MADD, a Novel Death Domain Protein That Interacts with the Type 1 Tumor Necrosis Factor Receptor and Activates Mitogen-activated Protein Kinase*

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The death domain of the type 1 tumor necrosis factor receptor (TNFR1) mediates interactions with several proteins involved in signaling the downstream effects of TNF. We have used the yeast interaction trap to isolate a protein, MADD, that associates with the death domain of TNFR1 through its own C-terminal death domain. MADD interacts with TNFR1 residues that are critical for signal generation and coimmunoprecipitates with TNFR1, implicating MADD as a component of the TNFR1 signaling complex. Importantly, we have found that overexpression of MADD activates the mitogen-activated protein (MAP) kinase extracellular signal-regulated kinase (ERK), and expression of the MADD death domain stimulates both the ERK and c-JUN N-terminal kinase (MAP) kinase ERK. Interestingly, expression of a truncated form of MADD, containing the C-terminal death domain, also activates several transcription factors, including NF-κB and c-JUN/AP-1, leading to the up-regulation of a large number of genes involved in the inflammatory response. The signaling elements involved in initiating these pathways were not discovered until recently, when the yeast two-hybrid system was used to identify proteins that associate directly with TNFR1. Briefly, TRADD (17) is a death domain-containing protein that interacts directly with the death domain of TNFR1. TRADD is believed to act as an adaptor protein that recruits two other proteins, TRAF2 and RIP, to the receptor (18, 19). TRAF2 has been implicated in the pathway leading to the activation of NF-κB (18, 20), while RIP seems to mediate both apoptosis and NF-κB activation (19, 21). TRADD also interacts with MORT1/FADD (22, 23), which in turn associates with the ICE-like protease MACH/FLICE (24, 25), providing a mechanism by which TNFR1 activates key downstream mediators of the apoptotic response. The death domain motif plays a central role in these interactions, mediating associations between TNFR1, TRADD, MORT1/FADD, and RIP.

We have performed a yeast interaction trap screen and isolated a 176-kDa protein called MADD, for MAP kinase-Activating Death Domain protein, which also interacts with TNFR1. Here we show that MADD associates with TNFR1 through a death domain-death domain interaction and that overexpression of MADD activates the mitogen-activated protein (MAP) kinase ERK. Interestingly, expression of a truncated form of MADD, containing the C-terminal death domain, activates both the ERK and JNK MAP kinases and induces the phosphorylation of cPLA₂. These data suggest that MADD provides a physical link between TNFR1 and the induction of MAP kinase activation and arachidonic acid release.

EXPERIMENTAL PROCEDURES

Yeast Interaction Trap—Screening was based on the methods of Gyuris et al. (31). Briefly, the death domain of TNFR1 (amino acids 326–413; TNFR1-DD) was subcloned into pEG202, resulting in a DNA-binding fusion between the bacterial repressor LexA and the TNFR1 death domain. One million transformants from U937 or WI38 cDNA libraries in pG4–5 (encoding proteins fused to the B42 transcriptional activation domain; see Ref. 26) were screened for TNFR1-DD binding proteins, using an EGY48 yeast reporter strain containing chromosomal lexAop-leu2 and carrying lexAop-lacZ on the plasmid pSH18-34.

Mutagenesis and Plasmid Construction—TNFR1-DD was obtained by PCR using four oligonucleotides encoding overlapping TNFR1-DD sequences. TNFR1-DD was subcloned into pEG202 for screening and M13mp18 (27) for mutagenesis. For mutagenesis, each of five death domains was mutated individually to alanine using the Mutagen M13 mutagenesis kit (Bio-Rad), and the mutations were con-
MADD Binds TNFR1 and Activates MAP Kinase

The yeast interaction trap was used to compare the ability of 27TU and 15TU (in pEG4–5) to interact with a variety of "baits" (in pEG202), including TNFR1-DD with domain (TNFR-DD), the death domain of the Fas antigen (Fas-DD), the intracellular domain of TNFR1 (TNFR1-IC), the intracellular domain of TNFR2 (TNFR2-IC), and the Bicoid protein. 27TU and 15TU were also tested for their ability to bind TNFR1-DD containing mutations at critical signaling residues. Pho-54, Leu-511, Gly-369, Thr-378, and Ike-408 were individually mutated to alanine and subcloned into pEG202 for testing in the interaction trap. Pluses represent relative β-galactosidase expression, as judged by the color intensity in plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

