

Metaxin is required for tumor necrosis factor-induced cell death

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Received March 8, 2001; revised and accepted May 21, 2001

We used retrovirus insertion-mediated random mutagenesis and tumor necrosis factor (TNF) selection to generate TNF-resistant lines from L929 cells. The *metaxin* gene, which encodes a protein located on the outer membrane of mitochondria, was identified to be the gene disrupted in one of the resistant lines. The requirement of metaxin in TNF-induced cell death of L929 was confirmed by the restoration of TNF sensitivity after ectopic reconstitution of metaxin expression. Analysis of the cell death induced by other stimuli revealed that metaxin deficiency-mediated death resistance was selective to certain stimuli. Studies using deletion mutants of *metaxin* showed that mitochondrial association of metaxin is required for the function of metaxin. Over-expression of truncated metaxin lacking the mitochondria anchoring sequence mimicked metaxin deficiency in wild-type cells. Interfering with metaxin prevented TNF-induced necrotic cell death in L929 cells and apoptosis in MCF-7 cells. Our work has thus defined a novel component in the death pathway used by TNF and some other death stimuli.

INTRODUCTION

Tumor necrosis factor α (TNF) is a proinflammatory cytokine originally identified and purified as a factor that leads to rapid hemorrhagic necrosis of an established tumor (Beutler and Cerami, 1988). TNF can induce cell death in numerous different tumor cell lines (Fiers *et al.*, 1999). Interestingly, the morphological characteristics differ markedly among TNF-treated cell lines: KYM, MCF-7, and PC60 tumor lines die apoptotically in response to TNF treatment (Fiers *et al.*, 1999), whereas L929 and WEHI 164 clone 13 cells die with necrotic phenotypes (Fiers *et al.*, 1999). The typical symptoms of apoptosis such as nuclear condensation and DNA fragmentation are barely detectable in

L929 cells after exposure to TNF, and cell death is accompanied by the loss of plasma membrane integrity (Fiers *et al.*, 1999). Moreover, the caspase inhibitor zVAD does not block TNF-induced L929 cell death, but in fact enhances TNF-induced cell killing (Vercaemmen *et al.*, 1998a).

Both apoptosis and necrosis are initiated by TNF receptor I (TNF-RI) clustering (Aggarwal *et al.*, 1987; Ashkenazi and Dixit, 1998; Kroemer *et al.*, 1998; Fiers *et al.*, 1999). The clustered TNF-RI recruits the death domain (DD)-containing adaptor protein TRADD to its intracellular death domains. One function of TRADD is to bind to FADD, which in turn stimulates caspase-8 autoactivation in the apoptosis pathway. Active caspase-8 either acts via cytochrome *c* (cyt *c*) release or by the direct activation of effector caspases to execute apoptosis. Trimerization of the DD of TNF-RI also recruits TRADD in L929 (Fiers *et al.*, 1999). It is known that neither the known pro-apoptotic caspases including caspase-8, nor cyt *c* release are involved in TNF-induced L929 cell death (Fiers *et al.*, 1999). TNF stimulates reactive oxygen species (ROS) generation in mitochondria and the necrotic death of L929 cells is dependent on mitochondrial ROS generation (Fiers *et al.*, 1999). The intracellular molecules linked to the TNF-induced necrosis are largely unknown.

We designed a functional gene identification procedure and applied it to L929 cells to search for the molecules required for TNF-induced necrotic cell death. As a result, we identified metaxin to be required for TNF-induced death of L929. Metaxin is a protein serendipitously identified in an attempt to establish a mouse model for Gaucher's disease by introducing an A to G mutation into the glucocerebrosidase gene (*GC*) using homologous recombination (Armstrong *et al.*, 1997). Because the *metaxin* gene is located near the *GC* gene, it was disrupted by this approach which led to the death of mice during early gestation.

