Targeted Disruption of the Mouse Caspase 8 Gene Ablates Cell Death Induction by the TNF Receptors, Fas/Apo1, and DR3 and Is Lethal Prenatally

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Summary

Homozygous targeted disruption of the mouse Caspase 8 (Casp8) gene was found to be lethal in utero. The Caspase 8 null embryos exhibited impaired heart muscle development and congested accumulation of erythrocytes. Recovery of hematopoietic colony-forming cells from the embryos was very low. In fibroblast strains derived from these embryos, the TNF receptors, Fas/Apo1, and DR3 were able to activate the J un N-terminal kinase and to trigger IκBα phosphorylation and degradation. They failed, however, to induce cell death, while doing so effectively in wild-type fibroblasts. These findings indicate that Caspase 8 plays a necessary and nonredundant role in death induction by several receptors of the TNF/NGF family and serves a vital role in embryonal development.

Introduction

Programmed cell death in metazoans involves a crucial role for the caspases, members of a cysteine protease family that are expressed in the living cell as inactive precursors and become activated upon death induction. Once activated, these proteases cleave a specific set of substrate proteins that act as regulators of the apoptotic mechanisms and thus set the apoptotic program in motion (reviewed in Nicholson and Thornberry, 1997; Villa et al., 1997). Several receptors of the TNF/NGF family, including Fas/Apo1 (CD95), the p55 TNF receptor (CD120a), and others, can induce programmed death in cells. Studies of the mechanisms of action of these receptors led to the identification of a member of the caspase family, Caspase 8 (MACH/FLICE), which is recruited to Fas/Apo1 and apparently also to the p55 TNF receptor through association of a duplicated N-terminal motif in this caspase, the death effector domain (DED), with a homologous motif in an adapter protein, MORT1/FADD (Boldin et al., 1996; Muzio et al., 1996). It has also been suggested that two other caspases can associate with these receptors through specific adapter proteins: Caspase 10 (Casp10) through binding to MORT1/FADD (Fernandes-Alnemri et al., 1996; Vincenz and Dixit, 1997) and Caspase 2 (Casp2) through binding to the adapter protein RAIDD/CRADD (Ahmad et al., 1997; Duan and Dixit, 1997). Evidence has also been presented suggesting that Fas/Apo1 can induce death in a less direct manner through recruitment of the adapter protein Daxx, which activates the serine/threonine kinase J un N-terminal kinase (Yang et al., 1997). Most of the indications that the above signaling pathways may be involved in death induction are rather indirect, being based on assessment of the functional consequence of overexpression either of proteins suspected to participate in these pathways or of their nonfunctional mutants in transfected cells. Knowledge of the in vivo functional significance of the activation of these pathways is limited. Rather solid evidence implicates the death-inducing activity of Fas/Apo1 in restricting the immune response, mainly through self-destruction of lymphocytes and also in killing cells that express foreign antigens (reviewed in Nagata and Golstein, 1995). Other than this, however, there is little knowledge of the functional significance of the cell-killing activity of the various receptors of the TNF/NGF family.

In this study, we attempted to gain more direct information about the role and functional significance of Caspase 8 (Casp8) in the cell death-inducing activity of the receptors of the TNF/NGF family by targeted disruption of the mouse Caspase 8 (Casp8) gene.

Results

The Mouse Caspase 8 cDNA and Gene

Using the human CASP8 cDNA as a probe, we cloned the corresponding mouse cDNA, which we then used to clone the mouse Casp8 genomic region. Detailed sequence analysis of this region and comparison with the cDNA sequence disclosed the existence of eight exons within the region: exon I encoding the first DED: exons II and III, the second DED; exon IV, the intermediate region between the DEDs and the protease region;
Figure 1. The Mouse Caspase 8 cDNA, Its Exon Structure, and Its Embryonic Expression in Midgestation

(A) Collinear amino acid sequence alignment of the mouse and human Caspase 8 gene. The residues are numbered to the right of each sequence. Dashes indicate gaps in the sequence to allow optimal alignment. The protease homology region is shown in light shading. Identical amino acids are shown in boxes. Within the region of protease homology, the residues putatively involved in catalysis are shown in dark shading and marked by closed circles below the alignment. The residues constituting the binding pocket for the carboxylate side chain of P1 Asp are less heavily shaded and are marked by open circles. The potential sites of cleavage are darkly shaded.

