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Immunity, Vol. 12, 611–620, June, 2000, Copyright ©2000 by Cell Press

Apo2L/TRAIL-Dependent Recruitment of Endogenous FADD and Caspase-8 to Death Receptors 4 and 5

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Summary

Fas (APO-1/CD95) and tumor necrosis factor receptor 1 (TNFR1) trigger apoptosis by recruiting the apoptosis initiator caspase-8 through the adaptor FADD. Fas binds FADD directly, whereas TNFR1 binds FADD indirectly, through TRADD. TRADD alternatively recruits the NF-κB-inducing adaptor RIP. The TNF homolog Apo2L/TRAIL triggers apoptosis through two distinct death receptors, DR4 and DR5; however, receptor overexpression studies have yielded conflicting results on the ligand's signaling mechanism. Apo2L/TRAIL induced homomeric and heteromeric complexes of DR4 and DR5 and stimulated recruitment of FADD and caspase-8 and caspase-8 activation in nontransfected cells. TRADD and RIP, which bound TNFR1, did not bind DR4 and DR5. Thus, Apo2L/TRAIL and FasL initiate apoptosis through similar mechanisms, and FADD may be a universal adaptor for death receptors.

Introduction

Members of the TNF (tumor necrosis factor) gene superfamily regulate development and function of the immune system (Gruss and Dower, 1995). TNF and most of its relatives are homotrimeric type II transmembrane proteins that interact with cognate members of the TNFR (TNF receptor) superfamily. A subset of the TNFR superfamily, known as "death receptors," share homology in a cytoplasmic region dubbed the "death domain" (DD) (Ashkenazi and Dixit, 1998). Fas (also called APO-1 or CD95) is critical for apoptosis during peripheral lymphocyte deletion (Nagata, 1997), while TNFR1 plays a key role in the inflammatory response, by modulating gene expression through NF-κB and AP-1 (Wallach et al., 1999).

The mechanisms of apoptosis initiation by Fas and TNFR1 have been well defined by biochemical and genetic methods (Nagata, 1997; Ashkenazi and Dixit, 1998; Thornberry and Lazebnik, 1998). FasL triggers a series of protein–protein interactions that leads to assembly of a death-inducing signaling complex (DISC) at the cytoplasmic DD of Fas (Kischkel et al., 1995). Upon ligation, Fas recruits the adaptor molecule FADD/Mort1, through homophilic DD interactions. In turn, FADD recruits the zymogen form of the apoptosis-initiating protease caspase-8, through homophilic interaction of "death effector domains." The proximity of caspase-8 zymogens facilitates activation through self-processing, leading to cleavage of downstream effector caspases that execute the apoptotic death program.

The mechanism of apoptosis signaling by TNFR1 is similar to that of Fas, though not identical (Ashkenazi and Dixit, 1998). Upon TNF binding, TNFR1 recruits the adaptor TRADD, through homophilic DD interactions. TRADD recruits FADD and caspase-8, initiating apoptosis. Alternatively, TRADD recruits the DD-containing adaptor RIP to activate NF-κB and the adaptor TRAF-2 to activate the JNK/AP-1 pathway. Cells from transgenic mice expressing dominant-negative FADD mutants (FADD-DN) or from FADD gene knockout mice resist apoptosis induction through Fas and TNFR1 (Newton et al., 1998; Yeh et al., 1998; Zhang et al., 1998; Zornig et al., 1998), as do cells deficient in caspase-8 (Juo et al., 1998; Varfolomeev et al., 1998). Thus, FADD and caspase-8 are essential for apoptosis signaling through Fas and TNFR1.

Apo2L/TRAIL (Apo2 ligand or TNF-related apoptosis-inducing ligand) was identified recently as an apoptosis-inducing member of the TNF gene superfamily (Wiley et al., 1995; Pitti et al., 1996). The biological roles of Apo2L/TRAIL are not fully understood, but there is evidence for its involvement in apoptosis modulation within the immune system, including T cell apoptosis (Ashkenazi and Dixit, 1999), target killing by natural killer cells (Johnsen et al., 1999) and macrophages (Griffith et al., 1999), and dendritic cell death (Wang et al., 1999). Apo2L/TRAIL interacts with four cellular receptors that form a distinct subgroup within the TNFR superfamily. Death receptor 4 (DR4) (Pan et al., 1997a) and DR5 (Pan et al., 1997b; Sheridan et al., 1997) (also called TRAIL-R2, TRICK2, or KILLER) (Screaton et al., 1997; Walczak et al., 1997; Wu et al., 1997) have cytoplasmic DDs and signal apoptosis. Decoy receptor 1 (DcR1) (Sheridan et al., 1997) (also called TRID, TRAIL-R3, or LIT) (Degli-Esposti et al., 1997a; Pan et al., 1997b; Mongkolsapaya et al., 1998), is a phospholipid-anchored cell surface protein that lacks a cytoplasmic tail. DcR2 (Marsters et al., 1997) (also called TRAIL-R4 or TRUNDD) (Degli-Esposti et al., 1997b; Pan et al., 1998) has a truncated death domain that does not signal apoptosis induction. Apo2L/TRAIL binds also to a more distantly related TNFR homolog, osteoprotegerin, which is a secreted, soluble receptor for the TNF homolog TRANCE/RANKL/OPGL (Emery et al., 1998).

Despite several investigations, the mechanism of apoptosis initiation by Apo2L/TRAIL remains unclear (Ashkenazi and Dixit, 1998). There are conflicting reports on the effect of FADD-DN overexpression on apoptosis induction by overexpressed DR4 or DR5. Some studies indicate inhibition (Chaudhary et al., 1997; Schneider et al., 1997; Walczak et al., 1997), while others do not (Pan et al., 1997a, 1997b; Sheridan et al., 1997). In addition,

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there is evidence that overexpressed FADD can associate with overexpressed DR4 or DR5 (Chaudhary et al., 1997; Schneider et al., 1997), but this was not observed in other studies (McFarlane et al., 1997; Pan et al., 1997a, 1997b). Thus, it is unknown whether FADD is a physiological adaptor for DR4 and DR5. Besides FADD, overexpressed TRADD (Schneider et al., 1997) or TRADD and RIP (Chaudhary et al., 1997) were reported to interact with overexpressed DR4 or DR5, suggesting that these receptors may assemble a signaling complex that resembles that of TNFR1. FADD-deficient mouse embryonic fibroblasts (MEFs) undergo apoptosis upon overexpression of human DR4, suggesting the existence of a FADD-independent pathway from DR4 to caspases (Yeh et al., 1998). Evidence for caspase-8 involvement in apoptosis induction by Apo2L/TRAIL is also conflicting. Caspase-8 is processed in cells undergoing apoptosis in response to Apo2L/TRAIL (Griffith et al., 1998; Muhlenbeck et al., 1998). In two independent studies, dominant-negative caspase-10 mutants blocked apoptosis induction by overexpressed DR4 or DR5, while blocking by caspase-8-DN was inconsistent (McFarlane et al., 1997; Pan et al., 1997b). Further, two different caspase-10 mutations in patients with type II autoimmune lymphoproliferative syndrome have been implicated in lymphocyte resistance to Apo2L/TRAIL (Wang et al., 1999), although subsequent study revealed that one of these is in fact a common polymorphism in the Danish population (Gronbaek et al., 2000). It is unknown whether endogenous caspase-8 or -10 physically associates with endogenous DR4 and DR5.