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metaxin gene (94 bp from the starting codon) and thus caused a truncation. We analyzed the *metaxin*-disrupted line (termed *Meta^{mut}*) together with the other four mutation lines by northern blotting using randomly ³²P-labeled double-strand DNA probe for the *neo* gene. As shown in Figure 1C, a common band was seen in all clones (indicated by an asterisk), which is most likely the transcript derived from the viral genome driven by the 5'-LTR. The fused *neo* mRNA driven by the CMV promoter from the opposite direction was of a different size, indicating that insertion of the viral genome was random. Only one fused gene was found in each of the cell lines including *Meta^{mut}* (lane 4). It is crucial to the efficiency of this protocol that multiple functional insertions in a single cell be avoided, since these events will cause difficulty in determining which disrupted gene is responsible for the functional alteration.

Northern blotting with a *metaxin* probe showed that the mRNA level of *metaxin* was significantly reduced in *Meta^{mut}* cells (Figure 1D), suggesting that a functional allele of the *metaxin* gene was indeed disrupted. In support of this, we found an mRNA with a larger mass detected in the *metaxin* line with the *metaxin* probe, indicating a probable *metaxin*-vector fusion product.

The cell viability after TNF treatment of wild-type parental L929, *Meta^{mut}*, *Meta^{mut}* cells transfected with *metaxin* expression vector (termed reconstituted), and *Meta^{mut}* cells transfected with empty vector (vector-*Meta^{mut}*), is shown in Figure 1E. Parental L929 cells were very sensitive to TNF-induced cell killing, while *Meta^{mut}* cells were resistant to TNF-induced cell death. Expression of *metaxin* in *Meta^{mut}* cells regained the TNF sensitivity to almost the same level as wild-type L929, while vector control had no effect. Thus *metaxin* is required for TNF-induced L929 cell death.

The TNF resistance of the *metaxin*-deficient line

To further evaluate TNF resistance in the *Meta^{mut}* line, we analyzed the TNF sensitivity of parental, *Meta^{mut}*, reconstituted, and vector-*Meta^{mut}* lines using several different criteria. Conversion of the yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to the dark-blue formazan measures the activity of various dehydrogenases and quantifies the reductive capacity of the cell. This assay has been widely applied to the measurement of the cytolytic response to TNF. We used the MTT assay to compare TNF-induced cell death in the four cell lines (Figure 2A). *Meta^{mut}* and vector-*Meta^{mut}* lines had a smaller reduction in their capacity to convert MTT compared to the parental and reconstituted lines, supporting the conclusion that *metaxin* mutation promotes resistance to TNF-induced cell death.

Translocation of phosphatidylserine (PS) from the inner part of the plasma membrane to the outer layer is a common early event in apoptosis. Annexin staining of PS was also observed in TNF-treated L929 and may represent a change in TNF-induced necrosis of L929 (Fiers *et al.*, 1999). Uptake of propidium iodide (PI) is an indicator of a loss in cell-membrane integrity which is widely used as a measurement of cell death. We used annexin and PI staining to analyze the death profile of the four lines in response to TNF treatment. As shown in Figure 2B and C, *metaxin* deficiency reduced TNF-induced annexin V and PI staining. Ectopic expression of *metaxin* in *Meta^{mut}* cells restored TNF sensitivity.

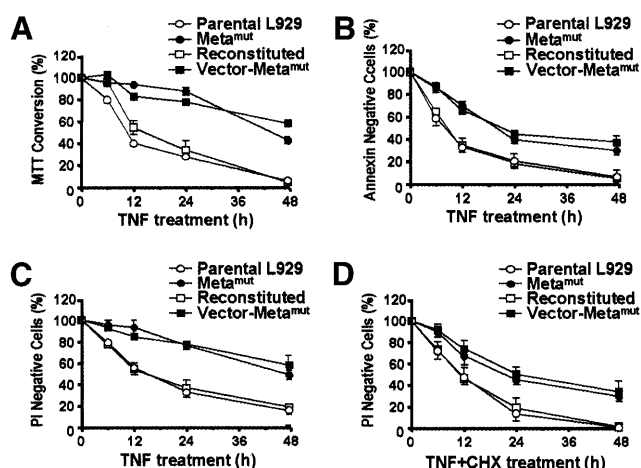


Fig. 2. TNF resistance in *Meta^{mut}* cells was demonstrated by different assays. Wild-type parental L929 cells (open circle), *Meta^{mut}* cells (filled circle), reconstituted cells (open square), and vector control cells (filled square) were treated with TNF or TNF + cycloheximide (CHX, 0.3 μg/ml) for different periods of time. Cell viability was measured by MTT assay (A), annexin staining (B), or PI staining (C and D).