(B) Splice junctions of the mouse Caspase 8 (Casp8) gene. Bases matching the splicing consensus sequence are noted in bold. The polyadenylation signal is underlined and the polyadenylation site is shaded.

(C) Analysis of the expression of Casp8 in a wild-type embryo at 10.5 dpc. Whole-mount mRNA in situ hybridization. Abbreviations: ba, bronchial arches; flb, forelimb bud; hlb, hindlimb bud; ht, heart. Arrow, area of the mid-hind brain junction. Arrowhead, region of the dorsal mesentery.

The mouse chromosomal location of the Casp8 locus was determined by interspecific backcross analysis using the 94 progeny in the Jackson BSS cross. Polymorphisms were detected upon PCR amplification of C57BL/6J and M. spreutus genomic DNAs. Linkage analysis revealed that the mouse Casp8 gene maps to the proximal region of chromosome 1 [Centromere-D1Mit4-17.02 ± 3.88 cM-Inpp1-1.06 ± 1.06 cM-Casp8, Cd152-4.25 ± 2.08 cM-Crygb-3.19 ± 1.81 cM-D1Mit7].

The human CASPASE 8 (CASP8), CASPASE 10 (CASP10), and caspase homolog (CASH) genes have been localized to chromosome 2, band q33–34, telomeric to the STS marker D2S116 (Fernandes-Alnemri et al., 1996; Han et al., 1997; Rasper et al., 1998; V. M. B. and E. E. V., unpublished data). This region is homologous to that found for the mouse Casp8 gene. It is consistent with the location of inositol polyphosphate-1-phosphatase (INPP1), cytotoxic T-lymphocyte-associated protein 4 (CTLA4), and gamma B crystallin (CRYGB), the human homologs of Inpp1, Cd152, and Crygb, respectively, which were found adjacent to mouse Casp8. Comparison with the location of several different known human EST and STS markers indicated that the human
Targeted Disruption of the Mouse Caspase 8 Gene

Results in a Lethal Embryonic Phenotype

Transcription Pattern of Caspase 8 in the Midgestation Mouse Embryo

Both whole-mount in situ hybridization (Figure 1C) and radioactive histological in situ hybridization (data not shown) at gestational ages between 9.5 and 12.5 days postcoitum (dpc) revealed widespread distribution of Casp8 transcripts. Elevated signals could be detected in the heart (especially in the primitive ventricle), the surface ectoderm of the fore- and hindlimb buds, the branchial arches, and the area of the mid–hind brain junction (see arrow in Figure 1C) as well as in the region of the dorsal mesentery (Figure 1C, arrowhead).

Targeted Disruption of the Caspase 8 Gene Results in a Lethal Embryonic Phenotype

Casp8 binds to MORT1/FADD through its two N-terminal DED motifs (Boldin et al., 1996; Muzio et al., 1996). To abolish signaling to death through this caspase, we transfected R1 embryonic stem (ES) cells with a targeting vector allowing replacement of exons I and II, which encode these motifs, with a neomycin resistance...
Figure 3. Histological Study of the Caspase 8<sup>−/−</sup> Embryos
Liver of (A) wild-type and (B) mutant (Casp8<sup>−/−</sup>) embryos at 11.5 dpc. (C) Lung of a mutant embryo at 10.5 dpc. (D) Intersomitic blood vessel of mutant embryo at 11.5 dpc. (E) Eye of mutant embryo at 12.5 dpc (note hyperemia in the lens capsule). Heart of (F) wild-type and (G) mutant embryos at 11.5 dpc. Heart of (H) wild-type and (I) mutant embryos (high magnification) at 12.5 dpc. Abbreviations: at, atrium of the heart; br, bronchus; ca, camera (common ventricle) of the heart; ec, erythrocytes; hc, presumptive hepatocytes; hm, presumptive heart muscle cells; hp, hematopoietic precursors; lu, lung; rc, round cells (lymphocytes?); tr, trabeculae of the heart ventricle. Arrows in (D), endothelial cells; arrowheads in (e), capsula lentis. Scale bars: (A and B), 40 μm; (C), 100 μm; (D), 20 μm; (E), 200 μm; (F and G), 100 μm; (H and I), 40 μm.

