Systematic Mutational Analysis of the Death Domain of the Tumor Necrosis Factor Receptor 1-associated Protein TRADD*

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Adam Park and Vijay R. Baichwal
From Tularik Inc., South San Francisco, California 94080

Tumor necrosis factor receptor 1 (TNF-R1) mediates most of the biological properties of TNF including activation of the transcription factor NF-κB and programmed cell death. An ~80-amino acid region within the intracellular domain of the receptor, termed the death domain, is required for signaling NF-κB activation and cytotoxicity. A TNF-R1-associated protein TRADD has been discovered that interacts with the death domain of the receptor. Elevated expression of TRADD in cells triggers both NF-κB activation and programmed cell death pathways. The biological activities of TRADD have been mapped to a 111-amino acid region within the carboxyl-terminal half of the protein. This region shows sequence similarity to the death domain of TNF-R1 and can self-associate and bind to the TNF-R1 death domain. We have performed an alanine scanning mutagenesis of TRADD’s death domain to explore the relationship among its various functional properties. Mutations affecting the different activities of TRADD do not map to discrete regions but rather are spread over the entire death domain, suggesting that the death domain is a multifunctional unit. A mutant that separates cell killing from NF-κB activation by the TRADD death domain has been identified indicating that these two signaling pathways diverge with TRADD. Additionally, one of the TRADD mutants that fails to activate NF-κB was found to act as dominant negative mutant capable of preventing induction of NF-κB by TNF-α. Such observations provide evidence that TRADD performs an obligate role in TNF-induced NF-κB activation.

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1 The abbreviations used are: TNF, tumor necrosis factor; TNF-R, TNF receptor; aa, amino acids; GST, glutathione S-transferase; IL, interleukin.

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that of TNF-R1 than FAS, and it does not interact with the death domain in FAS. Regions homologous to the death domains of TNF-R1 and FAS have been found in two other proteins including MORT1/FADD and RIP (19–21). Both of these proteins interact via their death homology regions with the death domain of FAS but only weakly with TNF-R1, and both induce apoptosis when overexpressed in mammalian cells. With FADD, the region that interacts with FAS is separable from the one that activates the cell death pathway. The carboxy-terminal part of MORT1/FADD is similar in primary amino acid sequence to the FAS death domain and is capable of direct interaction with it. However, expression of the amino-terminal 117 amino acids, lacking most of the death homology region of MORT-1/FADD, triggers cell death (20). First identified as a protein-protein interaction motif employed by members of the TNF-R1 family involved in signaling cell death and NF-κB activation, the death domains may be present in other proteins with diverse functions (22, 23). The rules governing interactions of the different death domains are unclear. Likewise it is not known how certain death domains signal cell death when overexpressed, whereas others do not.

The TRADD death domain performs multiple functions including self-association, binding to TNF-R1, activation of NF-κB, and induction of cell death (12). To determine whether these various activities specified by TRADD map to distinct regions within the death domain and to gain a better understanding of TRADD’s role in signal transduction by TNF-R1 we have performed an alanine scan mutagenesis of TRADD’s death domain. Alanine substitution mutants have been surveyed for their capacity to oligomerize and interact with TNF-R1 in binding assays and to activate NF-κB and induce cell death in transfection assays. The entire death domain appears to be involved in self-association of TRADD and binding to TNF-R1. It is shown that the regions of the death domain mediating NF-κB activation and induction of cell death overlap. However, one mutant was identified that kills cells but does not activate NF-κB. Furthermore, a TRADD mutant with alanines in positions 296–299 behaves as a dominant negative variant capable of preventing induction of a NF-κB reporter plasmid by TNF-α.

