Xenopus Death Receptor-M1 and -M2, New Members of the Tumor Necrosis Factor Receptor Superfamily, Trigger Apoptotic Signaling by Differential Mechanisms*

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Signaling through the tumor necrosis factor receptor (TNFR) superfamily can lead to apoptosis or promote cell survival, proliferation, and differentiation. A subset of this family, including TNFR1 and Fas, signals cell death via an intracellular death domain and therefore is termed the death receptor (DR) family. In this study, we identified new members of the DR family, designated xDR-M1 and xDR-M2, in Xenopus laevis. The two proteins, which show high homology (71.7% identity), have characteristics of the DR family, that is, three cysteine-rich domains, a transmembrane domain, and a death domain. To elucidate how members of xDR-M subfamily regulate cell death and survival, we examined the intracellular signaling mediated by these receptors in 293T and A6 cells. Overexpression of xDR-M2 induced apoptosis and activated caspase-8, c-Jun N-terminal kinase, and nuclear factor-κB, although its death domain to a greater extent than that of xDR-M1 in 293T cells. A caspase-8 inhibitor potently blocked this apoptosis induced by xDR-M2. In contrast, xDR-M1 showed a greater ability to induce apoptosis through its death domain than did xDR-M2 in A6 cells. Interestingly, a general serine protease inhibitor, but not the caspase-8 inhibitor, blocked the xDR-M1-induced apoptosis. These results imply that activation of caspase-8 or serine protease(s) may be required for the xDR-M2- or xDR-M1-induced apoptosis, respectively. Although xDR-M1 and xDR-M2 are very similar to each other, the difference in their death domains may result in diverse signaling, suggesting distinct roles of xDR-M1 and xDR-M2 in cell death or survival.

Apoptosis plays a crucial role in both the development and the homeostasis of metazoans. Cells die by apoptosis in the developing embryo during morphogenesis and in the adult during tissue turnover or at the end of an immune response. A number of molecules are known to be involved in apoptotic signaling. Intensive investigation of some members of the tumor necrosis factor receptor (TNFR) superfamily has led to a better understanding of the signaling pathways in apoptosis (1–3). In general, the TNFR superfamily is implicated in diverse biological activities, such as the regulation, not only of cell death, but also of cell proliferation, differentiation, survival, and cytokine production (4, 5). The receptors of this family share a common structural motif in their extracellular region that has been shown to be important for ligand binding; this motif consists of multiple cysteine-rich repeats of ~30–40 amino acids each (6). The death receptors (DRs), which make up a subset of the TNFR superfamily, contain in addition a homologous sequence in their cytoplasmic region termed the “death domain” that consists of ~80 amino acids and is involved in mediating apoptosis signaling (7). The best-characterized death receptors are Fas (also called CD95 or Apo1) (8, 9) and TNFR1 (also called p55) (10). Recently, a number of additional death receptors have been reported, such as DR3 (also called Apo3, WSL-1, or TRAMP) (11–14), DR4 (15), and DR5 (16).

The death receptors are believed to activate mainly three kinds of signaling cascades: (i) kinase cascades leading to the activation of nuclear factor-κB (NF-κB) (17), (ii) mitogen-activated protein kinase (MAPK) cascades, including the c-Jun N-terminal kinase (JNK) or p38 cascades (18), and (iii) protease cascades involving the sequential activation of caspases (19). For instance, the engagement of TNFR1 by its cognate ligand, TNFα, results in the formation of a death-inducing signal complex, which is comprised of the adapter molecules TNFR1-associated death domain protein (TRADD) (20), Fas-associated death domain/mediator of receptor-induced toxicity 1 (FADD/MORT-1) (21, 22), and caspase-8 (also called MACH or FLICE) (23, 24). The resulting complex facilitates the autolytic activation of caspase-8. The active caspase-8 then causes the proteolytic activation of downstream caspases that culminates in the irreversible commitment of the cells to apoptosis (19). More recently, evidence has accumulated that non-caspase proteases such as cathepsin and serine protease(s) are also involved in DR-induced apoptosis (25, 26). In contrast, with respect to stress signaling and the immune response, TNF interacts with TRADD, TNFR-associated factors (TRAFs) (27), and receptor-interacting protein (RIP) (28), leading to the activation of NF-κB or JNK. The subsequent activation...
of NF-κB-inducing kinase (NIK) (29, 30), a member of the MAPKKK family, activates the downstream kinases, IκB kinase α (IKKα) and IKKβ (31–33), leading to the phosphorylation and degradation of IκB and the activation of NF-κB. Alternatively, apoptosis signal-regulating kinase 1 (ASK1) (34), a member of the MAPKKK family, is recruited into the death-inducing signal complex, and JNK activation can then be elicited through the MAPK signaling pathway. It has been reported that RIP may bifurcate into two distinct signaling pathways, NF-κB and/or JNK activation (35).