( neo<sup>r</sup> ) cassette (Figures 2B, 2C, and 2D). Of 186 recombinant ES clones examined, 2 were positive for homologous recombination. Germline competent chimeras were generated by aggregation of the recombinant ES cells with morula-stage embryos, and progeny from heterozygous parents (Casp8<sup>+/−</sup>) were examined.

Although the heterozygous mice appeared phenotypically normal, no homozygous (Casp8<sup>−/−</sup>) mice could be detected in their intercross, whereas the Casp8<sup>+/−</sup> and Casp8<sup>+/+</sup> genotypes were represented in a ratio of 2 to 1, indicating that the homozygous disruption of Casp8 leads to prenatal death. Southern analysis of the genotypes of embryos collected between 9.5 and 11.5 dpc (Figure 2E) disclosed normal Mendelian segregation ratios. Until day 10.5, no gross morphological abnormalities could be detected. At day 11.5, however, 40% of the Casp8<sup>−/−</sup> embryos displayed the abnormal phenotype described below, and at day 12.5 almost all Casp8<sup>−/−</sup> embryos examined had this phenotype and some were found dead or close to death.

The most salient feature of the abnormal phenotype of the Casp8<sup>−/−</sup> mutant was marked hyperemia in the abdominal area (Figure 2F). In most mutant embryos this was accompanied by hyperemia in the superficial capillaries and other blood vessels, mainly in the umbilical and trunk area, including those in the intersomitic and interdigital blood vessels and to a lesser degree the face, head, and even the lens of the eye (Figure 3). Most mutant embryos were somewhat smaller than their wild-type littermates, but they were normally shaped and formed according to their gestational age. Histological examination revealed extensive erythrocytosis in the liver at day 10.5–13.5 dpc. Most of this organ was occupied by fully formed embryonic erythrocytes. Normal embryonic liver tissue, with its characteristic early hematopoietic elements, and early liver cells were visible only in the cortical area (compare Figures 3A and 3B). Hyperemia similar to that seen in the liver was observed in most major blood vessels and in many organs (for example, the lung [Figure 3C]); it was also seen in the intersomitic (Figure 3D) and intervertebral blood vessels, mesenchymal spaces, the retina, and under the capsule of the lens (Figure 3E). Examination at high magnification revealed that the erythrocytes were contained in larger and smaller blood vessel areas (Figure 3D), suggesting hyperemia and/or congestion rather than outright bleeding.

Detailed histological observation also revealed that the heart had developmental abnormalities. Although the heart was not appreciably larger than normal, the developing ventricular musculature was thin and in some cases not different from early mesenchyme. The trabeculae were thin and disorganized (compare Figures 3F and 3G as well as Figures 3H and 3I).

To further investigate the nature of the hyperemia, we assessed the numbers of hematopoietic precursors in...
the normal and mutant 11.5 dpc embryos. Whereas the cells of normal embryos yielded greater than 500 colonies (mostly myeloid) per input of $5 \times 10^6$ cells in the in vitro test, the same cell input yielded fewer than 10 colonies on average in the mutants (see Experimental Procedures). Disruption of the Casp8 gene thus appears to result in a dramatic primary or secondary depletion of the hematopoietic precursor pool.

The recombination event by which Casp8 was disrupted involved the introduction of a neo$^+$ gene flanked by two loxP recombination target sequences into the mouse genome (see Experimental Procedures). To confirm that the observed phenotype resulted from deficient Casp8 expression and not from distortion of the expression patterns of other genes by the neo$^+$ gene or its control elements, we excised the loxP-flanked insert by mating mice heterozygous for the recombination with PGK-Cre$^{loxP}$ (Lallemand et al., 1998) transgenic mice (Figure 5). They were equally resistant to killing by mouse recombinant GST-c-jun as substrate or analysed by Western blotting using phosphospecific anti-\(\alpha\)B or phosphorylation state-independent anti-\(\alpha\)B antibodies.

**Cultured Fibroblasts Derived from the Casp8$^{-/-}$ Mice Are Resistant to Death Induction by the TNF Receptors, Fas/Apo1, and DR3 but Are Sensitive to Death-Inducing Agents that Act from within the Cell**

To assess the functional consequences of the Casp8 mutation at the level of the individual cell, we established continuous fibroblast strains from wild-type and Casp8$^{-/-}$ embryos at 10.5 dpc. From each of the strains we also derived cell lines expressing a chimeric receptor comprised of the extracellular domain of the human p55 TNF receptor (CD120a) and the intracellular domain of DR3, a DD-containing receptor of the TNF/NGF family that induces cell death by a mechanism that seems closely related to that of CD120a (Chinnaiyan et al., 1996; Kitson et al., 1996; Marsters et al., 1996a; Bodmer et al., 1997).