To address whether new protein synthesis is involved in *metaxin* mutation-mediated TNF resistance, we treated cells with TNF in the presence of the translation inhibitor cycloheximide (CHX). CHX enhanced cell killing by TNF in both normal and *metaxin*-deficient cells (Figure 2D and C). As observed without CHX, a higher survival rate was seen in *metaxin*-deficient cells, suggesting that *metaxin* mutation-mediated death resistance is independent from new protein synthesis.

Meta^{mut} line is selectively resistant to some death stimuli

To establish whether *Meta^{mut}* is selectively resistant to different death triggers, we examined its sensitivity to several death stimuli. As shown in Figure 3, *Meta^{mut}* was resistant to TNF-, ceramide-, lonidamine- and sodium nitroprusside (SNP)-induced cell death. In contrast, *Meta^{mut}* was equally sensitive to H₂O₂-induced cell killing and was even more sensitive to staurosporine and etoposide. Both parental and *Meta^{mut}* lines were insensitive to dexamethasone, serum starvation, UV- and γ-irradiation (data not shown). Expression of Fas renders L929 cells sensitive to Fas ligation-induced cell killing (Vercaemmen *et al.*, 1998b). We transfected fas cDNA into both parental and *Meta^{mut}* cells and selected the clones that express Fas as described (Vercaemmen *et al.*, 1998b). Anti-Fas antibody treatment caused very quick cell death within 3 h, which was not influenced by *metaxin* mutation (Figure 3, bottom right panel). The different sensitivities of *Meta^{mut}* cells to different death stimuli supported the idea that there are multiple ways of cell death (Fiers *et al.*, 1999) and demonstrated that *metaxin* mutation-mediated TNF resistance is not due to a general promotion of cell survival, but an impairment of the death pathway used by TNF.

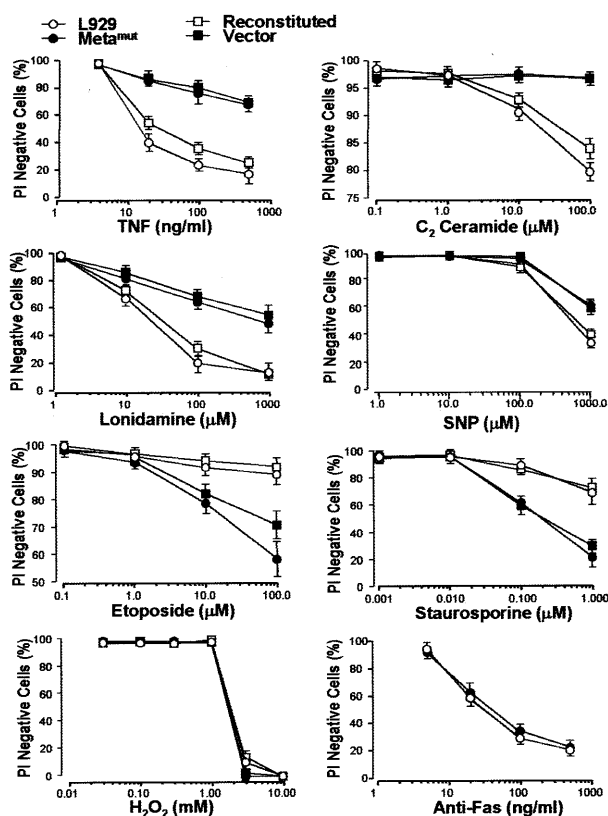


Fig. 3. The sensitivity to cell death of metaxin-deficient cells was decreased to certain death stimuli but increased to others. Wild-type parental L929 cells (open circle), *Meta^{mut}* cells (closed circle), reconstituted cells (open square), and vector control cells (filled square) were treated with different concentrations of TNF, C₂ ceramide, SNP, lonidamine, H₂O₂, etoposide or staurosporine for 24, 24, 48, 4, 8, 24 and 24 h, respectively. Parental and *Meta^{mut}* cells transfected with *fas* expression plasmid were treated with anti-Fas antibody (CH-11) for 3 h. Cell viability was measured by PI staining.