MATERIALS AND METHODS

Cell Culture and Transfections—The cell lines 293 and HtTA-1 (a HeLa derivative) were propagated as described (12). For NF-κB activation assays, 293 cells were transfected with 0.2 μg of E-selectin luciferase, 1 μg of PRK vector expressing TRADD mutants, 0.1 μg of cyto- megalovirus β-galactosidase, and 1.2 μg of pUC118 DNA by the calcium phosphate precipitation method as described (12). Thirty hours after transfection cells were either stimulated with 20 ng/ml recombinant human TNF-α (R&D Systems) or left unstimulated. Approximately 12 h later cells were harvested and assayed for luciferase. β-Galactosidase activity was measured with a chemiluminescent assay as follows. Cell extracts were incubated with 100 μl of a 1:100 dilution of Galacton (Tropix, Inc.) in phosphate-buffered saline with 1 mM MgCl₂ for 1 h. Samples were read on the luminometer as for the luciferase assay after addition of 60 μl of Troxip Inc.) diluted in phosphate-buffered saline with 0.2 M NaOH. β-Galactosidase activity was used to normalize luciferase activity. All transfections were performed in duplicate, and the data shown are the average of at least two independent transfections. Standard error for all measurements was <20%. For cell killing assays, HtTA-1 cells were transfected similar to 293 cells except that the selection vector in luciferase DNA was not included. Cells were stained for β-galactosidase expression 48 h after transfection, and the percentage of blue cells exhibiting a flat adherent morphology typical of live cells was determined. Blue cells that were rounded and loosely attached to the substratum were considered to be dead.

Plasmids—The NF-κB-dependent reporter plasmid E-selectin luciferase has −730 to +52 of the E-selectin promoter linked to luciferase coding sequences, and TNF-induced expression of this plasmid has been shown to require NF-κB binding sites (24). The plasmid pTRADD-(aa196–312) was constructed from the cyto-megalovirus immediate

early promoter-enhancer expression plasmid pRK5 (25). pTRADD-(aa196–312) encodes human TRADD starting at amino acid 196 with a myc epitope encoding MASMEQLISEEDELGIP at the amino terminus. The alanine substitution mutants were generated by polymerase chain reaction. To facilitate mutagenesis, three restriction sites unique to TRADD-(aa196–312) were introduced, which did not alter the encoded polypeptide sequence. To construct the mutants a primer with the desired nucleotide changes and overlapping a unique restriction site was used in combination with a primer flanking the TRADD sequences to amplify a portion of TRADD. The amplified fragment with the nucleotide changes was digested with appropriate restriction sites and substituted for the wild type sequence in TRADD-(aa196–312). Mutants are named with numbers corresponding to the amino acids that have been substituted. All mutants were confirmed by DNA sequencing. pTRADD-(aa25–312) and pTRADD-(aa112–312) were constructed from pTRADD-(aa1–312) by polymerase chain reaction-based mutagenesis. 296FL, 296L and 296S were constructed, respectively, from pTRADD-(aa1–312) by polymerase chain reaction-based mutagenesis. 296FL, 296L and 296S were constructed, respectively, from pTRADD-(aa1–312) by polymerase chain reaction-based mutagenesis. 296FL, 296L and 296S were constructed, respectively, from pTRADD-(aa1–312) by polymerase chain reaction-based mutagenesis.

In Vitro Binding and Co-immunoprecipitation Assays—Glutathione S-transferase (GST) fusion with TNF-R1 intracellular domain has been described (12) and contains amino acids 214–426 of human TNF-R1 fused to GST in a pGEX vector (Pharmacia Biotech). GST-TRADD is a similar fusion and has amino acids 9–312 of human TRADD (12). Binding assays were done with in vitro translated mutants and GST fusion proteins as described (12) after normalizing for the amount of protein synthesized in vitro. Binding of mutants was quantitated with a Phosphorlmager (Fuji Inc.) and is expressed relative to that of TRADD-(aa196–312). Co-immunoprecipitation assays were done as described (12). 293 cells (3 × 10⁵/cm² dish) were transfected with 10 μg of each DNA. Cells were harvested 24 h later, and lysates were used for immuno precipitation with the indicated antibodies. Protein immunoblots were developed with ECL reagents (Amersham Corp.).

RESULTS AND DISCUSSION

In Vitro Binding Properties of TRADD Death Domain Mutants—A derivative of TRADD extending from amino acids 195 to 312 retains all the activities associated with full-length TRADD including multimerization, binding to TNF-R1, and induction of NF-κB and of programmed cell death (12). We focused on amino acids 196–305 for mutagenesis, since the carboxy-terminal seven amino acids are dispensable for TRADD function (12). A series of 33 TRADD death domain mutants was generated in which groups of three or four contiguous amino acids were simultaneously changed to alanine (Fig. 1A). Alanine was chosen as the substituent amino acid, since changes to alanine eliminates the contribution from the side chain of the replaced amino acid with minimal alteration of the overall protein structure.