Gene activation by TNF and DR3 involves stimulation of the transcription factors NF-$\kappa$B and AP1 through protein kinase cascades that lead to phosphorylation of the AP1-component Jun (reviewed in Kyriakis and Avruch, 1996) and the NF-$\kappa$B inhibitory protein I\(\kappa\)B$\alpha$ (reviewed in Stancovski and Baltimore, 1997). Activation of Fas/Apo1 also leads to enhanced phosphorylation of J un (Lalini and Koretzky, 1996; Goillot et al., 1997; Lenczowski et al., 1997; Yang et al., 1997). As shown in Figure 4, these effects could be induced in the Casp8$^{-/-}$ fibroblasts just as effectively as in the wild-type cells. In cells derived from both sources, treatment with TNF, as well as ligation of Fas/Apo1 or the CD120a-DR3 chimera, induced increased Jun N-terminal kinase activity within 5 min of stimulation (Figure 4A); in addition, TNF treatment or CD120a-DR3 ligation induced phosphorylation of I\(\kappa\)B$\alpha$, which was promptly followed by I\(\kappa\)B$\alpha$ degradation (Figure 4B).

**Cultured Fibroblasts Derived from the Caspase 8$^{-/-}$ Mice Respond Normally to Noncytoidal Effects of the TNF Receptors, Fas/Apo1, and DR3**

Cultured fibroblasts just derived from Casp8$^{-/-}$ embryos were killed by human TNF when it was applied to these cells in the presence of the protein-synthesis blocker cycloheximide. They were also killed by antibody cross-linking of Fas/Apo1. In addition, the wild-type cells expressing the CD120a-DR3 chimera were killed by cross-linking these chimeric molecules using antibodies to human CD120a. In contrast, cells derived from the Casp8$^{-/-}$ embryos were completely resistant to death induction by these agents (Figure 5). They were equally resistant to killing by mouse immunoprecipitation by antibodies raised against JNK1 followed by in vitro kinase reaction using recombinant GST-c-Jun as substrate or analysed by Western blotting.
The resistance of Casp8−/− fibroblasts to death induction by several receptors of the TNF/NGF family, despite their normal responses to other, noncytoidal effects of the same receptors, implies that Casp8 plays a central and nonredundant role in the death-induction mechanism. This finding may appear to be at variance with studies suggesting that death induction by the receptors can also occur through other signaling pathways, namely, activation of Casp10 and Casp2 in a manner similar to the activation of Casp8 (reviewed in Villa et al., 1997) and activation of the Jun N-terminal kinase through the adapter protein Daxx (Yang et al., 1997). This apparent discrepancy could be accounted for in a number of possible ways: (1) although these other death-inducing pathways might contribute to death induction, their contribution perhaps depends on a permissive role of Casp8; (2) these alternative pathways might act in a cell-type-specific manner, having little involvement in death induction in fibroblasts, yet important roles in other cells; or (3) these pathways might not mediate death induction by the TNF receptors or Fas/Apo1, and the data suggesting otherwise are based on their involvement in death induction by other receptors, which act similarly to those utilizing Casp8 in their death-inducing function. Tests employing overexpression of dominant negative mutants of signaling molecules to assess the involvement of such molecules in signaling are prone to such misinterpretation, as illustrated by the conflicting reports on the mechanisms of death induction by TRAIL/Apo2-L. Unfortunately, the fibroblast lines established in the present study showed little death response to TRAIL/Apo2-L, precluding their use for assessing the involvement of Casp8 in death induction by TRAIL/Apo2-L.

While pointing to a crucial role for Casp8 in death induction by receptors of the TNF family, our data indicate that this caspase is not essential in death induction by agents like etoposide or staurosporine. These agents employ caspases in their death-inducing effects (Jacobson et al., 1996; Martins et al., 1997; Bossy-Wetzel et al., 1998), yet they apparently function through activation of intracellular mechanisms such as those triggered by cytochrome c upon its release from damaged mitochondria to the cytosol (Li et al., 1997).

