

Application of Promega's TNT[®] T7 Coupled Reticulocyte Lysate System in the Study of Fas-associated apoptosis

By Neal Cosby

Promega Corporation

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Chinnaiyan, A.M. et al. (1995) *FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis*. *Cell* **81**, 505.

Receptor-mediated control of programmed cell death -- apoptosis -- is one of the more interesting phenomena in cell biology. Apoptosis, characterized by cell shrinkage, membrane blebbing and chromatin condensation (1), is the subject of much current research. Binding of Fas ligand (FasL) or anti-Fas antibody to Fas (APO-1/CD95) receptor, or binding of tumor necrosis factor (TNF) to the TNF receptor (TNFR-1) rapidly induces cell death by an as yet undetermined mechanism. FasL and TNF target structurally similar receptors that belong to the TNFR superfamily. A unique cytoplasmic motif present in both TNFR-1 and Fas, the "death domain," is necessary for induction of cell death. The death domain is the site of protein:protein interaction, and researchers have exploited this motif for the cloning of novel interacting factors, especially by using the yeast two-hybrid system (2). The primary function of FasL and TNF, as recently postulated, is to mediate receptor aggregation (2). Therefore, a critical step is to identify proteins that bind directly to the cytoplasmic death domains of these receptors.

TNFR-1, a pleiotropic effector, signals activities such as fibroblast proliferation, resistance to chlamidiae, and prostaglandin E2 synthesis (3). Fas has been implicated in the activation-induced death of T cells (4-6). Research in this laboratory has demonstrated that Fas- and TNF-initiated activation of cell death occurs through a common pathway or signaling event. They found that the cowpox virus gene product, CrmA, acts as a potent blocker of both Fas- and TNF-induced cell death (7). CrmA, a viral serpin inhibitor (serine proteinase inhibitor), is only known to target interleukin-1beta (IL-1beta)-converting enzyme (ICE). ICE is a cysteine proteinase that acts in converting IL-1beta from the inactive to the active, mature form (8,9).

Although the mechanism underlying the cell signaling events that lead to apoptosis is not known, recent discoveries have identified some of the factors in various model systems, most notably the *ced-3* gene in *C. elegans* (10). The *ced-3* gene product (CED-3), the nematode homolog of ICE, is required for apoptosis (10), and ICE also can induce apoptosis (11). Chinnaiyan *et al.* suspected the mammalian CED-3-like proteinases to be distal effectors in the cell death pathway and were interested, consequently, in the more proximal effectors associated with the cell surface receptors.

In the article cited here, Chinnaiyan *et al.* identify and characterize FADD, a Fas-associated protein containing a novel death domain. These researchers used Promega's TNT[®]* T7 Coupled Reticulocyte Lysate System (Cat.# L4610) to synthesize ³⁵S-labeled FADD *in vitro* from a modified expression vector. Labeled FADD was incubated with wild type and mutant glutathione-S-transferase (GST)-Fas fusion proteins and a GST-TNF fusion protein. [Figure 1A](#) schematically represents the five GST fusion proteins used in the binding assays. The results of this experiment are presented in [Figure 1B](#). Briefly, FADD binds only the wild type Fas construct and the mutant Fas-FD5 construct, a mutant that possesses enhanced apoptotic activity compared to Fas. In a similar approach, these researchers transfected 293T cells with hemagglutinin (HA) epitope-tagged FADD (HA-FADD) and then metabolically labeled the cells with [³⁵S] methionine and cysteine. Cell lysates were incubated with the various GST fusion proteins as before, then the complexes were immunoprecipitated with anti-HA (alpha-HA) antibody and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). As is evident in [Figure 1C](#), FADD bound only wild-type Fas and the mutant Fas-FD5 constructs.

*U.S. Pat. No. 5,324,637 has been issued to Promega Corporation for coupled transcription/translation systems that use bacteriophage RNA polymerases and eukaryotic lysates.

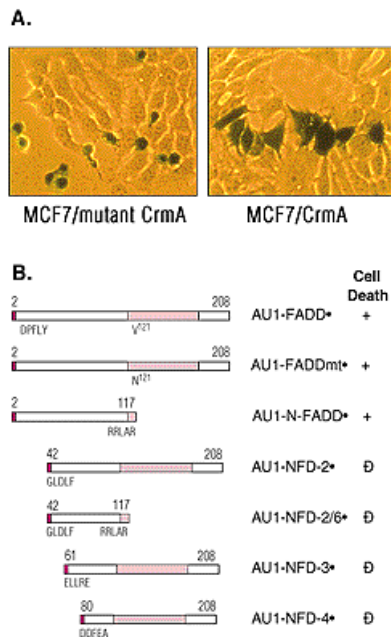


Figure 2. Functional effects of expression of FADD and FADD mutants in MCF7 cells. **Panel A:** Overexpression of FADD and beta-galactosidase in MCF7/mutant CrmA cells (left). Overexpression of FADD and beta-galactosidase in MCF7/ CrmA cells. Cells were stained with X-gal and examined by phase contrast microscopy (right). **Panel B:** Schematic representation of FADD and FADD mutants. Amino acid residues are given for selected junctions. The point mutation of FADD is represented (V¹²¹-->N¹²¹). The gray and black rectangles represent the death domain of FADD and an AU1 epitope tag, respectively. Ability of the various mutants to induce cell death in MCF7 cells is described to the right of the schematic and is based on data in Table 1 of the original article.

Chinnaiyan *et al.* present a number of interesting observations in support of the hypothesis that FADD may be a component in the Fas-mediated cell death pathway: FADD specifically binds Fas at the death domain; FADD binds, with enhanced affinity, a Fas mutant that exhibits increased cell killing; the overexpression of FADD (*in vivo*) induces apoptosis; FADD shares 25 to 35% amino acid (sequence) identity with Fas and TNF; FADD co-immunoprecipitates with Fas; and FADD possesses a death domain as illustrated by deletion mutation studies and sequence comparisons. Interestingly, though, it is a region in the amino-terminal portion of FADD, the death effector domain, that is the active domain and can induce apoptosis in the absence of the death domain and hence in the absence of binding to Fas.

The results clearly demonstrate the similar functional properties of the FADD products produced *in vitro* using Promega's TNT[®] T7 Coupled Reticulocyte Lysate System and *in vivo* using transfection assays, metabolic labeling and immunoprecipitation.

Editor's note: For more information on apoptosis, please refer to the article [Detection of Apoptotic Cells using the Apoptosis Detection System, Fluorescein](#) in Issue 57 of Promega Notes.

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