We first examined TRADD mutants for their ability to self-associate or bind TNF-R1. In vitro synthesized TRADD associates specifically with either TRADD or the intracellular domain of TNF-R1 fused to GST. The GST fusions are attached to glutathione-agarose beads, which facilitates separation of the TRADD bound to the fusion protein from unbound TRADD (12). This in vitro biochemical assay was used to compare the binding of TRADD-(aa196–312) and various substitution mutants to GST-TRADD and GST-TNF-R1. Mutations resulting in reduced binding to GST-TRADD and GST-TNF-R1 were distributed over the entire death domain, indicating that there were no subregions dedicated exclusively to TRADD multimerization or TNF-R1 binding (Fig. 1B). TRADD self-association may be essential for interaction with TNF-R1, since all mutations that reduced binding to GST-TRADD also compromised GST-TNF-R1 binding. Consistent with this interpretation, alanine substitutions at more positions affected binding to GST-TNF-R1 than to GST-TRADD. The TRADD-TNF-R1 interaction detected here may therefore reflect the binding of an oligomerized TRADD molecule to TNF-R1, and the interface
In vitro binding assays with TRADD death domain mutants. A, sequence of TRADD death domain from amino acid 196 to 305 (12). The amino acids substituted to alanine in the individual mutants are underlined. Numbers above the sequence correspond to the first amino acid changed in each mutant. B, percent binding of each mutant to GST-TRADD (top panel) and GST-TNF-R1-(aa214–426) (bottom panel) is shown as a bar graph. Binding was determined by precipitation of in vitro synthesized 35S-labeled TRADD mutant proteins with the GST fusion proteins coupled to glutathione-Sepharose. The precipitated proteins were resolved on a SDS-polyacrylamide gel, and the labeled protein brought down was quantitated with a Phosphorimager. The amount of in vitro translated TRADD-(aa196–312) bound to each wild type GST fusion protein was considered 100%. The numbers below the bottom panel correspond to amino acid positions in TRADD.

B activation independent of TNF-R1 when it is overexpressed in cells. Unlike mutant aa296–299, mutant aa296–298 substitution potently prevented induction of the NF-κB reporter gene by TNF. Unlike mutant aa296–299, mutant aa210 failed to block TNF-induced reporter gene expression.2 Taken together, these findings provide evidence that changing

The NF-κB killing activity of the mutants is expressed relative to it. A background cell killing observed with TRADD-(aa196–312) was set at 100%, and cell killing activity of the mutants is expressed relative to it. The numbers below the graph correspond to amino acid positions in TRADD. The upper panel presents the activity observed in unstimulated cells and the lower panel the activity seen in cells stimulated with TNF. The dashed line in the lower panel indicates the base-line activity observed in cells co-transfected with the E-selectin reporter and an expression vector lacking TRADD sequences and stimulated with TNF. TFNα enhanced expression of this reporter gene by approximately 20-fold. B, cell killing activity of TRADD death domain mutants presented as a bar graph. HITA cells were co-transfected with the various TRADD mutants and a β-galactosidase reporter plasmid, E-selectin luciferase. Luciferase activity was then determined from extracts of cells that were either stimulated with TNF for 12 h or left unstimulated. Expression of all mutants was confirmed by Western blot analysis with an antibody directed against the myc epitope tag present at the amino terminus of the proteins.2 As was observed with binding of the mutants to GST-TNF-R1 and GST-TRADD, mutations eliminating the capacity of the TRADD death domain to activate NF-κB were not restricted to any one part of the death domain (Fig. 2A). The NF-κB activation function is therefore dependent upon integrity of much of the ~100-amino acid region of TRADD. Five mutations (aa219–221, aa222–225, aa245–248, aa249–251, and aa269–271) that showed little or no detectable binding to GST-TNF-R1 in the in vitro binding assay were capable of inducing the NF-κB-dependent reporter to a similar extent as intact TRADD-(aa196–312) (Fig. 2A). If the failure of these mutants to interact with TNF-R1 in vitro reflects a similar lack of association with the receptor in cells, then this finding implies that the TRADD death domain can initiate NF-κB activation independent of TNF-R1 when it is overexpressed in cells. Alternatively, the large amount of TRADD protein produced in cells under conditions of transient transfection may result in a weak binding to the receptor sufficient to trigger NF-κB activation. In either case, there is no correlation between the extent of NF-κB activation and binding of TRADD mutants to TNF-R1 in vitro. It is therefore highly unlikely that induction of NF-κB by the overexpressed TRADD death domain is a mere consequence of it functioning as an intracellular ligand and clustering the receptor to stimulate other effector molecules.