Our findings also indicate that Casp8 is not involved in the induction of phosphorylation and degradation of
IxBa, nor in the induction of J un N-terminal kinase activation by the TNF receptors, Fas/Apo1, or DR3. Studies have indeed delineated caspase-independent signaling pathways through which these receptors can stimulate the protein kinases involved in these two effects (see Ting et al., 1996; Yeh et al., 1997; Kelliher et al., 1998, and references cited therein). Nevertheless, there is also evidence suggesting that J un N-terminal kinase activation by Fas/Apo1 can occur in a way that does depend on caspase activation (Chen et al., 1996; Lenczowski et al., 1997; Roulston et al., 1998). Again, while clearly excluded from operating in fibroblasts, such a Casp8-dependent signaling pathway may well be found to act in other cells.

The in vivo consequences of the targeted disruption of the Casp8 gene are quite different from those observed for knockout mutations of ligands or receptors known to employ Casp8 in their death-inducing pathways. Disruption of the genes of the TNF receptor Fas/Apo1 or its ligands had pronounced effects on immune functions that operate in the adult. Yet, in contrast to the lethal prenatal effect of Casp8 disruption, ablation of the expression of these ligands or receptors had almost no effect on the development of the mice, the only exception being defective Peyer’s patch organogenesis in mice with targeted disruption of the p55 TNF receptor (CD120a) (reviewed in Gruss and Dower, 1995; Matsumoto et al., 1997). This apparent discrepancy implies that, apart from signaling for the effects of the TNF receptors, Fas/Apo1, and DR3, Casp8 also mediates signaling by other stimuli that play vital roles in embryogenesis. One candidate for such a stimulus is the ligand for DR3 (Marsters et al., 1998), a receptor shown conclusively in this study to involve Casp8 in its signaling for death.

The Casp8−/− embryonic phenotype is characterized by two salient features: impaired heart muscle development and congested accumulation of erythrocytes. These features resemble the recently reported phenotype of mice with targeted disruption of the MORT1/FADD gene (Yeh et al., 1998; Zhang et al., 1998). MORT1/FADD−/− embryos died at about the same age as the Casp8-deficient mice. Moreover, like the Casp8-deficient embryos, they exhibited impaired heart muscle development and congested accumulation of erythrocytes. Conceivably, the death of the MORT1/FADD−/− embryos can be accounted for by the failure to activate Casp8.

The mechanism underlying the midgestation lethal Casp8−/− phenotype remains to be clarified. Congestion and edema frequently accompany heart failure. In our case, however, no generalized edema was observed. Moreover, a similar failure of midgestation heart development in the targeted mutation of neuregulin was not accompanied by congestion or hyperemia (Meyer and Birchmeier, 1995). Defects such as those observed in the Casp8−/− mice could be due to abnormal angiogenesis. However, despite detailed examination, we could not discern any defects in the small or large blood vessels of the mutants. Indeed, the MORT1/FADD−/− embryos were found to exhibit normal expression of flk-1, a major regulator of early angiogenesis (Yeh et al., 1998). Thorough investigation of additional markers of blood vessel formation should fully clarify whether or not defective blood vessel formation contributes to the phenotype. The extensive hyperemia could also have originated from a hematopoietic defect. Some kind of hematopoietic abnormality whose nature has yet to be clarified is indeed indicated by the dramatic decrease of hematopoietic precursors in the Casp8−/− mice. We cannot, however, exclude the possibility that this hematopoietic defect was secondary to poor circulation, nor can we exclude the possibility that some additional, less easily discernible consequence(s) of the disruption of the MORT1/FADD or Casp8 genes contributed to the death of the embryos. Assessment of the effect of MORT1/FADD disruption on the development of lymphocytes indicated that this adapter protein contributes not only to the regulation of death of these cells, but also to the induction of their growth (Zhang et al., 1998). Likewise, it may well be that the lethal effect of MORT1/FADD or Casp8 disruption on these embryos results not from insufficient death induction, but from deficient induction of some other effect (perhaps growth stimulation) through the MORT1/FADD–Casp8 signaling pathway.