Mitochondrial association is essential for metaxin function

Metaxin is a protein of 317 amino acids (aa) with an extra-mitochondrial domain (aa 1–271), a transmembrane domain (aa 272–294) and an Asp/Glu rich C-terminal domain inside the outer membrane of mitochondria (Figure 4A) (Armstrong *et al.*, 1997). Deletion of the C-terminal domain (ΔC) did not affect the mitochondrial location of metaxin, while further deletion of transmembrane domain ($\Delta TM/C$) disrupts its mitochondrial localization (Armstrong *et al.*, 1997). We examined whether the C-terminal intra-mitochondrial domain and the transmembrane domain are required for the function of metaxin in TNF-induced cell death. As shown in Figure 4B, full-length *metaxin* reconstituted TNF sensitivity of *Meta^{mut}* cells. While *Meta ΔC* had a partial reconstitution effect, *Meta $\Delta TM/C$* did not restore the TNF sensitivity in the *Meta^{mut}* line. We also over-expressed wild-type and truncated *metaxin* mutants in parental L929 cells. *metaxin* and *Meta ΔC* did not have a significant effect on TNF-induced parental L929 cell death (Figure 4C); however, transfection with *Meta $\Delta TM/C$* led to TNF resistance in the parental cells

(Figure 4C). The dominant-negative effect by the over-expression of *Meta $\Delta TM/C$* lacking its mitochondria-targeting sequence is likely to be elicited by binding to metaxin-interacting proteins and subsequently disrupting the function of the complex. Since *Meta $\Delta TM/C$* can act as a dominant-negative mutant, we tested whether over-expression of *Meta $\Delta TM/C$* in MCF-7 and MCF-7.3.28 [a cell line generated from MCF-7 by transfection of caspase-3 cDNA (Janicke *et al.*, 1998)] cells influenced TNF-induced apoptosis. Indeed, *Meta $\Delta TM/C$* expression inhibited TNF-induced MCF-7 and MCF-7.3.28 cell death (Figure 4D and data not shown). Thus, *metaxin* is required for TNF-induced apoptosis and necrosis.

Since free radical generation in mitochondria has an essential role in TNF-induced L929 cell death, we measured mitochondrial ROS levels in TNF-treated *Meta^{mut}* and parental lines. The ROS levels of *Meta^{mut}* and parental cells were similar at rest; ROS induction profiles in response to TNF stimulation were also comparable over a period of 24 h (data not shown). In addition, similar inhibition of TNF-induced cell death by the free radical scavenger butylated hydroxyanisole (BHA) was observed between the parental and *Meta^{mut}* cells; GSH/GSSG contents are also similar between these two lines (data not shown). Hence, metaxin should function at a position downstream of free radical generation.

Cell death is controlled by both death and survival pathways. We have determined by using electro-mobility shift assay and reporter gene assay that TNF-induced NF- κ B activation was comparable between the parental and *Meta^{mut}* cells (data not shown). The PI3K-Akt pathway is another well-studied survival pathway, but its role in TNF-induced cell death is unclear. We found that PI3K inhibitor LY294002 did not enhance, but rather inhibited TNF-induced L929 cell death (data not shown). The effect of LY294002 in both parental and *metaxin*-mutated cells was the same (data not shown). In addition, TNF did not induce any detectable change in Akt phosphorylation in either parental or *metaxin*-mutated cells. Thus, we did not have evidence to suggest that the survival pathway was affected by *metaxin* mutation at this moment.

