Molecular Cloning and Functional Analysis of the Mouse Homologue of the KILLER/DR5 Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIl) Death Receptor

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Abstract

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIl) and its receptor TRAIL selectively kills cancer cells but not normal cells. We report here the cloning of the mouse homologue of the TRAIL receptor KILLER/DR5 (MK). The cDNA of MK is 1146 bp in length and encodes a protein of 381 amino acids. MK contains an extracellular cysteine-rich domain, a transmembrane domain, and a cytoplasmic death-domain characteristic of Fas, tumor necrosis factor, and human TRAIL receptors. MK is highly homologous and binds TRAIL with similar affinity as human DR4 and KILLER/DR5. MK induces apoptosis in mouse and human cells and inhibits colony growth of NIH3T3 cells. Expression of MK is p53-dependent and up-regulated by tumor suppressor p53 and by DNA damaging agents in mouse cells undergoing apoptosis. This is the first report describing a mouse TRAIL receptor gene and also demonstrating that the p53-dependent regulation of KILLER/DR5-mediated apoptosis is conserved between human and mouse.

Introduction

In mammalian cells, apoptosis induction can be triggered by the interaction of a set of ligands and their corresponding death-domain-containing receptors belonging to the TNF family (1). Upon ligand binding, receptors become trimerized, and associated molecules are recruited to form the DISC (2, 3). FADD is directly recruited into the DISC in Fas-mediated apoptosis, while TNF-mediated apoptosis involves both FADD and TRADD (2, 3). Once the DISC is formed, it activates procaspases resulting in a caspase cascade leading to apoptosis. TRAIL is a member of the TNF superfamily that is expressed in most human and mouse tissues (4, 5). TRAIL selectively kills transformed cells but not normal cells, which suggests that TRAIL has a potential application for cancer therapy (4, 5). Thus far, four human TRAIL receptors have been identified: Death receptor 4 (DR4) or TRAIL-R1 (6), KILLER/DR5 (TRAIL-R2, TRICK2; 7–11), TRID (DcR1, TRAIL-R3 or LIT; 9, 12–14) and TRUNDD (DcR2 or Rhipid). Among them, DR4 and KILLER/DR5 are most human and mouse tissues (4, 5). TRAIL selectively kills transformed cells but not normal cells, which suggests that TRAIL has a potential application for cancer therapy (4, 5). Thus far, four human TRAIL receptors have been identified: Death receptor 4 (DR4) or TRAIL-R1 (6), KILLER/DR5 (TRAIL-R2, TRICK2; 7–11), TRID (DcR1, TRAIL-R3 or LIT; 9, 12–14) and TRUNDD (DcR2 or Rhipid). Among them, DR4 and KILLER/DR5 are

Apoptosis induction was determined by either β-galactosidase staining or by visible fluorescence under a UV microscope as described previously (8, 20). Briefly, for the β-galactosidase assay, at 48 h after transfection, cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside to assess the number of remaining blue cells as well as to observe

Materials and Methods

Cell Lines and Transfection Conditions. Mouse fibroblast NIH3T3 and the mouse embryonal carcinoma cell line F9 were obtained from the American Type Culture Collection (Rockville, MD). The human colon cancer cell line SW480 was maintained as described previously (8). The M3 murine lymphoma cell line expressing temperature-sensitive p53 has been described (18, 19). Cells were transfected using pCEP4 vector (Invitrogen, CA), pCEP4MK, pEGFP-N vector, and pEGFP-N-MK. In experiments involving transfection of pCMV-β-gal (Clonetech, CA) and the pCEP-expression vector, a 1:4 ratio of plasmid was used. For NIH3T3 cells, LipofectAMINE (Life Technologies, Inc., Gaithersburg) was used for the transfection. In the case of SW480 cells, Lipofectin was used for the transfection.

cDNA Cloning. BLAST search was performed with the human and mouse EST databases using a sequence of death domain of human TRAIL death receptor KILLER/DR5 (8). An EST clone (AI154278) showed a high degree of homology with death domains of DR4 and KILLER/DR5. On the basis of the EST sequence, primers (MO-F3: 5′-TGT CTG TGA GCA ACC ACT GCC GC-3′; MO4: 5′-GCC ACT TTC CGG TTC ACC GCC CAC-3′; MO8: 5′-GCC TGT AGC ACA GCA AGC AAC GC-3′; and MO9: 5′-CTC CGG CCG CTG TAG GCC AGC-3′) were designed and 5′-RACE-PCR was performed using a mouse kidney cDNA library (Clontech, CA) as a template according to the manufacturer’s instructions. To express MK, two vectors—pCEP4 (Invitrogen, CA) and pEGFP-N (Clonetech, CA)—were used. The cDNA of MK was obtained by PCR with primer pairs of 5′-CCG GCT TCT CTT CCT GAG ACC ATG GAT GCT CAC-3′ and 5′-CGC ACT GCT ATC GCT CCA-3′ for cloning into the pCEP4 vector or 5′-AGC CGG AAT TCC CAC CAT GGA GCC TCC AGG A-3′ and 5′-GCG CCT ACC AGC CAC TGA GAT CCT CAC-3′ for cloning into the pEGFP-N vector. PCR was performed using Pfu (Stratagene, CA) as enzyme and a mouse kidney library (Clontech, CA) as a template. The PCR conditions were: 35 cycles of 94°C for 30 s, 57°C for 1 min, and 72°C for 4 min, with one cycle of 72°C for 10 min for the final extension. The PCR product was purified after double digestion by HindIII/XhoI for pCEP4 or EcoRI/KpnI for pEGFP-N.