Until now, many members of the TNFR superfamily, including the DRs and nerve growth factor receptors, have been identified in mammals. However, only three members of the TNFR superfamily, xDR superfamily, xp75TRα, xp75TRβ (36), and full back (GenBank™ accession number AAD54072) have been cloned in Xenopus laevis. Although these molecules have death domains, there has been no evidence that they promote apoptosis. Here, to elucidate the signaling pathways of apoptosis or cell survival that occur through death receptors in Xenopus, new members of the death receptor family, xDR-M1 and xDR-M2, have been identified. Furthermore, this study reports that differential signaling on NF-κB and JNK activation is elicited by xDR-M1 and xDR-M2 and that xDR-M1 and xDR-M2 participate in apoptosis through distinct pathways.

**EXPERIMENTAL PROCEDURES**

**Materials**—The Alexa Fluor 546 anti-mouse IgG conjugate antibody and Alexa Fluor 546 anti-rabbit IgG conjugate antibody were purchased from Molecular Probes. Anti-FLAG monoclonal antibody, anti-rabbit IgG horseradish peroxide (HRP)-conjugated antibody, anti-mouse IgG HRP-conjugated antibody, and 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) were purchased from Sigma. Mouse anti-c-Myc monoclonal antibody, rabbit anti-His polyclonal antibody, and rabbit phospho-specific SAPK/JNK (Thr183/Tyr185) and p38 MAP kinase (Thr180/Tyr182) polyclonal antibodies were purchased from Roche Diagnostics, Santa Cruz Biotechnology, and Cell Signaling Technology, respectively. Z-IE TD-FMK was purchased from BD Biosciences.

**Isolation of xDR-M1 and xDR-M2 cDNAs**—A Xenopus immature cDNA library (37) was screened using a partial xDR-M1 cDNA fragment obtained as a highly expressed gene in the immature ovary by differential display between the immature ovary and testis. Hybridization was performed as described previously (38). The full-length xDR-M1 cDNA was subcloned by in vitro excision into pBluescript (Stratagene).

A Xenopus data base (Azxeldb) was screened for sequence homology with xDR-M1 cDNA by using the blastn algorithm. Fragments containing the 3′ end of the xDR-M2 cDNA were amplified from Xenopus ovary cDNA using a FirstChoice RLM-RACE kit (Ambion), according to the manufacturer’s instructions. Using 1/40th of the first-strand cDNA as a template, PCR was carried out for 30 cycles to amplify the xDR-M1 or xDR-M2 cDNA fragment with specific primer pairs: forward primer 5′-GTGTTTCGCTGTCCTCACCAG-3′ and reverse primer 5′-GCCCTTCTGCACTGCTC-3′ and reverse primer 5′-TGACCGGAACTGGAGTTCCG-3′, respectively. The forward and reverse primers for xDR-M1 correspond to the sequences containing exons 1 and 2 or exons 5, respectively.

**Cell Culture and Transfection**—X. laevis—A6 kidney epithelial cells were cultured in 70% Leibovitz’s L-15 medium containing 10% fetal bovine serum and antibiotics at 37 °C in a humidified incubator with 5% CO₂. A6 or 293T cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and antibiotics at 37 °C in a humidified incubator with 5% CO₂. At 12 h before transfection, the cells were plated at 1 × 10⁵ cells per 35-mm dish. Cells were transfected using FuGENE 6 (Roche Diagnostics), according to the manufacturer’s procedure.