An updated database search revealed that three proteins from *Caenorhabditis elegans*, *Drosophila* and fission yeast, shared sequence similarity with metaxin in the extra-mitochondrial domain, transmembrane domain, and Asp/Glu rich C-terminal residues. The hypothetical protein F39B2.11 from *C. elegans* (DDBJ/EMBL/GenBank accession No. T21992), CG9393 gene product from *Drosophila* (AAF54402), and a metaxin homolog from fission yeast (T40446), share 34, 30 and 27% identity, respectively, with metaxin in their extra-mitochondrial domain. The function of these proteins has not been studied. Metaxin also shares a low degree of sequence similarity with TOM37, a yeast protein which is located in the mitochondrial outer membrane and is a receptor subunit for general import pore (GIP) (Gratzer *et al.*, 1995). Armstrong *et al.* (1997) found that anti-metaxin antibody, when preincubated with mitochondria, partially inhibited the uptake of pre-adrenodoxin into mitochondria. Thus, metaxin was proposed to be a component of a pre-protein import complex in the outer mitochondrial membrane. However, the high degree of identity (24%) only occurs between the N-terminal halves of TOM37 and metaxin; TOM37 has an N-terminal mitochondrial targeting sequence, while metaxin has a C-terminal targeting sequence (Armstrong *et al.*

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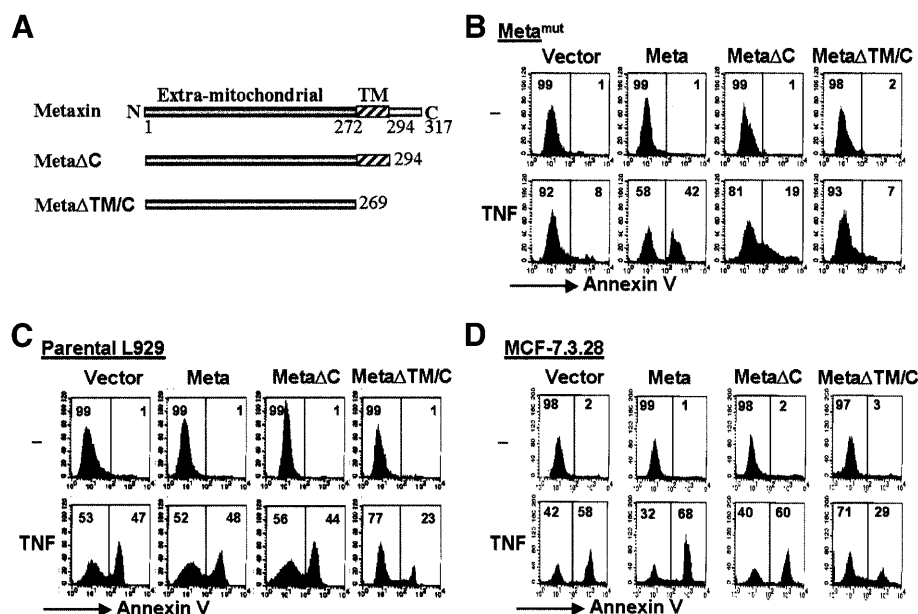


Fig. 4. Mitochondrial anchor is required for metaxin function. (A) Schematic representation indicates that metaxin is 317 aa in length, and its N-terminal extramitochondrial region (aa 1–272), transmembrane domain (TM, aa 272–294), and C-terminal region are inside the outer membrane (aa 295–317). Also shown here are the two deletion mutants of *metaxin*: *MetaΔTM/C* and *MetaΔC*. (B) *MetaΔTM/C* mutant fails to restore the sensitivity of *Meta^{mut}* cells to TNF, as assayed by annexin staining. *Meta^{mut}* cells were stably transfected with the empty vector, *metaxin*, *MetaΔTM/C*, or *MetaΔC*, respectively, and were treated with or without TNF. Cells were then analyzed for annexin staining using flow cytometry as above. (C) *MetaΔTM/C* exerts a dominant-negative effect on parental L929 cells in TNF sensitivity. (D) *MetaΔTM/C* expression leads to resistance to TNF-induced cell death in MCF-7.3.28 cells.