Our understanding of the in vivo significance of cell death lags significantly behind our knowledge of the mechanisms involved in this process. The current state of knowledge of the cell death–inducing function of TNF is a pertinent example. We have known for more than 30 years that TNFα and LTα (TNFα) can cause death of cells. Yet, while many other activities of these pleiotropic cytokines have been well placed in a physiological context, there is not yet even the slightest hint of the types of in vivo situations in which the direct cytotoxic effect of TNF is manifested. This function of TNF, like most other known activities of the TNF ligand family, presumably contributes to immune defense, but the way in which it does so is unknown. The targeted disruption approach provides a valuable tool for bridging this gap between molecular and physiological understanding, as it allows genes found to participate in the death process to be used as molecular probes for assessing the in vivo occurrence and functional consequences of the death process. The obligatory and specific role of Casp8 in death induction by receptors of the TNF/NFG family endows this molecule with particular value as such a probe. This value could not be fully exploited in the present study, since the prenatal death of the knockout embryos precluded analysis of the involvement of the Casp8 enzyme in functions manifested in the adult mouse. Restricting the disruption of the Casp8 gene to specific time periods during development or specific tissues (e.g., by creating mice chimeric for the recombination or by conditional disruption of the gene in a developmental stage-specific or cell lineage–specific manner) should allow further progress both in elucidating the role of this protein in embryonic development and in studying its function in the adult mouse.

Experimental Procedures

Isolation of the Mouse Caspase 8 cDNA

The mouse Casp8 cDNA was isolated from a λgt11 oligo(dT)-primed library derived from LPS-treated BAM3 mouse macrophage cells (kindly provided by S. Nagata; Watanabe et al., 1992) by screening
with a fragment of the human CASP8 cDNA. The inserts from the positive phages were subcloned into pBluescript vector and sequenced by the dideoxy chain-termination method. Sequence alignment of the mouse and human CASP8 cDNAs was performed, and their homology was evaluated using the alignment program in GeneAssist 1.1 b4 (Perkin-Elmer, Applied Biosystem Division).

Cloning of the Mouse Caspase 8 Gene and Adjacent Regions

A 129/Sv mouse genomic library (Stratagene) was screened with a mouse Casp8 cDNA probe. Of 1 million clones screened, 7 overlapping clones encompassing the full coding and the 3' UTR regions in the Casp8 gene were isolated. Their inserts were subcloned into the pBluescript vector using appropriate restriction enzymes and sequenced in both directions with dye terminators on ABI377T. The Sequencer program version 3.0 for Macintosh was used for analysis of the sequences and for final assembly of the sequence contig. To define the exon-intron boundaries of the Casp8 gene, its cDNA sequence was compared with that of the genomic contig using the alignment program in GeneAssist 1.1 b4 (Perkin-Elmer, Applied Biosystem Division). To obtain genomic clones that contain the 5' UTR region, we screened a mouse genomic library in P1 (MP1 Mouse P1 library number 703, Resource Center, Berlin-Charlottenburg, Germany) using a fragment at the 5' end of the sequence isolated from the phage library as a probe (the "5' probe" in Figure 2B). Further restriction nucleotide mapping of this clone and analysis of its structure were done by Southern DNA hybridization, using specific oligonucleotides of Tp, Sp6, CASHx, or Casp8 cDNA as probes.

Genetic Mapping of the Caspase 8 Gene

The chromosomal location of the mouse Casp8 gene was determined by linkage analysis with the BSS Backcross DNA Panel (Jackson Laboratory, Bar Harbor, ME). The panel consists of 94 genotyped progeny derived from a (C57BL/6j/Ei × SPRET/EiF1 × SPRET/Ei backcross) (Rowe et al., 1994). The allele pattern of Casp8 was compared using Map Manager (Manly, 1993) to those of the approximately 3200 previously mapped loci in the Jackson BSS cross, and the exact position determined by minimizing double crossovers (http://www.jax.org/resources/documents/cmda/).

For allele detection, Casp8 PCR primers (5'-GGGTACCTCGAGTTT GATCTCTGGAAACCAT-3' and 5'-CCGCTGACCTCAGTGTTTC TCTT-3') were used to amplify mouse genomic DNA purified from C57BL/6j/Ei and SPRET/Ei inbred strains (by denaturation for 45 s at 94°C, annealing for 1 min at 57°C, extension for 50 s at 72°C, 33 cycles, followed by further extension for 5 min at 72°C). PCR amplification of C57BL/6j/Ei DNA identified a 396 bp fragment and amplification of SPRET/Ei DNA identified a 310 bp fragment resolved on a 2% agarose gel. In the BSS crosses, the presence or absence of the 396 bp C57BL/6j/Ei-specific fragment was followed in the backcross mice. Homology data were retrieved from the Human Genome Database (GDB; http://www.gdb.org/) and the Mouse Genome Database (MGD; http://www.informatics.jax.org/).