**TABLE I**

| Specificity analysis | 15TU | 27TU |
|---------------------|------|------|
| TNFR1-DD            | ++   | +++  |
| Fas-DD              | -    | -    |
| TNFR1-IC            | -    | -    |
| TNFR2-IC            | -    | -    |
| Bicoid              | -    | -    |

**Mutational analysis**

| Specificity analysis | 15TU | 27TU |
|---------------------|------|------|
| TNFR1-DD            | ++   | +++  |
| F345A               | +    | +    |
| L351A               | +    | +    |
| E369A               | ++   | ++   |
| W378A               | ++   | ++   |
| I408A               | ++   | ++   |

**RESULTS**

**MADD Interacts with Critical Signaling Residues in the TNFR1 Death Domain**—The yeast interaction trap system (31) was used to identify proteins that interact with the death domain of the type 1 tumor necrosis factor receptor. The TNFR1 death domain (amino acids 326–413; TNFR1-DD) was fused to the C-terminal end of the LexA DNA binding domain as a "bait" in the interaction trap system. This construct was cotransformed into yeast along with a U937 cDNA library in which each cDNA was expressed as a fusion with the B42 transcriptional activation domain under the control of the GAL1 promoter. Approximately one million transformants were screened for their ability to express β-galactosidase and grow in the absence of leucine. 63 of the 340 LEU+ colonies isolated in the screen demonstrated a galactose-dependent phenotype. Fourteen of these, representing nine independent cDNAs, bound TNFR1-DD selectively, as assessed by comparing the interaction with TNFR1-DD to an unrelated bait, Bicoid.

Two of these clones were partial cDNAs encoding portions of a protein we have termed MADD. These partial clones, called 27TU and 15TU, encoded polypeptides of 607 and 320 amino acids, respectively. A partial MADD cDNA encoding a polypeptide of 410 amino acids was also isolated from a similar screen performed with a WI38 library (not shown). The yeast interaction trap was used to investigate the specificity of the interaction between TNFR1-DD and 27TU/15TU. As shown in Table I,
both 27TU and 15TU interacted strongly with the death domain of TNFR1, although neither interacted with the death domain of the Fas antigen. Both clones bound the intracellular domain of TNFR1 but not the type 2 TNF receptor.

In an effort to determine the amino acids within TNFR1-DD involved in this interaction, five of the six death domain residues previously shown to be critical for signaling TNF-induced cytotoxicity (3) were mutated individually to alanine. When assayed in the interaction trap, both N- and C-terminal mutations were found to affect MADD binding (Table I). Several other unrelated clones isolated in the screen did not show differential interaction (data not shown). The interaction of MADD with the death domain of TNFR1, and specifically with several critical signaling residues, supports the relevance of MADD in TNF signaling.

MADD Encodes a Protein of 1588 Amino Acids That Interacts with TNFR1 through a C-terminal Death Domain—Northern analysis revealed MADD mRNA to be expressed in a wide variety of tissues and cell lines as a 7-kilobase transcript (Fig. 1). A full-length MADD cDNA was assembled from partial clones isolated in three library screens. The open reading frame of MADD encodes a novel protein containing 1588 amino acids, with a predicted molecular mass of 176.4 kDa (Fig. 2A). Examination of the MADD sequence revealed several interesting features (Fig. 2B). The most striking is a C-terminal region that bears significant homology to the death domain of TNFR1. In addition, like other death domain-containing proteins (32), MADD contains regions rich in serine and threonine residues. Approximately 25% of the residues in these clusters are serine or threonine. The N terminus of MADD contains a consensus leucine zipper sequence (33), suggesting a mechanism by which MADD might dimerize or interact with other proteins.

Death domains mediate interactions between several proteins involved in TNF signaling. To examine the homology between the death domains of MADD and TNFR1, these sequences were aligned with each other and with Fas and TRADD (Fig. 3). The death domain of MADD is 17% identical to that of TNFR1, 12% identical to Fas, and 14% to TRADD. If conservative amino acid changes are considered, the degree of similarity between MADD and TNFR1, Fas, and TRADD increases to 30, 29, and 22%, respectively. For comparison, TNFR1 and Fas are 21% identical and 35% similar by this alignment. The six residues previously reported to be critical for TNFR1 signaling are indicated with asterisks. MADD is identical to TNFR1 at three of these positions.