To investigate the molecular basis for apoptosis initiation by Apo2L/TRAIL, we analyzed the DISC that this ligand assembles in *nontransfected* cells. We report the first direct evidence for Apo2L/TRAIL-induced recruitment of endogenous FADD and caspase-8 to endogenous DR4 and DR5 in multiple cell lines. We show that this ligand forms both homomeric and heteromeric complexes with DR4 and DR5 and that each receptor can recruit FADD and caspase-8 independently of the other receptor. These results indicate that Apo2L/TRAIL and FasL engage similar pathways to apoptosis and support the idea that FADD is a universal adaptor for death receptors.

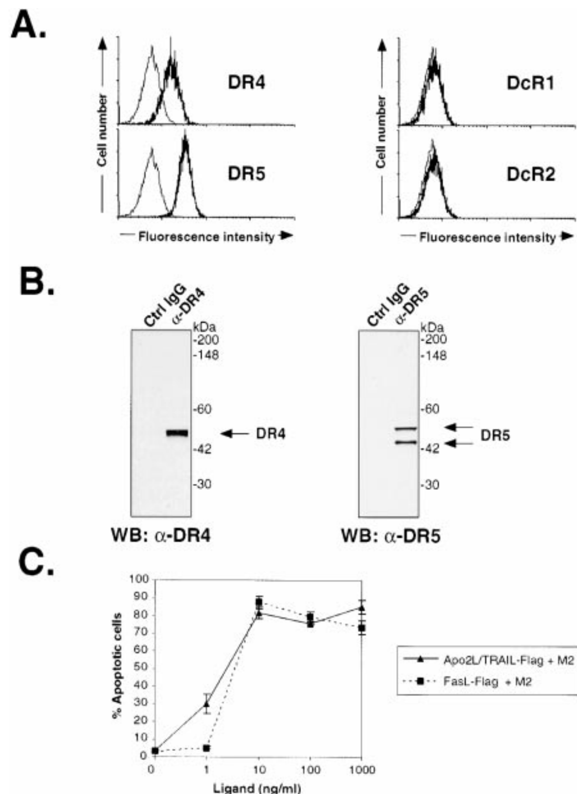

Figure 1. The B Lymphoma Cell Line BJAB Expresses DR4 and DR5 and Is Sensitive toward Apo2L/TRAIL-Induced Apoptosis

(A) BJAB cells were stained with antireceptor mAbs and analyzed by flow cytometry. Thin and thick lines show staining patterns of background and indicated antibody, respectively. Data are representative of three independent experiments.

(B) Characterization of endogenous DR4 and DR5. Immunoprecipitations (IP) with a control (Crtl IgG) and receptor-selective mAbs are shown. The IPs were resolved by SDS-PAGE and analyzed by Western blotting (WB) with the indicated antibody.

(C) Cells were incubated with 2 μg/ml anti-Flag (M2) mAb and various concentrations of Flag-tagged ligand for 24 hr and analyzed for apoptosis. Data are means ± SD of triplicates.

Results

BJAB Lymphoma Cells as a Model for Apo2L/TRAIL-Induced Apoptosis

To analyze the Apo2L/TRAIL DISC in comparison to the FasL DISC, we characterized the B lymphoma cell line BJAB, which is sensitive to both ligands (see below). Flow cytometry with receptor-selective monoclonal antibodies (mAbs) revealed cell surface expression of DR4 and DR5, but not DcR1 or DcR2 (Figure 1A). Analysis of BJAB cell supernatants by ELISA with anti-OPG mAbs indicated less than detectable (1 ng/ml) levels of OPG (data not shown). Immunoprecipitation (IP) and Western blot (WB) analysis of BJAB cell lysates with DR4 or DR5 mAb revealed a single DR4 protein band of ~45 kDa and two DR5 bands of ~49 kDa and ~43 kDa (Figure 1B). The DR5 data is consistent with results from a previous study, which also showed by peptide sequencing that the full-length 49 kDa form of DR5 is proteolytically degraded into the 43 kDa form (Walczak et al., 1997). Soluble FasL does not induce apoptosis efficiently; however, a Flag epitope–tagged version induces apoptosis upon aggregation with anti-Flag mAb (Tanaka et al., 1998; Huang et al., 1999). Although soluble homotrimeric Apo2L/TRAIL is biologically active (Ashkenazi et al., 1999; Walczak et al., 1999), to facilitate comparisons with FasL, we generated a Flag-tagged version of each ligand. Apo2L/TRAIL-Flag and FasL-Flag induced dose-dependent apoptosis in BJAB cells in the presence of the anti-Flag M2 mAb (Figure 1C).

Apo2L/TRAIL Stimulates Recruitment of FADD and Caspase-8 in BJAB Cells

To study DISC assembly, we stimulated BJAB cells with Apo2L/TRAIL-Flag plus M2 mAb for 10 min to enable ligand–receptor binding and recruitment of signaling molecules. We then lysed the cells and captured ligand-associated complexes through IP of the M2 mAb with