**Analysis of Apoptosis by Transient Transfection**—293T cells were grown in 35-mm dishes. Transfected cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min and then exposed to 0.2% Triton X-100 in PBS for 10 min. Unspecific binding was blocked by incubation with 5% skim milk in PBS for 30 min. The cells were then treated with primary antibodies in PBS for 1 h followed by the second and incubated with Alexa 488-labeled secondary antibodies. After extensive washing, the reaction was developed by enhanced chemiluminescent staining using Supersignal west femto Maximum sensitivity substrate (Pierce).

**Luciferase Reporter Assay**—A6 or 293T cells were seeded into 12-well dishes at 0.5 × 10⁵ cells per well. They were then transiently transfected with reporter constructs and various amounts of the testing plasmids. Assay of JNK Activity—293T cells were cotransfected with pcDNA3 His-S-JNK2 and the effecter plasmids. After 40 h, His-S-JNK2 was purified from the cell lysates using S-protein-linked agarose as described previously (40) and analyzed by immunoblotting with phospho-specific JNK antibody.

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RESULTS

Identification of xDR-M1 and xDR-M2 Belonging to a New Subfamily of Death Receptors—We obtained xDR-M1 cDNA fragments when we searched for a gene that was highly expressed in the Xenopus immature ovary. Full-length cDNA clones were then isolated using a Xenopus immature ovary cDNA library. Multiple alignments were performed using the ClustalW program. Identical amino acids of xDR-M1 and xDR-M2, and amino acids identical with them in other death receptors, are shown in white against black.
The cDNA was synthesized with 1 µg of total RNA isolated from various Xenopus tissues (A) or A6 cells (B). xDR-M1 or xDR-M2 cDNA fragment was amplified by PCR using one-forth of the cDNA with specific primer (see “Experimental Procedures”). 10 ng of pEF1 xDR-M1-Myc-His or pEF1 xDR-M2-Myc-His plasmid was used as templates to verify specific amplification of xDR-M1 or xDR-M2.

was obtained that showed 80.5% homology with part of the xDR-M1 cDNA. The full-length cDNA, which was designated xDR-M2, was then isolated by PCR from Xenopus ovary cDNA using specific primers corresponding to sequences that had been obtained from the data base and cloned RACE. The nucleotide and deduced amino acid sequences of xDR-M1 and xDR-M2 (DDBJ/GenBankTM/EBI Data Bank accession numbers AB111446 and AB111447, respectively) are shown in Fig. 1A. Each cDNA contained an ORF that encoded a protein consisting of 328 amino acid residues. The complete amino acid sequences of the proteins showed 71.7% identity. By analysis using the simple modular architecture research tool, each xDR-M was predicted to have a signal peptide, three cysteine-rich repeats, a transmembrane domain, and a death domain. Because a cleavage site for the signal peptide was found between lysine (at position -1 in Fig. 1A) and proline (at position 1 in Fig. 1A), xDR-M1 and xDR-M2 may undergo proteolytic processing to a mature protein with 298 and 295 amino acid residues, respectively. The transmembrane domains were located from positions 122–151 and 129–150 of xDR-M1 and xDR-M2, respectively. The predicted extracellular domains of the proteins contained the three cysteine-rich repeats that characterize the TNFR superfamily (Fig. 1A). These cysteine-rich domains contribute to the binding of the TNFR superfamily members with their specific ligands. The cytoplasmic regions of xDR-M1 and xDR-M2 included death domains that are typical of some TNFR superfamily members and are involved in apoptosis (Fig. 1A). Therefore, these molecules belong to the death receptor family within the TNFR superfamily.

The xDR-Ms displayed higher homology (71.7% identity) with each other than is seen among any other DRs, and their ligand-binding region (LBR) and death domains shared 83.3% and 70.3% sequence identity, respectively (Fig. 1B). Generally, the DR family members have 30–50% homology with one another. Because the LBRs of the xDR-Ms were also quite similar in primary structure, the xDR-Ms are likely to represent a new subfamily, which we term the xDR-M subfamily.