To detect endogenous MADD, a fusion protein between partial clone 27TU and maltose binding protein (MBP-27TU) was used as antigen to generate polyclonal antibodies 6007 and 6008. MADD was immunoprecipitated from various cell lines with antibody 6008 and detected by immunoblotting with antibody 6007. As shown in Fig. 4, MADD is widely expressed and migrates as a protein of approximately 200 kDa, similar to overexpressed MADD (see Fig. 5C). The 100-kDa band is

![Figure 1](http://www.jbc.org/)

**FIG. 1.** Northern analysis of MADD mRNA. Northern blots containing 2 μg/lane poly(A) mRNA from various human tissues (upper panel) and cell lines (lower panel) were probed with partial MADD cDNA 27TU. Kb, kilobase pairs.

![Figure 2](http://www.jbc.org/)

**FIG. 2.** A, the predicted amino acid sequence of MADD. The C-terminal death domain and N-terminal leucine zipper are underlined, with consensus leucine zipper residues shown in bold. The clones isolated from the interaction trap screen, 27TU and 15TU, begin at residues Glu-982 (E982) and Phe-1269 (F1269), respectively, as indicated by arrows. B, diagrammatic representation of MADD. The leucine zipper and death domain homology sequences are shown by the stippled and hatched boxes, respectively. Black boxes represent regions rich in serine and threonine residues.
nonspecific and the additional bands (at 170 and 85 kDa) immunoprecipitated from U937 cells represent degradation products of MADD (as judged by immunoprecipitation with preimmune antiserum, data not shown).

As discussed above, the death domain is a protein-protein interaction motif found in several proteins implicated in TNFR1 signaling. Both 27TU and 15TU contain this motif, suggesting a mechanism by which they bind the death domain of TNFR1. To investigate whether the death domain of MADD was indeed sufficient for interaction with the death domain of TNFR1, a fragment encoding the 76-amino acid region of death domain homology, residues 1281–1356, was tested in the interaction trap for binding to TNFR1-DD. A death domain deletion mutant of clone 15TU (15\textsubscript{DD}) was used as a control. As shown in Table II, the MADD death domain is sufficient for interaction with the death domain of TNFR1. Conversely, deletion of the death domain abolishes the ability of 15TU to interact. These data establish that the MADD death domain mediates association with TNFR1. Interestingly, the MADD death domain also mediated interaction with itself, as well as with the death domain of TRADD (Table II). In contrast, no interaction was detected with the death domain of Fas. The observation that MADD can associate with TNFR1 and TRADD, but not with Fas, is consistent with the possibility that MADD is a component of the TNFR1 signaling complex.

To confirm the association between MADD and TNFR1, in vitro binding experiments were performed (Fig. 5, A and B). The death domain of TNFR1 was expressed as a fusion protein with glutathione S-transferase (GST-DD) and tested for interaction with MBP-27TU. GST or GST-DD immobilized on glutathione-agarose beads was incubated with purified MBP-27TU or MBP alone. Immunoblotting with α-MBP antibody revealed that MBP-27TU bound to GST-DD (Fig. 5A) but not GST, nor did MBP associate with GST-DD.

To test whether MADD associated with TNFR1 in mammalian cells, COS cells were transfected with a plasmid encoding full-length MADD fused at its N terminus to the FLAG epitope (FLAG-MADD). Endogenous TNFR1 was immunoprecipitated using a polyclonal antibody to TNFR1, and the immunoprecipitates were immunoblotted with anti-FLAG antibody. As shown...
in Fig. 5C, MADD coimmunoprecipitated with TNFR1 immune, but not preimmune, serum. Proteins immunoprecipitated from cells transfected with the pED-FLAG vector (V) showed no immunoreactivity to the FLAG epitope. The coimmunoprecipitation of MADD and TNFR1 was observed in both untreated and TNF-treated cells, suggesting that MADD is constitutively associated with TNFR1.