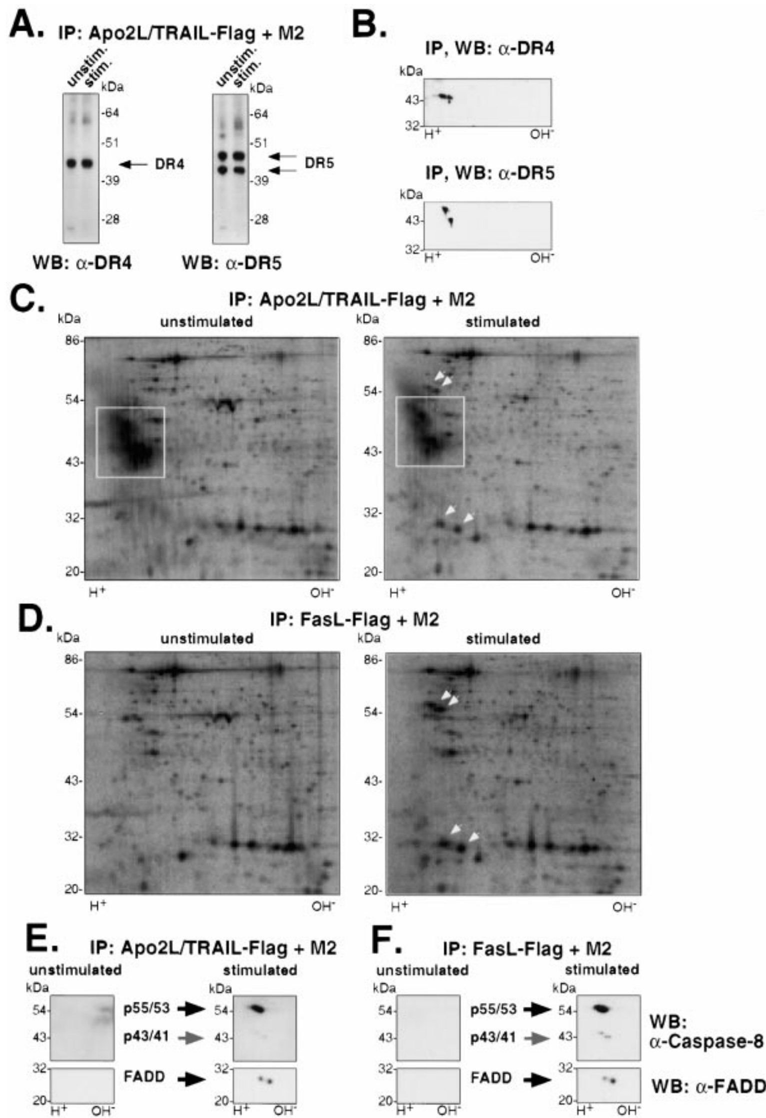

Figure 2. Apo2L/TRAIL Recruits DR4, DR5, FADD, and Caspase-8 into the DISC in BJAB Cells

Ligands (1 μg/ml) plus M2 mAb (2 μg/ml) were added either after cell lysis (unstimulated) or 10 min before cell lysis (stimulated).

(A) IP of DR4 or DR5 through Apo2L/TRAIL. Each receptor was visualized by WB.

(B) Characterization of DR4 and DR5 by direct IP and 2D gel/WB analysis.

(C–F) 2D gel analysis of the Apo2L/TRAIL and FasL DISC by metabolic labeling (C and D) and subsequent WB (E and F). White arrowhead pairs indicate ^{35}S-labeled pro-caspase-8 isoforms (p55/53, upper doublet) and FADD (lower doublet). Black arrows mark the corresponding doublets of spots in the WB analysis. Gray arrows indicate the processed forms of caspase-8 (p43/41). White boxes surround ^{35}S-labeled DR4 and DR5 spot areas.

protein A/G beads. Recruitment of cytoplasmic components to the DISC requires intact cells. Therefore, to control for stimulation-independent components, we added the ligand and M2 mAb after lysis of the cells for IP (unstimulated control). Apo2L/TRAIL associated with DR4 and DR5 (Figure 2A); as expected, ligand–receptor association was unaffected by cell lysis, because this interaction does not require cytoplasmic factors.

To examine DISC components closely, we analyzed IPs from unstimulated or stimulated cells by two-dimensional (2D) gel electrophoresis. As a prelude for DISC analysis, we determined the positions of DR4 and DR5 on 2D gels by IP and WB with receptor-selective mAbs. DR4 migrated as a series of spots of ~45 kDa (Figure 2B), probably representing differentially glycosylated forms of the protein, which contains a potential N-linked glycosylation site (Pan et al., 1997a; Sheridan et al., 1997). DR5 migrated as two less diffuse spots of ~49 kDa and ~43 kDa (Figure 2B), consistent with the absence of N-linked glycosylation sites in this receptor and its previously observed degradation (Pan et al., 1997b; Sheridan et al., 1997; Walczak et al., 1997).

Next, we labeled BJAB cells metabolically with [^{35}S]cysteine and [^{35}S]methionine and analyzed Apo2L/TRAIL-induced DISC formation by 2D gel analysis and autoradiography. We detected both DR4 and DR5 by this method (Figure 2C, white boxes, compare with Figure 2B). Two additional unique doublets of spots appeared in a stimulation-dependent manner (Figure 2C, white arrowheads). This pattern was identical to the pattern of spots in 2D gel analysis of FasL-stimulated cells (Figure 2D), suggesting association of the same DISC components. Fas was detectable only with prolonged exposures (data not shown), consistent with published results (Kischkel et al., 1995). Previous 2D gel studies on the Fas DISC identify the two doublets of spots as FADD and caspase-8 (Kischkel et al., 1995; Scaffidi et al., 1997). To confirm the identity of the spots in the Apo2L/TRAIL DISC, we probed the membranes that contained the electroblotted ^{35}S-labeled proteins with anti-FADD and anti-caspase-8 mAbs (Figures 2E and 2F). The 2D gel positions of the immunodetected molecules matched the positions of the ^{35}S-labeled proteins, identifying the upper doublet of spots as the two

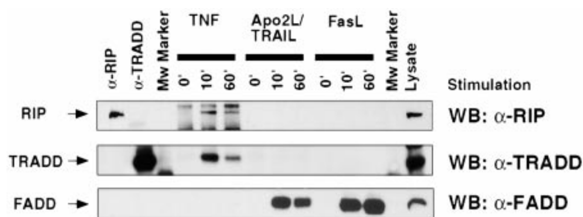

Figure 3. Apo2L/TRAIL Does Not Recruit Endogenous TRADD or RIP in BJAB Cells

RIP, TRADD, or FADD in BJAB cell lysates were detected either by IP/WB (two left lanes) or by direct WB (last lane on right). Apo2L/TRAIL-Flag or FasL-Flag (1 μg/ml) plus M2 (2 μg/ml), or TNF (0.1 μg/ml) were added as indicated for 0, 10, or 60 min, and the cells were lysed and DISCs analyzed by IP with anti-TNFR1 for TNF or through M2 for Apo2L/TRAIL or FasL. Signaling components in the IPs were visualized by WB.