Mutant aa296–299 showed detectable interaction in vitro with both GST-TNF-R1 and GST-TRADD yet did not activate NF-κB in transiently transfected cells (Figs. 1B and 2A). Moreover, expression of the E-selectin luciferase reporter was consistently lower when cells transfected with this mutant were stimulated with TNF than in cells transfected with a control vector lacking TRADD sequences. These observations indicate that the mutant might interfere with TNF-induced NF-κB activation, perhaps by preventing endogenous TRADD and/or TNF-R1 from interacting with downstream effector molecules. The aa296–299 change was reconstructed in a TRADD protein starting at amino acid 25 to confirm and extend the findings with the mutant in the death domain background. Both TRADD-(aa196–312) and TRADD-(aa225–312) with the aa296–299 substitution potently prevented induction of the NF-κB reporter gene by TNF in a dose-dependent manner without significantly affecting induction by IL-1 (Fig. 3A). As a control, another mutant that does not activate NF-κB (aa210) was tested for its capacity to prevent induction of the NF-κB reporter plasmid by TNF. Unlike mutant aa296–299, mutant aa210 failed to block TNF-induced reporter gene expression.2
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A

B

C

Fig. 3. Effect of mutant aa296–299 on NF-κB activation. A, mutant aa296–299 blocks NF-κB activation induced by TNF but not IL-1. 293 cells were co-transfected with the NF-κB-dependent reporter plasmid and varying amounts of TRADD (aa196–312) with alanines at amino acids 296–299 (296L) or TRADD (aa25–312) with alanines at amino acids 296–299 (296L) as indicated. For each data point the amount of expression plasmid was made up to 1 μg by addition of expression vector lacking TRADD sequences. Forty hours after transfection, cells were stimulated with TNF (IL-1) or IL-1β for 8 h, and the cells were then harvested and assayed for luciferase activity. The fold increase in reporter gene expression observed when 1 μg of empty expression vector was co-transfected is set at 100 for both TNF (IL-1) and IL-1β treatment. The actual increase observed was 20.5-fold for TNF and 8.5-fold for IL-1β. B, activation of NF-κB-dependent reporter gene expression by amino-terminal truncations of TRADD and TRADD with the aa296–299 substitution. The E-selectin luciferase reporter was co-transfected with full-length (aa312), truncated (aa25–312), or just the death domain (aa196–312) of TRADD with the native sequence or with alanines at aa296–299 (296FL, 296L, and 296S). Luciferase activity was determined in unstimulated (∼TNF) or cells stimulated with TNF for 8 h (∼TNF). The numbers given are relative to the luciferase activity observed for cells transfected with the empty expression vector and left unstimulated. The asterisks denote the substitution of alanines at amino acids 296–299. C, co-immunoprecipitation of TRADD having the aa296–299 substitution with TNF-R1 and native TRADD. Expression vectors lacking TRADD sequences were co-transfected into 293 cells along with expression vectors encoding TRADD (lanes 1–3) or encoding myc epitope-tagged TRADD (lanes 4–6) with the wild type sequence (WT) or with alanines at aa296–299 (296FL, 296L, and 296S). Cell lysates were immunoprecipitated with a polyclonal rabbit antiserum to TNF-R1 (lanes 1–3) or anti-flag monodonal antibody (lanes 4–6). Immunoprecipitates were resolved on a SDS-polyacrylamide gel, and the myc epitope-tagged TRADD brought down was detected in a protein immunoblot with an anti-myc monoclonal antibody.

Amino acids 296–299 to alanine results in a dominant negative TRADD mutant. To demonstrate that TRADD with the aa296–299 substitution could associate with native TRADD and interact with TNF-R1 in cells, a co-immunoprecipitation experiment was performed (Fig. 3C). 293 cells were co-transfected with expression vectors for myc epitope-tagged TRADD having the wild type sequence or alanine substitution at aa296–299 and expression vectors for either TNF-R1 or flag epitope-tagged TRADD. TNF-R1 and flag-TRADD were immunoprecipitated from the transfected cells, and the myc-tagged TRADD brought down with these proteins was determined with a Western blot (Fig. 3C). Immunoprecipitation with irrelevant antibodies confirmed that co-precipitation of myc-tagged TRADDs was specific for the anti-TNF-R1 and anti-Flag antibodies.2 Alkaline substitutions at 296–299 did not affect association with TNF-R1 or TRADD. Taken together the results of the transfection assays confirm the involvement of TRADD in the NF-κB activation pathway and furthermore demonstrate an obligate role for TRADD function in TNF-initiated NF-κB activation.

