The Ubiquitin-homology Protein, DAP-1, Associates with Tumor Necrosis Factor Receptor (p60) Death Domain and Induces Apoptosis*

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The tumor necrosis factor receptor, p60 (TNF-R1), transduces death signals via the association of its cytoplasmic domain with several intracellular proteins. By screening a mammalian cDNA library using the yeast two-hybrid cloning technique, we isolated a ubiquitin-homology protein, DAP-1, which specifically interacts with the cytoplasmic death domain of TNF-R1. Sequence analysis reveals that DAP-1 shares striking sequence homology with the yeast SMT3 protein that is essential for the maintenance of chromosome integrity during mitosis (Meluh, P. B., and Koshland, D. (1995) Mol. Biol. Cell 6, 793–807). DAP-1 is nearly identical to PIC1, a protein that interacts with the PML tumor suppressor implicated in acute promyelocytic leukemia (Boddy, M. N., Howe, K., Etkin, L. D., Solomon, E., and Freemont, P. S. (1996) Oncogene 13, 971–982), and the sentrin protein, which associates with the Fas death receptor (Okura, T., Gong, L., Kamitani, T., Wada, T., Okura, I., Wei, C. F., Chang, H. M., and Yeh, E. T. (1996) J. Immunol. 157, 4277–4281). The in vivo interaction between DAP-1 and TNF-R1 was further confirmed in mammalian cells. In transient transfection assays, overexpression of DAP-1 suppresses NF-κB/Rel activity in 293T cells, a human kidney embryonic carcinoma cell line. Overexpression of either DAP-1 or sentrin causes apoptosis of TNF-sensitive L929 fibroblast cell line, as well as TNF-resistant osteosarcoma cell line, U2OS. Furthermore, the dominant negative Fas-associated death domain protein (FADD) protein blocks the cell death induced by either DAP-1 or FADD. Collectively, these observations highly suggest a role for DAP-1 in mediating TNF-induced cell death signaling pathways, presumably through the recruitment of FADD death effector.

Tumor necrosis factor (TNF) is one of the key inflammatory cytokines that modulates normal and pathological immune responses due to its pleiotropic activities on cell proliferation and apoptosis. Two types of TNF receptors were identified: TNF-R1 and TNF-R2, with approximate molecular masses of 60 and 80 kDa, respectively. TNF-R1 has diverse biological activities including the induction of apoptosis and NF-κB/Rel activation (5). These biological activities are mediated by several TNF-receptor associated signaling proteins. Among these, the TNF-receptor associated death domain protein (TRADD) (5, 6), Fas-associated death domain protein (FADD) (7), and RIP (8) are responsible for transducing cell death signals through the activation of caspases. The TNF-receptor associated factors (TRAFs) transduce cell proliferation and survival signals through the activation of c-Jun NH2-terminal kinase, AP-1, and NF-κB/Rel transcription factors (6, 9–13). In particular, TRAF2 was shown to be essential for c-Jun NH2-terminal kinase, but not NF-κB/Rel, activation and regulates lymphocyte proliferation and survival (14, 15). Whereas RIP was found to be required for NF-κB/Rel activation and for suppression of TNF induced cell death in vivo (16). TNF-R1 interacting proteins also include the TRAF-interacting protein (17), PIP5K (18), FAN (19), and others (20–22). In addition, TNF-R1 induces sphingomyelinase activity and ceramide generation, leading to activation of cell death machinery (23, 24).

The ubiquitin-proteosome pathways are utilized in the regulation of many intracellular functions. These include NF-κB/Rel activation (25, 26), cell cycle control (27), DNA repair (28), and protein localization (29). Recently, many proteins with homology to ubiquitin were identified. These ubiquitin-homology (UbH) proteins constitute an expanding protein family with diverse biological activities. For instance, RAD23 is involved in DNA excision repair (30), SIII p18 elongin protein is necessary for efficient transcription elongation (31), PIC1 is present in a protein complex containing the PML tumor suppressor protein (3), and sentrin is one of the Fas death receptor associated proteins (4).