isoforms of pro-caspase-8 (p55/p53) (Scaffidi et al., 1997), and the lower doublet as phosphorylated (top) and unphosphorylated (bottom) FADD (Kischkel et al., 1995; Scaffidi et al., 2000). Immunodetection revealed also the existence of two proteolytically processed forms of caspase-8 (p43/p41) (Figures 2E and 2F, gray arrows), which are formed during caspase-8 activation (Medema et al., 1997; Scaffidi et al., 1997). These caspase-8 spots were weakly detectable in ^{35}S-labeled gels from FasL-stimulated cells, but were not visible in Apo2L/TRAIL-stimulated cells, probably because of masking by the nearby DR4 and DR5 spots (Figure 2C). Thus, Apo2L/TRAIL and FasL similarly induce recruitment of endogenous FADD and caspase-8 to their death receptors in BJAB cells, leading to activation of caspase-8 by cleavage. We obtained similar results by stimulation of BJAB cells with non-cross-linked trimeric Apo2L/TRAIL and IP through the ligand or through DR4 or DR5 (data not shown).

Absence of Endogenous TRADD or RIP in the Apo2L/TRAIL DISC in BJAB Cells

Next, we examined whether Apo2L/TRAIL stimulates recruitment of endogenous TRADD or RIP through endogenous DR4 and DR5 in BJAB cells (Figure 3). Both TRADD and RIP were readily detectable in BJAB lysates by direct WB or by IP/WB analysis, as was FADD. TNF stimulated the recruitment of both TRADD and RIP to TNFR1. In contrast, Apo2L/TRAIL and FasL each induced recruitment of FADD, consistent with the data in Figure 2, but not recruitment of TRADD or RIP. TNF did not stimulate recruitment of FADD to TNFR1, perhaps because of dominant activation of the NF-κB pathway. Thus, in the absence of overexpression of receptors and adaptors and at least under the conditions used in these experiments, TNF induces receptor recruitment of TRADD and RIP, while Apo2L/TRAIL and FasL do not.

Apo2L/TRAIL Stimulates Recruitment of FADD and Caspase-8 in Various Cells

To assess whether recruitment of FADD and caspase-8 to the Apo2L/TRAIL DISC was unique to BJAB cells, we investigated a panel of cell lines (Figure 4). Flow cytometry showed frequent coexpression of DR4 and DR5, and low or undetectable expression DcR1 and DcR2 (Figure 4A). Quantitative reverse transcriptase-PCR analyses of receptor mRNA expression revealed similar results, with two exceptions: (1) DR5 appeared even more abundant then the other receptors at the mRNA level, and (2) HCT116 and NCI-H460 cells showed low yet detectable decoy receptor mRNA levels (data not shown). Apo2L/TRAIL induced marked levels of apoptosis in the tested cell lines (Figure 4B). Albeit with some variation, Apo2L/TRAIL stimulated recruitment of FADD and caspase-8 as well as caspase-8 processing in all cases (Figure 4C). Some cell lines (e.g., MDA-MB-231) showed a much more prominent DISC at 10 min than 60 min, suggesting that in these cells caspase-8 processing is more rapid. These results indicate that Apo2L/TRAIL recruits FADD and caspase-8 and induces caspase-8 activation in multiple cell types.

DR4 and DR5 Can Form Homomeric and Heteromeric Complexes

Recent crystallographic studies demonstrate that soluble Apo2L/TRAIL is a homotrimeric molecule that is stabilized by a unique zinc binding site (Hymowitz et al., 2000). The homomeric complex between soluble Apo2L/TRAIL and soluble DR5 extracellular domain contains one ligand homotrimer and three DR5 molecules (Hymowitz et al., 1999; Mongkolsapaya et al., 1999). It is unknown, however, whether Apo2L/TRAIL can form heteromeric complexes with both DR4 and DR5 at the cell surface. To investigate this possibility, we incubated BJAB cells with buffer or Apo2L/TRAIL-Flag (without M2 mAb), lysed the cells, and subjected the lysates to IP with DR4 or DR5 mAbs. We used antibodies that do not block ligand binding to capture both ligated and nonligated receptor, and mAbs that block ligand binding to capture just the nonligated receptor. We detected DR5 in a ligand-dependent manner in DR4 IPs generated with nonblocking mAb (Figure 5A, bottom left panel). We detected DR4 in a similar fashion in DR5 IPs (Figure 5A, top right panel). Neither DR4 nor DR5 was detectable in blocking mAb IPs of the alternate, nonligated receptor, indicating that heterocomplex formation was ligand dependent. More uncleaved DR5 was coimmunoprecipitated with the nonblocking than the blocking DR4 mAb (Figure 5A), suggesting that DR5 was less susceptible to degradation when complexed with ligand. The amount of heteromeric complexes appeared to be smaller than the amount of homomeric complexes of ligated receptor (compare right lane of top and bottom left panels, or right lane of top and bottom right panels, of Figure 5A). This raises the possibility that the ligand favors homomeric over heteromeric complexes; however, our experimental system might underestimate heteromeric complexes, because the nonblocking mAbs recognize both ligated and nonligated receptors. Regardless, Apo2L/TRAIL induces at least some formation of heteromeric receptor complexes.

DR4 and DR5 Can Recruit FADD and Caspase-8 Independently of Each Other

Given the ability of DR4 and DR5 to form heterocomplexes and their coexpression in many cell lines, we