Construction of a Caspase 8 Targeting Vector and Production of Knockout Mice

A targeting vector was constructed in the pPNT vector (Samuel Lunenfeld Research Institute, Toronto, Canada) by replacing a 4.8 kb fragment of Casp8 encompassing the first two coding exons with a PGK-neo-polyadenylate (poly A) cassette bordered by loxP sites (derived from the pLoxpNeo vector, Samuel Lunenfeld Research Institute, Toronto, Canada). The construct contained two DNA stretches derived from the 129 mouse genome: a 2.4 kb fragment placed 5' of the neo' cassette and a 3.6 kb fragment, encompassing exons III, IV, and V, placed 3' of the neo' cassette. The neo' gene was introduced in the opposite transcriptional orientation to Casp8 (Figure 2C).

R1 embryonic stem (ES) cells (Nagy and Rossant, 1993) were transfected with the targeting vector linearized with NotI. Recombinant ES cell clones were selected by the positive-negative technique (Mansour et al., 1988). The genotype of positively selected clones was evaluated by Southern analysis using genomic DNA probes from regions upstream of the 5' arm and downstream of the 3' arm of the targeting construct (Figure 2B).

Chimeric mice were produced by aggregation as described (Nagy and Rossant, 1993). Germline transmission in mice generated by mating chimeric males with F1 females was detected by coat color and reconfirmed by Southern analysis of tail DNA. In a fraction of the population, the "floxed" neo' cassette was deleted by mating Casp8 +/- mice with the early "deleting" transgenic mouse strain Cre(+) (Lallemand et al., 1998).

Whole-Mount mRNA In Situ Hybridization and Histological Analysis

Whole-mount mRNA in situ hybridization in 10.5 dpc embryos was performed as described (Conlon and Herrmann, 1992) using single-strand digoxigenin-UTP-labeled mouse Casp8 sense and antisense RNA probes. Radioactive in situ hybridization was as described previously (Orr-Urtreger et al., 1993). Histological investigations were performed in Paraplast sections (7 µm) and H-E staining.

In Vitro Hematopoietic Colony Assay

Semisolid cultures were established in order to determine the levels of hematopoietic progenitors in wild-type and Casp8-/- embryos. Yolk sacs and total embryos were dissected, mechanically disrupted, and filtered through 15 µm nylon mesh. For determination of the genotype of the embryos, DNA was isolated from embryonic limbs and subjected to Southern analysis as described above. Cell viability was determined by trypan blue staining. Samples of 5 × 10^6 viable cells were plated in RPMI medium containing 0.9% methylene blue (Sigma, St. Louis, MO), 30% fetal calf serum (FCS), 5 × 10^-3 M β-mercaptoethanol, 50 ng/ml SCF, 100 ng/ml FLT3 ligand (Immunex, Seattle, WA), 6 U/ml erythropoietin (EPO, Ott Bio Tech, Don Mills, ON, Canada), and murine IL-3 (culture medium of the IL-3-producing cell line X63/O kindly provided by M. Oren) applied at a dilution of 1:100 in 6 CM culture dishes. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO2 and scored 7 days later for myeloid, erythroid, megakaryocytic, and mixed colonies identified according to morphological criteria. More than ten embryos of each kind, obtained from seven pregnancies, were examined.

Establishment of Embryonic Fibroblast Cell Strains

Fibroblasts derived from 10.5 dpc embryos by trypsinization (Todaro and Green, 1963) were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM), supplemented with 10% FCS, nonessential amino acids, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cell strains from individual embryos of mice whose genome did not contain the neo' cassette were established by their transformation with an SV40 T antigen-expressing retroviral vector (Almazan and McKay, 1992). To assess the effect of DR3 signaling in the T antigen-transformed fibroblasts, a cDNA encoding a CD120a-DR3 chimera, produced by fusion of the extracellular domain of human CD120a (amino acids 1-168) with the transmembrane and intracellular domains of human DR3 (amino acids 197-417), was subcloned into the pBabe-Hygro retroviral vector (Morgenstern and Land, 1990) and transfected into the BOSC 23 retroviral packaging line (Pear et al., 1993). Supernatants of these cells were applied to the T antigen-transformed fibroblasts, followed by selection for hygromycin-resistant colonies, as described (Morgenstern and Land, 1990). All tests of DR3 function were performed using the CD120a-DR3 chimera-expressing cells.