Analysis of NF-κB activation by aa296–299 mutants provided an additional insight into TRADD function. A full-length TRADD protein with the aa296–299 substitution (296FL) produced enhanced expression from a co-transfected NF-κB reporter plasmid to a small extent, approximately 6-fold compared with 37-fold with native TRADD, even though this same mutation inactivated the death domain (Fig. 3B). Similar results were obtained with the mutation aa300–302. In the context of the death domain alone substituting amino acids 300–302 with alanine inactivated TRADD; however, full-length TRADD with the same substitutions showed low levels of NF-κB-inducing activity.2 Removal of 24 amino acids from the amino terminus of 296FL abolishes the residual NF-κB inducing activity and unmasks the dominant negative effect of this mutation on TNF-induced NF-κB activation. This supplementary role of the amino terminus is most readily observed in mutants that compromise the activity of the death domain. However, amino-terminal deletions of wild type TRADD also show a 2–4-fold reduction in NF-κB activation confirming the importance of this region (Fig. 3B). All of these observations suggest that the amino-terminal half of TRADD contributes to NF-κB activation by the death domain. Additional experiments are needed to determine whether the amino-terminal sequences facilitate NF-κB activation by stabilizing the interaction of effector proteins with the death domain or by being a site for interaction of additional factors.

Induction of Cell Death by TRADD Mutants—TRADD over-expression in cells induces apoptosis (12). We used a co-transfection assay (26) with a β-galactosidase expression vector to monitor the apoptotic effect of TRADD. HTA cells were transfected with the various TRADD mutants and a plasmid encoding β-galactosidase, and 40 h after transfection β-galactosidase-expressing cells were visualized by staining. The percentage of stained cells that exhibited a flat adherent morphology characteristic of live cells was determined. As shown in Fig. 2, there was a good correlation between the capacity of mutants to induce cell killing and activate NF-κB. With one exception all mutants that activated NF-κB-dependent reporter expression induced cell death, and all mutants that failed to activate expression did not kill. Mutant aa241–244 consistently failed to activate expression of the NF-κB-dependent reporter (less than 1.5-fold activation) but was as effective at inducing cell death as the TRADD death domain. The cowpox virus gene product, CrmA, which inhibits TNF- and TRADD-induced cell death (12, 27), also prevented killing by the mutant aa241–244,2 suggesting that similar cell killing pathways were being used by the mutant as by TRADD. The demonstration that cell killing and NF-κB-inducing activity of TRADD are separable functions implies that the two TNFα-activated pathways are distinct and suggests that they may bifurcate at TRADD.

In summary, our mutational analyses indicate that the TRADD death domain cannot be subdivided into discrete regions that are individually responsible for the different functions of TRADD. It appears therefore that this domain is not composed of independent subdomains that are individually dedicated to different functions of TRADD such as oligomerization, TNF-R1 binding, NF-κB activation, and cell killing but rather that the TRADD death domain is a multifunctional unit performing all of these functions. Further insight into a structure-function relationship will require knowledge of the three-dimensional structure of the TRADD death domain. The protein interfaces involved in multimerization of TRADD and binding to TNF-R1 appear distinct but overlapping. TRADD self-association is likely to be a prerequisite for binding to TNF-R1 suggesting that a multimer of TRADD interacts with TNF-R1. TNFα is thought to induce receptor clustering on binding to TNF-R1, and it has been proposed that TRADD may preferentially bind to aggregated TNF-R1 (12). It is possible
then that interaction between TRADD and TNF-R1 involves binding of two multimerized death domains, and, in general, self-association of a death domain is different from the interaction of death domains from two different proteins.

The amino acid residues involved in signaling NF-κB activation and apoptosis are largely coincident, but these two activities are separable. The tight link between NF-κB activation and induction of cell death may reflect the interaction with TRADD of distinct but related proteins to mediate the two functions or alternately the existence of a single complex that can execute the signals required to activate both pathways. Most importantly, the mutagenesis has identified a dominant negative mutant, which indicates that the TNF-induced NF-κB activation pathway proceeds through TRADD. Finally, the TRADD mutants described here should prove useful in deciphering the role of any new TRADD-interacting proteins that emerge from future studies.

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