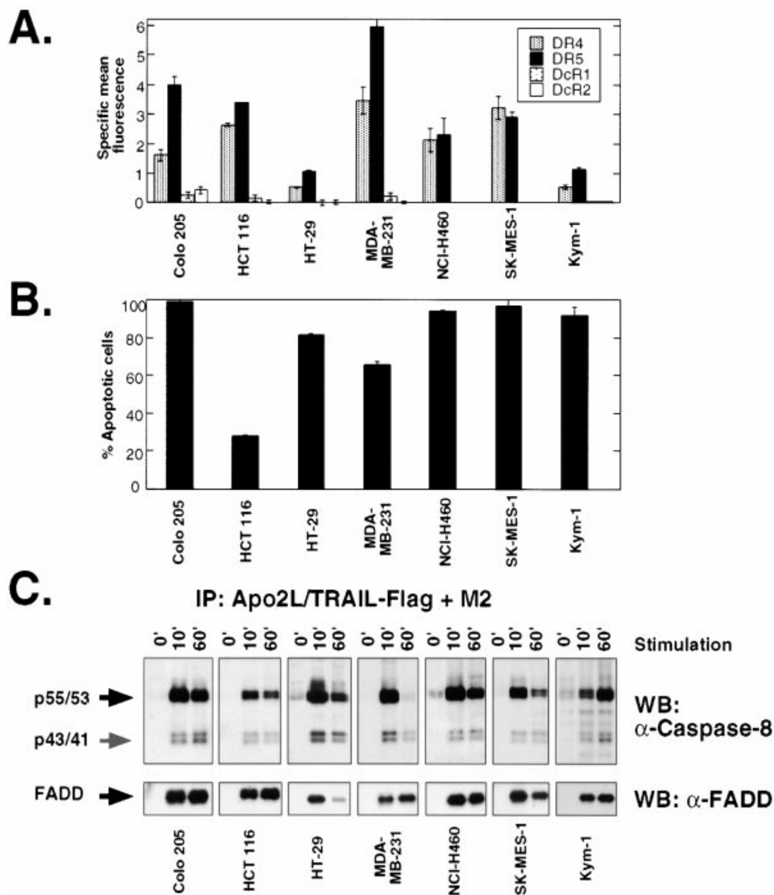

Figure 4. Apo2L/TRAIL Recruits FADD and Caspase-8 in Multiple Cell Lines

(A) Expression of DR4, DR5, DcR1, and DcR2 as analyzed by flow cytometry. Data are means ± SD of duplicates, and represent at least two independent experiments. Specific mean fluorescence was determined by subtraction of background fluorescence as measured with secondary antibody alone.

(B) Apoptosis induction. Cells were incubated with 1 μg/ml Apo2L/TRAIL-Flag + 2 μg/ml M2 for 24 hr and then analyzed for apoptosis in triplicates.

(C) One-dimensional Apo2L/TRAIL DISC analysis. Cells were incubated with Apo2L/TRAIL-Flag (1 μg/ml) plus M2 (2 μg/ml) for 0, 10, or 60 min, lysed, and subjected to IP through M2 and WB analysis.

investigated whether heterocomplex formation is required for DISC assembly. In a survey of additional cell lines, we identified some that expressed DR5 without DR4, but none that expressed DR4 without DR5. Flow cytometry showed that the glioma lines G55 and G142 expressed DR5, but not DR4, DcR1, or DcR2 (Figure 5B). In both cell lines, Apo2L/TRAIL induced apoptosis (Figure 5C), as well as recruitment of FADD and caspase-8 and caspase-8 processing (Figure 5D). Hence, DR5 is sufficient for recruiting these molecules into a functional DISC. To study DR4 selectively, we stimulated BJAB cells with a DR4 mAb that induces apoptosis upon cross-linking with anti-Fc antibodies (Figure 5E). Cross-linked DR5 mAb also induced apoptosis, and the combination of cross-linked DR4 and DR5 mAbs induced a roughly additive amount of cell death at the concentrations used (Figure 5E). Cross-linked DR4 mAb induced recruitment of FADD and caspase-8 (Figure 5F); DR5 was not detectable in DR4 IPs, confirming DR4-specific function. Thus, taken together, these findings indicate that DR4 and DR5 can recruit FADD and caspase-8 and initiate apoptosis independently of each other.

Discussion

FADD May Be a Universal Adaptor for Death Receptors

Since the cloning of the two death receptors for Apo2L/TRAIL, several studies have explored the mechanism of apoptotic signaling through DR4 and DR5. This work implicated the adaptors FADD, TRADD, and RIP in Apo2L/TRAIL signaling; however, the evidence for involvement of these molecules was based entirely on overexpression. Moreover, conflicting results were obtained by different groups (Ashkenazi and Dixit, 1998). Compelling evidence that FADD may not be required for apoptosis signaling by DR4 comes from experiments with FADD-deficient MEFs (Yeh et al., 1998). In these cells, overexpression of human DR4, but not of Fas, TNFR1, or DR3, induced apoptosis.

To identify molecules that transmit the apoptosis signal emanating from Apo2L/TRAIL, we used a nonbiased rather than a candidate-based approach. By metabolically labeling cells and co-IP of the DISC through ligand, we detected cellular components that associate with the DISC in a stimulation-dependent manner. We found that Apo2L/TRAIL induces recruitment of FADD and caspase-8 to the DISC, and processing of caspase-8, which indicates its activation. Apo2L/TRAIL stimulated recruitment of FADD and caspase-8 in all cell lines tested, and both DR4 and DR5 were independently able to recruit these factors. Since the same DISC components were recruited when either DR4 or DR5 or both receptors were stimulated, it is likely that heterocomplexes of DR4 and DR5 are functionally equivalent to homomeric receptor complexes. Thus, we conclude that FADD and caspase-8 frequently constitute an apoptosis signaling pathway for Apo2L/TRAIL. While we did not establish that FADD or caspase-8 are necessary for Apo2L/TRAIL