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4 The abbreviations used are: TNF, tumor necrosis factor; DISC, Death Inducing Signaling Complex; TRAIL, TNF-related apoptosis-inducing ligand; TNFR, TNF receptor; EST, expressed sequence tag; RACE-PCR, rapid amplification of cDNA ends-PCR; MK, mouse (homologue of the TRAIL receptor) KILLER/DR5.

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MOLECULAR CLONING AND FUNCTIONAL ANALYSIS OF MK

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[1]:

Northern Blot Analysis. The expression pattern of mouse TRAIL death receptor MK was determined through hybridization to a mouse multiple tissue blot (Clontech, CA) using a full-length cDNA of MK as probe. The results revealed that TRAIL can bind MK with similar affinity as DR4 and KILLER/DR5 (Fig. 2C, Lanes 2-4), supporting the hypothesis that MK is a mouse TRAIL receptor.

Table 1 Homology between death receptors and MK

<table>
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<th>Homologue</th>
<th>DD</th>
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<tr>
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<td>18/40</td>
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<td>13/28</td>
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<td>Mouse TNFR-1</td>
<td>11/28</td>
<td>19/32</td>
<td>13/28</td>
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<td>TRID</td>
<td>13/28</td>
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The (%) identity/similarity values were obtained from a Clustal protein alignment using the MacVector 6.0.1 program. ORF indicates Open Reading Frame. The following amino acids (aa) were included within the death domain (DD) of each family member: DR4 aa 74–169; KILLER/DR5 aa 81–178; DR4 aa 132–230; TRUNDD aa 83–181; and Mouse TNFR-1 aa 381–449. The residues included within the death domain (DD) of each family member: DD CRD Full ORF

74 –161; and mouse TNFR-1 aa 79–211. For full open reading frame (ORF) comparison, all ORF were included within the cysteine-rich domain (CRD) of each family member: MK aa 74–169; KILLER/DR5 aa 81–178; DR4 aa 132–230; TRUNDD aa 83–181; and Mouse TNFR-1 aa 381–449.

Apoptosis Induction by MK in Human and Mouse Cell Lines. Overexpression of the two proapoptotic human MK receptors DR4 and KILLER/DR5 can rapidly induce apoptosis in human cells (6–9).

Results

Cloning of MK. On the basis of the information from EST clone (AI154278), which showed a high degree of homology with the death domains of DR4 and KILLER/DR5, we performed RACE-PCR and obtained the complete sequence for the mouse TRAIL death receptor KILLER/DR5 cDNA (Fig. 1). The sequence predicts an open reading frame of 1146 bp encoding a protein of 381 amino acids. The protein contains a cytoplasmic COOH-terminal death domain, a transmembrane domain, and a cysteine-rich extracellular NH2-terminal domain characteristic of the TNFR family (2). At the protein level, the death domain of MK shows high homology with human TRAIL receptors DR4 (76%) and KILLER/DR5 (79%; Fig. 2B and Table 1). Overall, the whole sequence of MK shares 43 and 49% of similarity with DR4 and KILLER/DR5, respectively (Fig. 2A and Table 1) as well as high homology with two decoy receptors TRID, TRUNDD (Table 1). MK is more closely related to TRAIL receptors than either Fas or TNFR-1 (Table 1). Analysis of the DNA sequence indicated that MK has a Kozak sequence upstream of the initiating methionine 5’-GACAC-CATGG-3’ (data not shown).

MK Is a TRAIL Receptor. Because MK is more closely related to human TRAIL receptors than other death domain-containing receptors such as Fas or TNFR-1, we suspected that MK may be a novel TRAIL receptor. To test this possibility, we fused the extracellular domain of MK to the Fc portion of mouse IgG and then performed a TRAIL-binding assay (Fig. 2C). The results revealed that TRAIL can bind MK with similar affinity as DR4 and KILLER/DR5 (Fig. 2C, Lanes 2-4), supporting the hypothesis that MK is a mouse TRAIL receptor.