Expression of xDR-M1 and xDR-M2 mRNA—RT-PCR analyses were performed to determine the level of xDR-M1 or xDR-M2 mRNA expression in various tissues. The amplified fragments were specific to xDR-M1 or xDR-M2 cDNA, because the sequences of the 3’ ends of the primers used for xDR-M1 and xDR-M2 (see “Experimental Procedures”) were different. Furthermore, the sequence of the forward primer for xDR-M1 was designed to contain exons 1 and 2 based on the information obtained for the xDR-M1 gene, and the reverse primer for xDR-M1 included the sequence for exon 5 (data not shown). Although the genomic structure of the xDR-M2 gene has not been identified, it may be similar to that of the xDR-M1 gene, since genomic structures tend to be conserved among TNFR superfamily genes. The PCR fragments were cloned and con-
Investigated by RT-PCR in A6 cells, which are derived from X. laevis kidney. In these cells, the amplified fragments for xDR-M1 and xDR-M2 have no effect on the xDR-M2-induced apoptosis in A6 cells. Both caspase inhibitors were able to significantly protect the cells from the xDR-M2-induced cell death (Fig. 5). The deletion mutant of xDR-M2 containing its death domain also activated caspase-8, but the mutant lacking the death domain did not, indicating a significant role of the death domain in activating caspase-8. However, in A6 cells neither xDR-M1 nor xDR-M2 caused detectable cleavage of caspase-8 domain in activating caspase-8. However, in A6 cells neither xDR-M1 nor xDR-M2 brought about apoptosis (Fig. 3C).

Activation of Caspase-8 or Serine Protease(s) Is Required for the Apoptosis Induced by xDR-M2 or xDR-M1. Death receptors recruit caspase-8 in a manner mediated by adapter molecule(s), such as FADD and/or TRADD, and activate caspase-8. This in turn leads to the cleavage of other downstream caspases in a caspase cascade and death (8). To determine whether the activation of caspase-8 plays a crucial role in the initiation of xDR-M-triggered apoptosis, we examined the autolytic cleavage of caspase-8 induced by the overexpression of xDR-M. For the analysis of caspase activation, 293T or A6 cells were transfected with expression vectors containing FLAG-Xenopus caspase-8 and xDR-M1 or xDR-M2, and the caspase-8 activation was determined by Western blot analysis with anti-FLAG antibodies. As shown in Fig. 4, overexpression of xDR-M2 but not xDR-M1 activated caspase-8 in 293T cells. The deletion mutant of xDR-M2 containing its death domain also activated caspase-8, but the mutant lacking the death domain did not, indicating a significant role of the death domain in activating caspase-8. However, in A6 cells neither xDR-M1 nor xDR-M2 caused detectable cleavage of caspase-8 (data not shown). To determine whether the activation of caspase-8 is essential for the apoptosis induced by xDR-M2, we examined the effects of a caspase-8 inhibitor, Z-IETD-FMK, and a cytokine response modifier A (Crma), a product of the cowpox virus, on the cell death induced by xDR-M2 in 293T cells. Both caspase inhibitors were able to significantly protect the cells from the xDR-M2-induced cell death (Fig. 5A and data not shown). Unexpectedly, the caspase inhibitors were likely to have no effect on the xDR-M2-induced apoptosis in A6 cells (Fig. 5B and data not shown).

On the other hand, the xDR-M1-induced apoptosis in 293T or A6 cells was scarcely blocked by the caspase inhibitor as shown in Fig. 5, A or B, respectively. Recent study has reported that tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-triggered apoptosis can be blocked by a general serine protease inhibitor, AEBSF, in adenocarcinoma cell line, SNU-16 (26). Therefore, we investigated whether activation of serine protease(s) is required for the apoptosis by xDR-M1. The serine protease inhibitor, AEBSF, could prevent the apoptosis induced by xDR-M1 both in 293T and A6 cells (Fig. 5, C and D, respectively). In contrast, the inhibitor AEBSF had little effect on the apoptosis induced by xDR-M2 in A6 or 293T cells (Fig. 5, C or D, respectively).