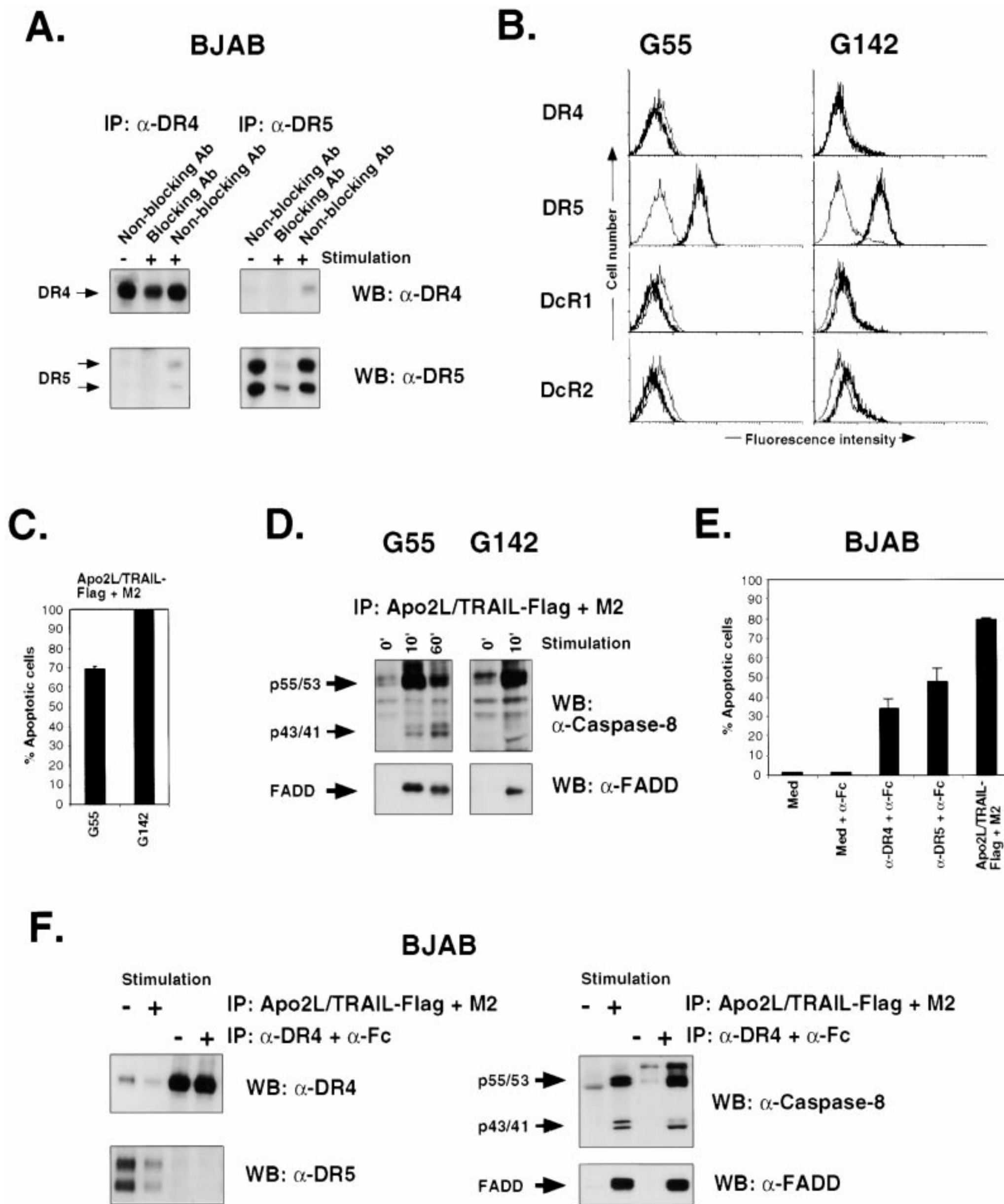

Figure 5. DR4 and DR5 Can Form Heteromeric Complexes, but Each Can Recruit FADD and Caspase-8 Independently of the Other

(A) Ligand-dependent formation of receptor heterocomplexes in BJAB cells. Cells were stimulated with 1 μg/ml Apo2L/TRAIL-Flag for 10 min (+) or left untreated (−). DR4 and DR5 were captured by IP with nonblocking (4G7 and 5C7, respectively) or blocking (1H5 and 3F11, respectively) mAb and visualized by WB analysis.

(B) G55 and G142 cells express DR5 but not DR4, DcR1, or DcR2. Cells by flow cytometry as in Figure 1. The data is representative of three independent experiments.

(C) Apo2L/TRAIL induces apoptosis in G55 and G142 cells. Cells were treated and analyzed as in Figure 4B.

(D) Apo2L/TRAIL stimulates recruitment of FADD and caspase-8 through DR5. Cells were treated and analyzed as in Figure 4C.

(E) Fc-cross-linked anti-DR4 or anti-DR5 mAbs induce apoptosis in BJAB cells. Cells were incubated with DR4 (4H6) or DR5 (3F11) mAb or both mAbs (1 μg/ml) plus anti-Fc antibody (0.5 μg/ml), or Apo2L/TRAIL-Flag (1 μg/ml) plus M2 (2 μg/ml) for 24 hr and analyzed for apoptosis.

(F) DR4 cross-linking recruits FADD and caspase-8. BJAB cells were treated with cross-linked DR4 mAb (5×10^7 cells per lane) or Apo2L/TRAIL-Flag plus M2 (1×10^7 cells per lane) as in (E) either after (−) or 10 min before (+) cell lysis. Lysates were subjected to IP with protein A/G beads and WB analysis with the indicated antibodies.

function, the equal prominence of these factors in the Apo2L/TRAIL and FasL DISC, together with their requirement for apoptosis induction by FasL (Juo et al., 1998; Varfolomeev et al., 1998; Yeh et al., 1998; Zhang et al., 1998), suggests that these molecules are similarly important for signaling by both death ligands.

Previous studies have yielded conflicting results with respect to inhibition of apoptosis signaling by DR4 and DR5 by dominant-negative FADD and association of FADD with these receptors (Chaudhary et al., 1997; McFarlane et al., 1997; Pan et al., 1997a, 1997b; Schneider et al., 1997; Sheridan et al., 1997; Walczak et al.,

1997). Common to all these studies was the use of overexpression systems, in which the receptors and/or FADD were present at nonphysiologically high levels. It is possible that under such conditions the subcellular localization and the stoichiometry of the overexpressed components were abnormal, hence skewing the ability of receptor and adaptor molecules to interact. Notwithstanding, our data do not rule out the possibility that other signaling molecules that may not be detected by our methods also may link DR4 or DR5 to apoptosis. It has been proposed that at least one mechanism that bypasses FADD can link Fas to apoptosis (Yang et al., 1997); however, the complete resistance of FADD knockout cells to Fas-mediated apoptosis suggests that this pathway is not dominant (Yeh et al., 1998; Zhang et al., 1998). There is an apparent discrepancy between the broad utilization of FADD by Apo2L/TRAIL and the observation that DR4 kills FADD-deficient MEFs (Yeh et al., 1998). Perhaps DR4 overexpression unmasks a FADD-independent death pathway in MEFs that DR4 can use more effectively than Fas. The physiological significance of such a pathway is unclear, however, since MEFs are naturally resistant to Apo2L/TRAIL (Varfolomeev et al., 1998).

Like TNFR1, the death receptor DR3 appears to bind FADD indirectly through TRADD, although the evidence for this comes from overexpression experiments (Ashkenazi and Dixit, 1998). Here we establish that FADD interacts with DR4 and DR5 under physiologic conditions. Thus, FADD may be a universal adaptor for death receptors. It is notable in this context that mice deficient in FADD activity display not only defects in lymphocyte apoptosis, but also lethal defects in embryonic development and in T cell proliferation in response to antigen (Newton et al., 1998; Yeh et al., 1998; Zhang et al., 1998; Zornig et al., 1998). In contrast, mice deficient in FasL or Fas activity show defects in apoptosis, but not proliferation (Nagata, 1997). Hence, death receptors other than Fas may modulate nonapoptotic functions of FADD. The interaction of DR4 and DR5 with FADD places them among the candidates for this latter function.