Tissue Expression of the MK Gene. Northern blot analysis using the MK probe revealed the expression of a single MK TRAIL death receptor transcript of approximately 3.4 kb in the mouse multiple tissue blot (Fig. 2D). MK is predominantly expressed in heart, lung, and kidney (Fig. 2D). In humans, two proapoptotic TRAIL receptors DR4 and KILLER/DR5 are expressed in almost all tissues (6, 9), as are the two decoy receptors TRID and TRUNDD (9, 13, 15, 16). This preferential expression of MK in heart, lung, and kidney suggests that MK may play a specialized role in these tissues as compared with other tissues in which MK expression is low or undetectable.

Apoptosis Induction by MK in Human and Mouse Cell Lines. Overexpression of the two proapoptotic human MK receptors DR4 and KILLER/DR5 can rapidly induce apoptosis in human cells (6–9).
Fig. 2. Homology, TRAIL binding, and tissue expression of the MK gene. A, amino acid homology between MK and its closest human DR4 and KILLER/DR5. * = identity; · = similarity. The multiple alignment was carried out using the MacVector 6.0 Clustal W (1.4) program (Oxford Molecular Group).

B, homology in the death-domain regions of human TRAIL receptors DR4 and KILLER/DR5 is shown. A multiple sequence alignment algorithm was carried out as in A. Boldface, a predicted death-domain consensus. The following amino acids were included: MK aa 286 – 353, DR4 aa 356 – 404, KILLER/DR5 aa 324 – 393.

C, the extracellular domain of MK binds TRAIL. A conditioned media from the culture of 293T cells transfected with Fas-Fc, DR4-Fc, KILLER/DR5-Fc, and MK-Fc were incubated with purified soluble TRAIL. TRAIL-binding assay was carried out as described previously (7).

D, tissue expression pattern of the MK gene. Poly(A)+ RNA was obtained from different tissues (as indicated) and analyzed by Northern blotting as described in “Materials and Methods.” Hybridization to the mouse MK cDNA (upper panel) or mouse β-actin probe (lower panel) is shown. RNA size markers are indicated in kb (left).
Fig. 3. Expression of MK induces apoptosis and suppresses growth of human and mouse cells. Transfections with mammalian expression vectors in mouse NIH3T3 cells and human SW480 cells, apoptosis, and colony assays were carried out as described in “Materials and Methods.”

A, cells were transfected with pCEP4 (a and b, upper panel) and pCEP4MK (c and d, lower panel) along with CMV/β-gal and stained with 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside on day 2 after transfection. Arrows in c and d, round apoptotic blue cells in MK-transfected cells; arrows in a and b, healthy nonapoptotic blue cells transfected by the vector control. B, cells were transfected with pEGFP-N (e and f, upper panel) or pEGFP-N-MK (g and h, lower panel). Arrows in g and h, cells with bright fluorescence and characteristic nuclear fragmentation undergoing apoptosis; arrows in e and f, cells with bright fluorescence have no characteristic nuclear fragmentation. C, MK suppresses colony growth. NIH3T3 cells were transfected with pCEP4 (left) or pCEP4MK (right), selected with 0.2 μg/ml hygromycin for 10 days and stained with Coomassie Blue as described in “Materials and Methods.”
On the basis of its death domain-containing structure, we predicted that MK may be a proapoptotic death receptor. To investigate whether MK could induce apoptosis, we transfected MK-expressing vectors into mouse or human cells. As shown in Fig. 3, overexpression of pCEP4MK or pEGFP-N-MK rapidly induces apoptosis of SW480 cells (a human colon cancer cell line), NIH3T3 cells (a mouse fibroblast line), and 293 T cells (data not shown), which suggests that MK is a proapoptotic receptor.

**MK Inhibits Colony Formation in Mouse NIH3T3 Cells.** The evidence presented above suggested that MK is a mouse homologue of the human TRAIL receptors DR4 and KILLER/DR5. We previously showed (8) that overexpression of KILLER/DR5 can eliminate colony formation of human cancer cell lines. To test the possibility that MK can inhibit colony formation, we transfected an empty vector pCEP4 or the MK-expressing vector pCEP4MK into NIH3T3 cells. After 10 days of selection in the presence of hygromycin B, we found that MK is a potent suppressor of colony formation (Fig. 3, A and B). In pCEP4MK transfected cells, there were extremely rare surviving colonies as compared with empty vector pCEP4 transfected cells (Fig. 3, C). A similar result was obtained in the human colon cancer cell line SW480 (data not shown). Inhibition of colony formation by overexpression of the mouse TRAIL receptor MK is probably due to apoptosis induction in these cells (Fig. 3, A and B).