MADD Clones Activate MAP Kinase and Induce the Phosphorylation of cPLA2—An important signaling pathway in the cellular response to TNF is the activation of the MAP kinases ERK and JNK. These enzymes phosphorylate and activate several transcription factors, including AP-1 (34), ATF2 (35), and ELK-1 (36), leading to the increased transcription of a number of genes involved in inflammation (37–39). ERK has also been shown to activate cPLA2 by phosphorylation, leading to the release of arachidonic acid (40). To explore whether MADD might be involved in these signaling pathways, we tested whether overexpression of this protein stimulated MAP kinase activity. COS cells were cotransfected with HA-epitope tagged ERK2 or JNK1 and cDNAs encoding either intact MADD or partial clone 15TU. After transfection, MAP kinase was immunoprecipitated with anti-HA antibody, and its activity was assessed using myelin basic protein or GST-c-JUN (amino acids 1–79) as substrates for ERK and JNK, respectively. As shown in Fig. 6A, MADD expression stimulated both basal and TNF-induced ERK activity. Immunoblotting of lysates from vector- and MADD-transfected cells with anti-HA antibody confirmed equivalent HA-ERK expression (not shown). Expression of 15TU caused an even greater activation of ERK (Fig. 6B). In addition, 15TU induced a significant activation of JNK even in the absence of TNF, although the stimulation was less robust than that seen with ERK. Expression of intact MADD at levels comparable to 15TU did not stimulate JNK activity (data not shown), possibly because a more modest stimulation would be difficult to detect in this assay. Transfection of the vector (V) or clones unrelated to MADD (not shown) had no effect. The ability of MADD clones to activate ERK and JNK indicates that MADD plays a role in the signaling pathway(s) between TNFR1 and the MAP kinase family of enzymes.

In many cell types, treatment with TNF results in the phosphorylation of cPLA2, a critical step in the activation of this enzyme (41). One of the kinases that can phosphorylate and activate cPLA2 is ERK (40). To test whether MADD might be involved in the signaling pathway leading to cPLA2 phosphorylation, perhaps as a consequence of its ability to activate ERK, we tested whether overexpression of the partial MADD clone 15TU induced the phosphorylation of cPLA2. After cotransfection with cPLA2 and 15TU, COS cells were treated with TNF and the lysates immunoblotted for cPLA2. As phosphorylation significantly reduces the electrophoretic mobility of cPLA2, the phosphorylation state of cPLA2 can be assessed by observing the ratio between the upper and lower bands (42).

As shown in Fig. 7, in cells transfected with the vector (V), the majority of cPLA2 was dephosphorylated and became phosphorylated with increasing TNF treatment. Quantitation by phosphoimage analysis revealed the percentage of cPLA2 in the upper band to be 35, 66, 92, and 100% at 0, 0.4, 2, and 10 ng/ml TNF, respectively. In contrast, cPLA2 was heavily phosphorylated in cells expressing 15TU, with 86% of the cPLA2 found in the upper band even in the absence of TNF. The induction of cPLA2 phosphorylation by 15TU suggests that MADD may be involved in the activation of the arachidonic acid cascade by TNF.

DISCUSSION

We have identified a protein, MADD, that provides a link between TNFR1 and the activation of MAP kinases and cPLA2. Like TRADD, MADD and TNFR1 associate through a protein-protein binding motif known as the death domain. The interaction between MADD and TNFR1 was first described in the yeast interaction trap and was also demonstrated in in vitro binding experiments using purified proteins. This association was confirmed in mammalian cells by the coprecipitation of MADD with endogenous TNFR1. Two additional lines of evidence implicate MADD as a TNFR1 signaling protein. First, mutation of TNFR1-DD at residues critical for the activation of downstream signaling pathways decreased the ability of MADD to associate, suggesting that the sites of MADD interaction correspond to important signaling residues. Second, and most importantly, overexpression of MADD mimics TNF-induced MAP kinase activation. This effect appears to be mediated through the death domain, as 15TU induced the activation of both ERK and JNK as well as cPLA2 phosphorylation. MADD is the only TNFR1-associated protein reported to activate all of these signaling pathways.
MADD Binds TNFR1 and Activates MAP Kinase

FIG. 7. Effect of 15TU overexpression on the phosphorylation of cPLA2. A, COS cells were cotransfected with 2 μg of pmt-2EMC-cPLA2 and 10 μg of either pED-FLAG (Vector) or FLAG-15TU (15TU). Two days after transfection, cells were treated with TNF and the lysates immunoblotted for cPLA2. B, expression of 15TU. Lysates from transfected cells blotted in A were probed with anti-FLAG antibody.

MADD is a 176-kDa protein that interacts with TNFR1 through their respective C-terminal death domains. The death domain is a familiar protein-protein interaction motif in TNF signaling, mediating associations between TNFR1, TRADD, MORT1/FADD, RIP, and now MADD. Like other death domains, the death domain of MADD mediates self-interaction as well as interaction with other proteins. This region does not allow indiscriminant binding, however, as evidenced by the ability of MADD to interact with TRADD but not Fas. The interaction of MADD with TNFR1 and TRADD, but not Fas, suggests that MADD is specific to the TNFR1 signaling complex. Interestingly, the N terminus of MADD contains a well-conserved leucine zipper sequence, suggesting a mechanism by which MADD might interact with downstream effector proteins.

