2 family protein with nuclear translocation. More experiments are needed to understand the biological meaning of this unique localization of zfMcl-1a.

In conclusion, we have cloned and characterized a novel member of the Mcl-1 class of the Bcl-2 family from the zebrafish, Danio rerio. This protein, denoted as zfMcl-1a, was unique in the following aspects. First, compared with other Mcl-1 proteins, zfMcl-1a has a very short N-terminal region. Second, although it probably does not have a BH4 domain, zfMcl-1a contains a predicted α helix at aa 85–95, which might define another functional element in zfMcl-1a. And finally, zfMcl-1a is the only known nuclear Bcl-2 family protein. Together with the embryonic expression pattern of zfMcl1 mRNA, our data suggested that zfMcl1 is likely to be an important apoptotic regulator during normal zebrafish embryonic development. Further experiments...
are underway to determine the role of zfMcl-1a in zebrafish embryogenesis.

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