Caspase-8 Is Involved in Apoptosis Signaling by Apo2L/TRAIL

Previous work suggested that caspase-10, but not caspase-8, interacts with DR4 and DR5 (Pan et al., 1997b), while other studies implicated both caspases (McFarlane et al., 1997; Wang et al., 1999). The similar phenotype of mice deficient in FADD (Yeh et al., 1998; Zhang et al., 1998) or caspse-8 (Varfolomeev et al., 1998) suggests that both molecules act in a linear pathway and that caspase-10 cannot substitute for caspase-8. The presence of caspase-8 in the Apo2L/TRAIL DISC indicates that this caspase is involved in signaling by DR4 and DR5; however, it does not rule out the possibility that caspase-10 is involved as well. To date, physical association of endogenous caspase-10 and death receptors has not been shown. Caspase-10 has several isoforms, the functions of which are unknown (Ng et al., 1999), and mAbs that distinguish these isoforms have not been developed. Direct studies on the involvement of endogenous caspase-10 in Apo2L/TRAIL signaling await better characterization of this enzyme.

TRADD and RIP Are Not Prominent Components of the Apo2L/TRAIL DISC

The comparable prominence of FADD in the Apo2L/TRAIL and FasL DISCs suggests that DR4 and DR5 recruit FADD directly, similar to Fas. It has been proposed on the basis of overexpression experiments that DR4 and DR5 bind FADD indirectly, through TRADD (Chaudhary et al., 1997; Schneider et al., 1997), and that TRADD also recruits RIP to these receptors (Chaudhary et al., 1997). We examined these interactions in nontransfected BJAB cells. Whereas TNF stimulated the recruitment of endogenous TRADD and RIP to its death receptor, Apo2L/TRAIL and FasL did not. These results support the possibility that DR4 and DR5 bind FADD directly, rather than through TRADD, and suggest that the reported recruitment of TRADD and RIP to overexpressed DR4 and DR5 may be artifactual. Thus, apoptosis signaling by Apo2L/TRAIL resembles signaling by FasL rather than TNF.

Mechanisms that May Control Sensitivity to Apo2L/TRAIL

DR4 and DR5 bind Apo2L/TRAIL with similar affinities (Degli-Esposti et al., 1997a). Our data suggest that DR4 and DR5 are also similar with respect to apoptosis signaling. While these receptors are often coexpressed and are capable of forming heteromeric complexes with Apo2L/TRAIL, they each can signal apoptosis independently. The patterns of DR4 and DR5 mRNA expression in human tissues are different, though not without overlap. One reason for the existence of two similar receptors for Apo2L/TRAIL might be to enable tighter regulation of responsiveness in tissues. It is conceivable also that DR4 and DR5 have additional signaling functions that may be distinct.

The biological roles of Apo2L/TRAIL are not fully defined, although there is evidence implicating this ligand in several aspects of apoptosis in the immune system (Ashkenazi and Dixit, 1999; Griffith et al., 1999; Johnsen et al., 1999; Wang et al., 1999). The Apo2L/TRAIL mRNA is expressed in many human tissues, as are DR4 and DR5 transcripts; yet most normal cells are resistant to apoptosis induction by this ligand (Ashkenazi et al., 1999; Walczak et al., 1999). Many tumor cell lines including the ones studied here express DR4 and DR5, but little or no DcR1 and DcR2 protein, suggesting that in cancer cells, the decoy receptors are generally not important regulators of sensitivity to Apo2L/TRAIL. In contrast, several normal tissues express DcR1 and/or DcR2, which suggests that these receptors may contribute to the physiologic resistance of certain cells to Apo2L/TRAIL. An additional factor that may modulate sensitivity to Apo2L/TRAIL is the initiator-caspase inhibitor, c-FLIP (Wallach, 1997). C-FLIP expression levels correlate with sensitivity to Apo2L/TRAIL in certain melanoma cell lines (Griffith et al., 1998). There is no evidence, however, for a causal connection between c-FLIP expression and resistance to Apo2L/TRAIL. Further, c-FLIP expression did not correlate with resistance to Apo2L/TRAIL in colon, lung, or breast cancer cell lines (D. A. L. and A. A., unpublished data). In addition, resistance of T cells to FasL during early phases of antigen–receptor stimulation appears to be independent of c-FLIP (Scaffidi et al., 1999). Future studies should examine whether other

antiapoptotic molecules such as Bcl-2 or IAP family members contribute to modulation of sensitivity to Apo2L/TRAIL. Our identification of the endogenous signaling factors that mediate apoptosis initiation by Apo2L/TRAIL should facilitate the identification of potential modulators that act at the level of the DISC.

Experimental Procedures

Cell Lines, Antibodies, and Reagents

The following human cell lines were used: B cell lymphoma BJAB (gift from E. Humke, Genentech); colon carcinomas Colo 205, HCT 116, HT-29; breast carcinoma MDA-MB-231; lung carcinomas NCI-H460, SK-MES-1 (gift from E. Sauseville, NCI, Bethesda, MD); gliomas G55, G142 (gift from M. Westphal, Universitaetskrankenhaus Eppendorf, Hamburg, Germany); rhabdomypsarcoma KYM-1D4 (gift from A. Meager, NIBSC, WHO, Hertfordshire, UK). BJAB cells were cultured in RPMI 1640 + 10% heat-inactivated fetal bovine serum (FBS) + 1000 U/ml Penicillin–Streptomycin (Life Technologies, Rockville, MD), whereas the other cell lines were grown in 50:50 media (50% HAM's F-12; 50% DMEM) + 10% FBS + 1000 U/ml Penicillin–Streptomycin (Life Technologies). The primary antibodies anti-FADD, anti-RIP, and anti-TRADD were purchased from Transduction Laboratories (Lexington, KY), anti-Caspase-8 from Upstate Biotechnology (Lake Placid, NY), and anti-FLAG (M2) from Sigma (St. Louis, MO). Anti-DR4 (1H5, 4H6, 4G7, 3G1, 4E7), anti-DR5 (3F11, 5C7, 3H1), anti-DcR1 (6D10), anti-DcR2 (1G9) mAbs were generated by using receptor–Fc fusion proteins as antigens. As secondary reagents, we used: horseradish peroxidase (HRP) –conjugated goat α-mouse IgG2a and IgG2b from Southern Biotechnology Associates, Inc. (Birmingham, AL), HRP-conjugated goat anti-mouse IgG1 from PharMingen (San Diego, CA), HRP-conjugated streptavidin (Amersham Pharmacia Biotech, Inc., Piscataway, NJ), fluorescein isothiocyanate (FITC) –conjugated $F(ab')_2$ goat anti-mouse IgG + IgM from Jackson ImmunoResearch Labs, Inc. (West Grove, PA), and goat anti-mouse IgG γ-specific (anti-Fc) from Roche Molecular Biochemicals (Mannheim, Germany). The ligands were purified over an M2-Agarose column (Apo2L/TRAIL-Flag [amino acids 114–281] in pFLAG-MAC [Sigma] expressed in *E. coli* and FasL-Flag [amino acids 131–281] in pCMV-1 [Sigma] expressed in CHO cells). Recombinant human TNF was produced by Genentech. As substrates for immunodetection, either ECL (Amersham) or SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL) were used.