In Vitro J NK1 Assay

Cells (4 × 10⁴) were seeded into 6 cm plates and, after overnight incubation, were treated with human TNFa (2000 U/ml; Genentech, South San Francisco, CA), the 02 anti-mouse Fas/Apo1 antibody (2 µg/ml; Pharmingen, San Diego, CA), or (when using cells expressing the CD120a-DR3 chimera) monoclonal antibodies against the human CD120a (20 µg/ml of the antibodies produced by clones 18 and 20 [Engellmann et al., 1990] at a 1 to 1 ratio) for the indicated time periods. They were then lysed, J NK1 was immunoprecipitated from the cell lysate using the anti-JNK1 C-17 polyclonal antibody (Santa Cruz Biotechnology, CA), and in vitro kinase reaction using purified glutathione 5-transferase GST-C-jun (S-89) as substrate.
was performed as described (Kerkhoff and Rapp, 1997). 

**Western Blot Analysis of TNF- and DR3-Induced IκBα Phosphorylation and Degradation**

Cells were seeded and treated with human TNFα or anti-human CD120a for the indicated periods as described above for the in vitro JNK1 assay. The cells were then lysed in SDS-PAGE lysis buffer and analyzed by SDS-PAGE followed by Western blot analysis, using phosphospecific anti-IκBα (Ser32) or phosphorylation state-independent anti-IκBα antibodies (New England Biolabs, Beverly, MA) and the ECL kit (Amersham, UK).

**Cytotoxicity Assays**

The cytotoxic activity of tested agents was determined as described (Wallach, 1984). Briefly, 12 hr prior to assay cells were seeded in 96-well plates at a density of 2.5 × 10⁴ cells/well. Human recombinant TNF and mouse recombinant TNF (Genentech, South San Francisco, CA), the J2 anti-Fas/Apo1 monoclonal antibody, and the mouse anti-p55 TNF receptor monoclonal antibodies 18 and 20, at a 1 to 1 ratio (Engelman et al., 1990), were applied to the cells in the presence of cycloheximide (50 μg/ml), Ceramide (C₂, D-erythro-ceramide; Matreya, PA), etoposide (Sigma), staurosporine (Sigma), VSV (Indiana strain, ATCC VR-158 grown on WISH cells), and UV-C treatment were applied to the cells in the absence of cycloheximide. After treatment for 12 hr (for TNF, anti-Fas, anti-CD120a, and staurosporine) or 24 hr (for all others), cell viability was assessed by measuring the uptake of the dye neutral red (Finter, 1969).

To assess the death resulting from serum deprivation, cells were washed three times with phosphate-buffered saline and then incubated for 48 hr with DMEM supplemented with various FCS concentrations.

**Acknowledgments**

We thank Ahuva Knisnisky and Tatiana Burakova for assistance in the gene targeting experiments, Niv Tutka for animal care, Adela Dibman for assistance in tissue culture work, Dr. Rebecca Haffner-Krausz for assistance in whole-mount in situ hybridization, Esther Arman for useful advice in ES cell culture and selection, Yuri Chernajovsky for advice on establishment of SV40 transformed cell lines and use of retroviral expression vectors, Lucy Rowe for assistance with chromosomal mapping data, Françoise Gay for expert technical help, and Shirley Smith for editorial assistance. This work was supported in part by grants from Inter-Lab Ltd., Ness Ziona, Israel; Ares Trading S. A., Switzerland; and from the Israeli Ministry of Arts and Sciences (E. E. V., M. S., I. L. M., D. R., V. M. B., O. C. K., D. S., T. G.), and from the Wellcome Trust Research Trust Laboratory for Gene Targeting, established by the Israel Ministry of Science (V. L. and P. L.); the Israel Science Foundation, founded by the Israel Academy of Sciences and Humanities—Charles H. Revson Foundation (T. S. and K. B. A.); and the Association Française contre les Myopathies (N. C. and J. S. B.). M. S. is a recipient of a fellowship of the Deutsche Forschungsgemeinschaft.

Received May 28, 1998; revised July 13, 1998.

**References**