MADD coprecipitated with TNFR1 in a TNF-independent manner, suggesting that MADD is constitutively associated with the receptor. However, MADD may exist in an inactive form in the absence of TNF and be activated upon TNF binding, perhaps by aggregation or post-translational modification. Consistent with the latter possibility, MADD is heavily phosphorylated on serine residues.\(^2\) We have also found that the high molecular weight band observed upon immunoblotting of 15TU (Fig. 7B) is due to ubiquitination (as assessed by immunoblotting with anti-ubiquitin antibody, not shown). Whether these modifications regulate MADD activity is under investigation. It should be noted that it is also possible that MADD association with the receptor is TNF-dependent under physiological conditions but that the TNFR1 antibody used for immunoprecipitation mimics TNF treatment by inducing aggregation of the receptor.

As discussed above, overexpression of TRADD, through its recruitment of MORT1/FADD, TRAF2 and RIP, induces signaling pathways leading to the initiation of the apoptotic response and the activation of NF-κB. No obvious effect on NF-κB or cell death was observed upon expression of either full-length or partial MADD clones (data not shown). In addition to these downstream events, TNF has been reported to elicit several, more immediate, cellular responses. The activation of kinases, including the MAP kinases ERK, JNK and p38, and phospholipases, including phospholipase C, neutral and acidic sphingomyelinase and cPLA2, are some of the significant early events in TNF signaling. Overexpression of intact MADD stimulates ERK activity. As mentioned above, this effect is retained in 15TU, a deletion mutant primarily comprised of the death domain. The observation that 15TU activated ERK more potently than did MADD suggests that the N terminus of MADD functions as a negative regulatory domain. 15TU was also observed to induce cPLA2 phosphorylation and JNK activation. Interestingly, although TRAF2 and RIP have recently been shown to stimulate JNK activity, none of the signaling proteins in the TNF receptor complex was able to activate ERK when overexpressed (44). These data, taken together, suggest that MADD is involved in the pathway linking TNFR1 to MAP kinase activation and may play a central role in the stimulation of ERK activity by TNF.

The observation that expression of 15TU is sufficient to stimulate MAP kinase activity suggests that effector proteins for this pathway bind to the MADD death domain. Many proteins involved in the activation of ERK and JNK have been described including the small GTP-binding proteins, such as RAS, CDC42, and RAC (45–49). Examining the relationship between MADD, the small G proteins, and the other proteins in the TNFR1 signaling complex will provide a better understanding of the mechanism(s) by which MADD regulates TNF-induced MAP kinase activation.

As discussed above, phosphorylation of cPLA2 stimulates the intrinsic enzymatic activity of this enzyme (40), leading to the release of arachidonic acid. This phosphorylation can be mediated by ERK (40) and possibly other kinases (50–52). The ability of 15TU to induce cPLA2 phosphorylation may result from its activation of ERK. The induction of cPLA2 phosphorylation by the MADD death domain implicates MADD as a potential regulator of TNF-stimulated arachidonic acid release. Interestingly, in addition to its role in inflammation, cPLA2 has been implicated in TNF-induced apoptosis (53). The activation of JNK has also been proposed to be important in this process (54). The observation that 15TU stimulates both JNK activity and cPLA2 phosphorylation without triggering apoptosis, however, suggests that the activation of other components, such as MACH/FLICE, are required for initiation of this pathway. This is consistent with recent findings demonstrating that JNK activation is insufficient for triggering apoptosis (44).

The identification of MADD adds a new member to the group of death domain-containing proteins in the TNFR1 signaling complex. Clearly, death domains play a central role in regulating the diverse signaling cascades that are initiated when TNF binds its receptor. The death domain of TRADD mediates the recruitment of both RIP and MORT1/FADD to TNFR1, which (with TRAF2) signals the activation of NF-κB and the initiation of apoptosis. The effect of MADD expression, in contrast, implicates this protein in the pathway(s) leading to the activation of MAP kinases, particularly ERK, and the release of arachidonic acid. These data, taken together, support the concept that individual death domains mediate signaling through distinct intracellular pathways. The recruitment of this diverse group of proteins to the TNFR1 signaling complex provides a mechanism by which TNF exerts such pleiotropic effects.

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\(^2\) A. R. Schievella and L.-L. Lin, unpublished data.
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