et al., 1997). We found the TOM37-disrupted yeast strain obtained from Dr N. Pfanner was as sensitive as the parental strain to Bax-induced cell death (data not shown). Thus, metaxin may have a different function from that of TOM37. Supporting this, anti-metaxin 2 antibody did not have an effect on pre-adrenodoxin uptake (Armstrong *et al.*, 1999). Despite the function of metaxin in pre-protein import still needing further investigation, the possibility that the TNF resistance in the *Meta^{mut}* line is due to inefficient protein transport into mitochondria cannot be completely excluded. However, we have found no difference between parental and *metaxin*-mutant cells in ATP levels, mitochondrial succinate dehydrogenase enzyme complex (complex II) activity, creatine kinase activity, mitochondrial quantity or the major protein amount and composition in the mitochondria (data not shown). The selective resistance of *metaxin*-mutant cells to certain death stimuli, but not to others (Figure 3), further supports the idea that metaxin deficiency-mediated death resistance does not result from a generalized deleterious effect of cellular physiology, but a specific defect of a cell death pathway used by TNF. The early embryonic lethality (<9 days) in mice lacking metaxin demonstrates a requirement of metaxin in development, and may be caused, at least in part, by an impaired process of cell death.

METHODS

Cell culture. L929 murine fibrosarcoma cells from ATCC were cultured in RPMI-1640 media as described (Vercammen *et al.*, 1998a). Stable cell lines derived from L929 cells were

established either by retrovirus infection or plasmid transfection by GenePorter™ transfection reagent (GTS Inc., San Diego, CA). Selection of transfected clones was performed by adding 1 mg/ml G418 or 10 μg/ml blasticidin S (Invitrogen, Carlsbad, CA) to the culture medium.

Plasmid construction. The pDisrup retroviral vector was constructed based upon MMLV retroviral vector pLNCX as backbone. The splicing donor and acceptor were designed according to human adenovirus type 2 major late mRNA intron sequence. Details of the plasmid as well as its construction are available upon request. *metaxin* and *fas* cDNA were subcloned into the expression vector pcDNA6 (Invitrogen) with blasticidin resistance. Metaxin truncation was made as described by Armstrong *et al.* (1997).

Northern blotting. The entire coding region of the G418-resistant gene was used as a probe in the detection of neo-fusion mRNA. *metaxin* cDNA was used for detecting *metaxin* mRNA levels in L929 and *Meta^{mut}* lines. The probes were labeled by Prime-It kit (Stratagene, La Jolla, CA) with [α-³²P]dCTP.

3'-RACE. The portion of endogenous gene fused with *neo* gene was amplified by a 3'-RACE technique. Total RNA was isolated and reverse transcription was performed with an RT primer (5'-CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGC(T)₁₇-3'). A nested PCR was performed with the RT product by separately using primers P1/Q1 (5'-ATGGGCTGACCGCTTCT-3'/5'-CCA-GTGAGCAGAGTGACG-3') and P2/Q2 (5'-GACGAGTTCTTCT-GACTAGCTAG-3'/5'-GAGGACTCGAGCTCAAGC-3'). P1 and P2 are located in the *neo* resistance gene while Q1 and Q2 are on

the anchor sequence of QT. The PCR fragments were subcloned into the TA-cloning vector (Stratagene) and sequenced.

Cell death/survival assays. Mouse recombinant TNF was used with a concentration of 100 ng/ml unless otherwise indicated. Plasma membrane integrity was determined according to the exclusion of PI (Vercammen *et al.*, 1998a). Annexin V-FITC kit (Roche Molecular Biochemicals) was used for measuring PS flipping according to the manufacturer's protocol, and MTT assay was carried out as described previously (Vercammen *et al.*, 1998a).

ACKNOWLEDGEMENTS

We would like to thank Dr Alan G. Porter for MCF-7.3.28 cells and Dr Nikolaus Pfanner for yeast strains and Ms Janet V. Kuhns for excellent secretarial assistance. This is publication No. 13864-IMM from the Department of Immunology, The Scripps Research Institute, La Jolla, CA. This work was supported by grants from the National Institutes of Health and the California Cancer Research Program.

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DOI: 10.1093/embo-reports/kve135