Another yeast UbH protein, Smt3p, is involved in centro-mere stability during mitosis (1, 2). It was demonstrated by Johnson et al. (2) that the activation of Smt3p can be activated by two enzymes with homology to the ubiquitin-activating enzyme, E1. Activation of Smt3p by the E1-like enzymes leads to cleavage and exposure of the diglycine residues near the carboxyl-terminal, which permit conjugation of Smt3p to the protein substrates. Although activation of the UbH protein pathway occurs in a similar fashion to that of the ubiquitin-proteosome pathways, they remain distinct in that activation of the UbH proteins, such as Smt3p, results in substrate conjugation instead of protein degradation. This distinction suggests that these parallel pathways are likely to be regulated independently.

It remains obscure as to how these UbH proteins are regulated in response to external stimuli and whether some of these UbH proteins are associated with receptor-signaling mecha-
Fig. 1. Evaluation of various TNF-R1 receptor bait constructs. A. TNF-R1 receptor bait constructs. Four different DNA fragments encoding the mouse TNF-R1 cytoplasmic domain corresponding to amino acids 207–333 (Construct A), amino acids 207–403 (Construct B), amino acids 261–425 (Construct C), and amino acids 334–425 (COOH-terminal) (Construct D) were subcloned into the pEG202 (His<sup>1</sup>) plasmid in fusion with LexA DNA binding domain. The black box represents the death domain. These constructs and the reporter plasmid PSH18–34-LacZ (Ura<sup>+</sup>) were transfected into yeast to test for potential intrinsic transcription activity. The transfected yeast were plated on Glu/CM-Ura,-His drop out
nisms, such as the TNF receptor system. Due to the diversity of TNF biological activities, it is conceivable that multiple intracellular molecules are involved in its signal transduction. Here, we report the identification of a novel UbH protein, DAP-1, which specifically interacts with the death domain of TNF-R1 and can trigger programmed cell death in a variety of cell lines.

EXPERIMENTAL PROCEDURES

**Plasmid Constructs and HeLa cDNA Library—**All TNF-R1-pEG202 constructs containing various portions of TNF-R1 cytoplasmic domain were generated by polymerase chain reaction and subcloned into the EcoRI and NotI sites of the pEG202 vector so that the TNF-R1 cytoplasmic fragment was in-frame with the LexA DNA binding domain (32, 33). Constructs A, B, C, and D encode peptides corresponding to amino acids 207–333, 207–403, 261–425, and 334–425 of the TNF-R1 cytoplasmic domain, respectively. The HeLa cDNA library, kindly provided by Dr. Roger Brent, was generated by cloning cDNA into the EcoRI and XhoI sites of PG04–5 vector in fusion with the LexA transcription activation domain (32, 33).

DAP-1, sentrin, and FADD cDNAs were subcloned into the XbaI and HindIII sites of the CDMS expression vector with the hemagglutinin tag (HA tag) at the NH2 terminus of the cDNAs. The FADD-DD-C2D and pBMN-LacZ expression constructs were kindly provided by Dr. Yongwon Choi (Rockefeller University) and Garry Nolan (Stanford University), respectively. The pCMV-LacZ construct contains β-galactosidase gene under the regulation of CMV promoter.

**Yeast Two-hybrid Screening—**To evaluate any intrinsic transcription activity of the four bait constructs, the bait plasmids TNF-R1/pEG202/his+) were cotransfected with the reporter plasmid PSH18–34/LacZ(ura+) into yeast strain EGY48, by MeSO and heat shock method (32, 33). Transfectants were plated on Glu/CM-Ura,-His dropout plates, and incubated at 30 °C overnight. For each construct, 12 colonies were streaked onto Glu/CM-Ura,-His dropout plates. Colonies were further streaked on LB/Amp^- plates for plasmid DNA isolation and sequencing analysis using the Qiaqen plasmid mini-prep kit.

Sequencing analysis was performed by mixing 2 μg of plasmid DNA with 2 μl primer and 4.5 μl of premix sequencing reagents (containing Taq polymerase, nucleotides) included in the Applied Biosystems Dye Terminator Cycle DNA sequencing kit (Perkin-Elmer, Applied Biosystems 402080) and amplified for 28 cycles by polymerase chain reaction. The final polymerase chain reaction samples were applied to the Applied Biosystems automatic sequencer. A sequence homology search was performed by accessing to the National Institutes of Health BLAST data base.