**MK Is Regulated by the p53 Tumor Suppressor Gene.** We previously showed that the human TRAIL death receptor KILLER/DR5 is a candidate p53 target gene (8). After DNA damage, KILLER/DR5 was induced in cells with a functional p53 but not in cells with a mutant p53 or where p53 is degraded. To investigate whether MK is also regulated by p53, we used M3 murine lymphoid cells harboring a temperature sensitive-p53 to examine MK expression in the presence or absence of DNA damage. When the temperature is shifted from 37°C to 32°C, p53 adopts a wild-type conformation and M3 cells undergo massive apoptosis (18, 19). As shown in Fig. 4, MK is induced upon p53 activation at 32°C but not at 37°C (Fig. 4, A and B). In the presence of wild-type p53 at 32°C but not mutant p53 (37°C), expression of MK is further elevated after exposure of M3 cells to the DNA-damaging agent Adriamycin (Fig. 4, Lane 2 versus Lane 4). These results indicate that MK is a putative p53 target gene.
target gene and that DNA damage induces MK expression in a p53-dependent manner. Previous studies (22, 23) performed with wild-type p53-expressing F9 teratocarcinoma cells have demonstrated that these cells undergo apoptosis upon exposure to DNA-damaging agents. Because we have found that the human KILLER/DR5 is induced only in cells undergoing p53-dependent apoptosis but not in cells undergoing p53-mediated cell-cycle arrest, we examined the expression of MK after cells undergo apoptosis upon exposure to DNA-damaging agents. Be-CA expressing F9 teratocarcinoma cells have demonstrated that these p53-dependent apoptosis inducible

We report the isolation and characterization of a cDNA encoding the mouse receptor (MK) for TRAIL. Like the human proapoptotic TRAIL receptors DR4 and KILLER/DR5, the mouse TRAIL receptor MK displays all of the features characteristic of the TNFR family. In humans, there are four TRAIL receptors identified, and they are classified into two categories based on their ability to induce or inhibit apoptosis (2). Several lines of evidence support the conclusion that MK is a mouse homologue of KILLER/DR5:

(a) MK rapidly induces apoptosis of human and mouse cell lines and inhibits colony formation (Fig. 3);
(b) MK has more similarity to the human TRAIL receptor KILLER/DR5 than to DR4 (79 versus 76%, respectively; Fig. 2);
(c) MK is able to bind TRAIL, which suggests that it may in fact be a TRAIL receptor (Fig. 2C); and
(d) MK is induced upon p53 activation, and this induction is further elevated in the presence of DNA-damaging agents.

In humans, KILLER/DR5 but not DR4 is regulated by the tumor suppressor gene p53 (Wu et al. and data not shown). Induction of MK in cells upon p53 activation and by DNA damage is consistent with our previous observation in humans that KILLER/DR5 seems to be a p53 target gene (8) and suggests that MK may be the mouse homologue of KILLER/DR5. On the basis of this reasoning, we conclude that MK is a DNA damage-inducible p53-regulated mouse TRAIL death receptor.

Interestingly, the expression of MK is particularly abundant in heart, lung, and kidney, which is different from its human counterpart KILLER/DR5 as well as from DR4, TRUNDD, and TRID, which are expressed in most tissues (7, 9, 10, 13). It is notable that the expression of MK in immune tissues such as spleen is not appreciable (Fig. 2D). In mice, TRAIL is highly expressed in spleen, prostate, and lung but not in brain, liver, and testes (4, 5). Expression of MK is also not observed in brain, liver, and testes. Therefore, the high expression of both TRAIL and its receptor in the lung suggests a possibly important role for TRAIL and its receptor in this particular tissue.

In addition to providing a tool for studying the role of KILLER/DR5 in p53-dependent apoptosis in mouse cell lines and tissues, the isolation of MK makes possible a number of other future experiments. Among these is the possibility to determine whether other TRAIL receptors exist in mice. If in the future such homologues are not found through long stringency hybridization or database searching, the inhibition of MK expression through knockout or antisense strategies should provide a way to determine whether MK is the sole determinant of TRAIL sensitivity. The isolation of MK also makes it possible for the first time to examine the role of TRAIL receptors in tumor development in different genetic backgrounds, e.g., in p53-null or other death receptor-null mice.

In summary, we have isolated the mouse TRAIL death receptor KILLER/DR5 (MK) whose expression appears to be p53-dependent and is induced in cells undergoing p53-dependent apoptosis. Overexpression of MK rapidly induces apoptosis of mouse and human cells and suppresses colony growth. Future experiments will focus on elucidating the role of MK in p53-dependent apoptosis and mouse embryonic development.

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