**Fig. 5.** Effects of caspase-8 and serine protease inhibitors on apoptosis induced by xDR-M1 and xDR-M2. A and C, 293T cells were transfected with pEF1 expression plasmid of xDR-M1, xDR-M2, or Fas under the same condition with the experiments of Fig. 3A. B and D, A6 cells were transfected with pCI xDR-M1-DD, pCI xDR-M2-DD, or pCI empty vector under the same condition with the experiments of Fig. 3C. They were analyzed with the same way as described above in A or C, except that a final concentration of AEBSF was 0.2 mM.

**Fig. 6.** Activation of JNK by xDR-M1 and xDR-M2. 293T cells were cotransfected with 0.5 μg of pcDNA3 His-S-JNK2 and 1.0 μg of pEF1 xDR-M1-Mye-His, pEF1 xDR-M2-Mye-His, pEF1 Fas-Mye-His, or pEF1 Mye-His empty vector. After 40 h, 9/10 of the cell lysates were precipitated with S-protein-linked agarose (S-PA) and analyzed by immunoblotting with phospho-specific-JNK antibody (upper panel) and anti-His antibody (middle panel). Expression of the death receptors was examined by immunoblotting 1/10 of the cell lysates with anti-Myc antibody (lower panel).
**JNK Is Activated by Overexpression of xDR-M2**—The apoptosis induced by death receptors is frequently accompanied by the activation of JNK or p38. We examined whether there is a difference in JNK signaling between xDR-M1 and xDR-M2. For the analysis of JNK activation, 293T cells were cotransfected with the expression vectors for JNK and xDR-M1 or xDR-M2, and the activation was determined by Western blot analysis with an anti-phospho-JNK antibody. xDR-M2 had a greater ability to activate JNK than did xDR-M1 or Fas (Fig. 6). The JNK inhibitor, SP60125, had no significant effect on the apoptosis induced by xDR-M2 (data not shown). In A6 cells, activation of JNK or p38 induced by xDR-M1 or xDR-M2 was not detected in PathDetect trans-reporting system (Stratagene) or Western blotting analysis using phospho-JNK or p38 antibodies (data not shown).

**xDR-M1 and xDR-M2 Differentially Activate NF-κB**—An important response induced by members of the death receptor family is the activation of the transcription factor NF-κB (17). To investigate the signaling pathway leading to NF-κB activation, *Xenopus* A6 cells and human 293T cells were transiently cotransfected with an NF-κB-luciferase reporter plasmid and xDR-M1 or xDR-M2 expression vector. In 293T cells, the overexpression of xDR-M1 moderately induced NF-κB activation (~10-fold), whereas that of xDR-M2 or Fas markedly activated NF-κB (~200-fold) (Fig. 7A). Both receptors activated NF-κB in a dose-dependent manner. Analyses of deletion mutants indicated that the death domains of xDR-M1 and xDR-M2 were likely to contribute to the activation of NF-κB (Fig. 7). These death domain-driven NF-κB activation was inhibited by the coexpression of an NF-κB super repressor, IxBa S32/36A (data not shown). In A6 cells, overexpression of xDR-M1 and its death domain also activated NF-κB (~30- and ~40-fold, respectively). Unexpectedly, xDR-M2 showed a weaker ability to activate NF-κB than did xDR-M1 (Fig. 7B).

**DISCUSSION**

In this study we have isolated cDNAs from *Xenopus* ovary designated xDR-M1 and xDR-M2, which encode new members of the TNFR superfamily. The xDR-Ms share the highest homology (71.7% identity) with each other among the known members of the TNFR superfamily (Fig. 1B). The extracellular domains of TNFR superfamily members contain cysteine-rich repeats that play a crucial role in binding to their cognate ligand (1). Importantly, LBRs of xDR-M1 and xDR-M2 are especially similar to each other (81.3% identity), compared with their other functional domains. In mammals, TNFRI and TNFRII or DR4 and DR5 are believed to bind the same ligand, TNFα or TRAIL, respectively (41). It is possible that the same ligand may trigger the signaling mediated by xDR-M1 or xDR-M2.

The expression patterns of xDR-M1 and xDR-M2 mRNAs were similar to each other in various adult tissues (Fig. 2). In contrast, xDR-M1 mRNA was detected in the A6 cell line derived from *Xenopus* kidney by RT-PCR, while xDR-M2 mRNA was not. These results suggest that xDR-M1 and xDR-M2 may be differentially expressed during development or due to extracellular circumstances. To investigate these possibilities, it will be necessary to determine the distributions of xDR-M1 and xDR-M2 by in situ hybridization or immunohistochemical analyses.