Flow Cytometry

Cells (10^6) were washed once with PBS and resuspended in 1 ml cold FACS buffer (1× PBS, 3% FBS) containing the primary antibody (2 μg/ml of anti-DR4 [4E7], 10 μg/ml of anti-DR5 [3F11], and 2 μg/ml of anti-DcR1 or anti-DcR2). Cells were stained on ice for 60 min, then washed with 3 ml cold FACS buffer, and incubated with the secondary antibody (1:100 dilution of FITC-conjugated goat anti-mouse IgG + IgM) at 4°C for 60 min in the dark. After an additional 3 ml wash with FACS buffer, 10,000 cells per sample were analyzed using an Elite-ESP flow cytometer (Beckman-Coulter, Miami, FL).

Immunoprecipitation and DISC Analysis

If not otherwise stated, cells (10^7) were either stimulated for 10 min with ligand (1 μg/ml Apo2L/TRAIL-Flag or FasL-Flag + 2 μg/ml M2 or 100 ng/ml TNF) or left untreated (unstimulated). After one wash with PBS, the cells were lysed for 30 min on ice with lysis buffer (1% Triton X-100, 150 mM NaCl, 10% glycerol, 20 mM Tris-HCl [pH 7.5], 2 mM EDTA, 0.57 mM PMSF, protease inhibitor cocktail [Complete; Roche Molecular Biochemicals]) and centrifuged at 15000 × g for 15 min at 4°C. The postnuclear supernatants were collected and rotated at 4°C for 4–16 hr in the presence of 20 μl protein A/G beads (Pierce) alone or with an indicated antibody. After seven washes with the lysis buffer, the IPs were analyzed on one or 2D gels followed by electroblotting and detection either through WB or phosphorimager analysis (see below).

Metabolic Labeling and 2D Gel Analysis

Cells (3×10^7) were washed twice with PBS and resuspended in 15 ml RPMI media without cysteine and methionine (ICN, Costa Mesa, CA, USA) + 10% FBS + 1000 U/ml Penicillin–Streptomycin (Life Technologies). After 30–60 min of incubation at 37°C, 0.5 mCi of a ^{35}S-labeled mix of cysteine and methionine (Tran^{35}S-Label, ICN) was added into the media. The cells were labeled for up to 24 hr at 37°C, washed once with PBS, and lysed, and the lysates were subjected to IP as described above. The first dimension isoelectrofocusing (IEF) was performed with the precast Immobiline DryStrips in an IPGphor apparatus essentially following the manufacturer's instructions (Amersham Pharmacia Biotech, Inc.). Briefly, the IP was mixed for 30 min at 30°C with reducing sample buffer (9.8 M urea, 4% nonidet P-40, 100 mM dithiothreitol [DTT], 2% IPG buffer and bromophenol blue) and electrofocused in 7 cm ImmobilineR DryStrips (pH 3–10 nonlinear) with the IPGphor. The following focusing protocol was used: 50 μA per strip at 20°C; (1) 30 V for 16 hr (step and hold); (2) 200 V for 3 hr (step and hold); (3) 3500 V for 1 hr 30 min (gradient); (4) 3500 V for 1 hr 30 min (step and hold). The settings resulted in a total of 9105 Vh. After electrofocusing, the strips were either stored at −70°C or shaken for 10 min at ambient temperature with equilibration buffer (50 mM Tris-HCl [pH 7.5], 6 M urea, 30% glycerol, 2% SDS, 10 mg/ml DTT) and subjected to SDS-PAGE. The second dimension was performed with a precast 10% Bis Tris NuPAGE gel system, and the gels were then electrotransfered on nitrocellulose membranes following the manufacturer's instructions (Novex, San Diego, CA). The blots were air dried for 2 hr at 37°C and exposed to BAS-III imaging plates (Fuji Photo Film, Inc., Greenwood, SC). The plates were read using the BAS-2000 IP Scanner with the Image Reader V1.2 software and then analyzed with the MacBAS V.2.4 software (Fuji Photo Film, Inc., Greenwood, SC).

Immunodetection

One-dimensional analysis of IPs was identical to the second dimension of the 2D gel analysis, omitting the IEF. The membranes were rehydrated in PBS containing 0.1% Tween-20 and blocked with 5% milk in PBS. The concentration of all primary antibodies was 1 μg/ml. The dilution for the secondary antibodies and the developing reagent are indicated in brackets. For caspase-8 and FADD analysis, the membrane were cut at about 32 kDa and the upper part was probed with anti-caspase-8 (1:20000 anti-IgG2b-HRP, ECL), whereas for the lower part anti-FADD (1:10000 anti-IgG1-HRP, SuperSignal) was used. Other blots were treated with α-TRADD (1:10000 anti-IgG1-HRP, SuperSignal), anti-RIP (1:10000 anti-IgG2a-HRP, SuperSignal), biotinylated anti-DR4 (3G1) and anti-DR5 (3H1) (1:5000, ECL and SuperSignal).

Apoptosis Assay

Cells were treated with indicated reagents for 24 hr. Percentage of apoptotic cells was determined by quantitation of DNA fragmentation, through analysis of propidium iodide stained nuclei by FACScan using the CELLQuest software (Becton Dickinson, Franklin Lakes, NJ), as described (Nicoletti et al., 1991).

Acknowledgments

We thank E. Sauseville, M. Westphal, and A. Meager for cell lines, S. Marsters and R. Pitti for reagents and suggestions, K. Totpal for activity assays, R. Pai and S. Leung for help with purifications and fermentations, D. Arnot for help with 2D gel methods, S. Schilbach for DNA sequencing, and M. Vasser, P. Jhurani, and P. Ng for DNA synthesis.

Received February 29, 2000; revised April 27, 2000.

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