It has been believed that the sequential activation of caspases following the release of active caspase-8 is likely to be crucial for apoptosis by death receptors (8). However, recent studies have revealed that other types of proteases may also participate in apoptosis triggered by TNFα or TRAIL in some cell lines (25, 26). From the analyses using inhibitors of caspase-8 and serine protease(s) (Fig. 5), we have shown that xDR-M1 and xDR-M2 is likely to induce apoptosis by activation of some serine protease(s) and caspase-8, respectively, suggesting distinct roles of xDR-M members in apoptotic signaling. It is possible that TNFα receptors TNFRI and TNFRII or TRAIL receptors DR4 and DR5, may play distinct roles in cysteine and serine protease pathways on apoptotic signaling as xDR-M1 and xDR-M2 may do.

The activation of death receptors upon the binding of specific ligands is known to induce the JNK and NF-κB signaling pathways in addition to the protease pathways. The JNK signaling is known to activate proapoptotic signals (42). However, the activation of JNK is not required for death-receptor-induced apoptosis (43, 44). In 293T cells, a high activation of JNK was observed when xDR-M2 was overexpressed (Fig. 5). However, JNK activation does not appear to induce the apoptosis elicited by xDR-M2, because a JNK inhibitor had no significant effect on the xDR-M2-induced apoptosis (data not shown). The activation of NF-κB is believed to be a survival signal against apoptosis that leads to the transcription of anti-apoptotic genes, including IAP-1, IAP-2, XIAP, NDED, and c-FLIP, which block caspase activity (45–47). Although overexpression of xDR-M2 induced a strong activation of NF-κB, this activation did not affect the rate of apoptosis, even when the coex-
pression of a repressor of NF-κB signaling, IκBα (S32/36A), had suppressed the signaling (data not shown). Other members of the death receptor family can also induce the activation of NF-κB concomitantly with apoptosis (11–14, 16). Thus, the role of NF-κB activation in death receptor-induced cell death or survival remains controversial.

The ability of xDR-M1 to activate NF-κB was stronger than that of xDR-M2 in A6 cells, whereas the former was weaker than the latter in 293T cells (Fig. 7). The activation induced by xDR-M1 or xDR-M2 was mostly inhibited by the dominant negative form of IκBα in the both cell lines (data not shown). Therefore, this cell-type difference may be caused by exquisite combinations of amounts and properties of some adapter proteins that can be recruited into xDR-M1 or xDR-M2 signaling complex.

The activation of caspase-8, JNK, or NF-κB by xDR-M receptors depended on its death domain (Figs. 4, 6, and 7 and data not shown). These results demonstrated that the differences in caspase-8, JNK, and NF-κB signaling mediated by xDR-M1 and xDR-M2 are attributable to the death domains in their cytoplasmic regions. In mammals, death receptors associate with adapter proteins, such as FADD and TRADD, through their death domains. The difference in the death domains of xDR-M1 and xDR-M2 might affect their affinity for the adapter molecule(s), resulting in the induction of diverse signaling. The similarity between the death domains of xDR-M1 and xDR-M2 is less than that between their ligand-binding regions (Fig. 1B); thus, it is easy to imagine that each death domain could associate with adapter proteins in a distinct manner, leading to the differential signaling of xDR-M1 and xDR-M2. We have isolated adapter molecules from Xenopus that share homology with mammalian FADD and RIP. It will be interesting to investigate the roles of these molecules in xDR-M signaling.

Finally, it is possible that xDR-M1 and xDR-M2 play distinct roles in the intracellular signaling for apoptosis or survival, resulting in the generation of diverse effects from the same extracellular signals. It is of great interest to determine how xDR-M1 and xDR-M2 are related to metamorphosis, during which both xDR-M1 and xDR-M2 mRNA were expressed (data not shown). To elucidate the physiological functions of the two receptors, it will be necessary to study the expression patterns of xDR-M1 and xDR-M2 during development and to identify their cognate ligand(s).

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