**TNF-R1 and DAP-1 Interaction Assay—**To generate the TNF-R1/GST construct, the DNA fragment encoding amino acids 334–425 of TNF-R1 region was subcloned in-frame with the GST fragment in the medium and subsequently tested for β-galactosidase activity in X-gal-containing Z buffer. Construct D exhibited no intrinsic transcription activity and was subsequently utilized in the yeast two-hybrid screening. Yeast clones interact with TNF-R1 Construct D were identified in yeast two-hybrid system. Construct D (see Fig. IA) was utilized as a bait to identify interacting proteins in HeLa cDNA library using the yeast two-hybrid screening method (see “Experimental Procedures”). Blue colonies represent a specific interaction between the bait and the cDNA-derived peptides, whereas white colonies indicate no direct physical interaction between the bait and the cDNA-derived peptides.
**FIG. 3. Sequence analysis of DAP-1 cDNA.** A, sequences of DAP-1 cDNA clones and the LexA transcription activation domain. The LexA transcription domain in the PJG4–5 vector begins at the ATG start codon (amino acid 1) and ends at the EcoRI cloning site (underlined; amino acid 106). This fusion domain also contains SV40 nuclear localization sequence (PPKKKRKVA; underlined) and the HA epitope tag (YPYDVPDYA; underlined). The DAP-1 clones contain an open reading frame from amino acids 108–248. Clones 7 and 16 have an additional 14 amino acids as compared with clone 3. B, comparison of DAP-1 with homologous cDNAs. Using the BLAST GenBank™ search, several cDNAs with homology to DAP-1 cDNA were identified. All of these proteins have high homology to the ubiquitin-homologous proteins of various species. DAP-1 shares about 50% identical (75% homology) to human SMT3A, SMT3B EST sequences, and the yeast Smt3p protein. DAP-1 is 95% similar to the PDI sequence. Except for the first 40 amino acids, it is identical to senclin.
pEBG vector (34). The TNF-R1/GST and DAP-1/CDM8(HA tag) plasmids were cotransfected into 293T cells by calcium phosphate transfection method. Transfectants were lysed into the CDMS plasmids with 0.3 µg of IgG-luciferase reporter construct (34) and pBMN-LacZ. Transfectants were plated on six-well plates and incubated at 37 °C for 48 h. Cells were then lysed with 500 µl of luciferase lysis buffer. To normalize the transfection efficiencies, an aliquot of each lysate was assayed for luciferase activity by mixing 10 µl of lysis with 50 µl of LacZ buffer and 50 µl of o-nitrophenol-D-galactoside in 500 µl total final volume. Reactions proceeded at 37 °C for 10 min and were stopped with 500 µl of 1 M Na2CO3, prior to analysis by spectrophotometry at OD 420 nm. Normalized amounts of lysates (50–70 µl) were then incubated with 20 µl of luciferase substrate and analyzed by β counter.

Expression Assay —The test constructs, including DAP-1/CDMS, sentrin/CDMS, FADD/CDMS, FADD-DN-CD2, or in combination, were cotransfected with pCMV-LacZ reporter plasmid at 2:1 ratio into either L929, 293T, or U2OS cell lines. All transfections were performed by calcium phosphate method in triplicates in six-well plates with total 12 µg of DNA/well. Where indicated, some transfecants were treated with 10 ng of murine TNF-α at 3 h post-transfection. Cells were harvested at 48 h after transfection and fixed with 0.02% glutaraldehyde in phosphate-buffered saline prior to incubation with the X-gal staining solution. Cells were analyzed by phase contrast microscopy. Several hundred to two thousand blue cells were counted depending upon the transfection efficiency. Wrinkled blue cells with condensed nuclei were scored as dead cells, whereas flat blue cells with intact morphology were scored as live transfected cells. The percentage of apoptotic cells was calculated as 100 × (dead cell count/total transfected cell count). All transfections were repeated in at least three independent experiments.

RESULTS

Cloning of a Novel TNF-R1 (p60)-Binding Protein, DAP-1 —We utilized the yeast two-hybrid system to isolate proteins that interact with the TNF-R1 cytoplasmic domain (32, 33). In this system, the TNF-R1 DNA fragments were subcloned into the EcoRI and NcoI sites of the pEG202 plasmid, in which it produced a bait protein with LexA DNA binding domain (amino acids 1–222) at the NH2-terminal in fusion with the TNF-R1 cytoplasmic peptide at the COOH-terminal. The HeLa cDNA library was cloned into the EcoRI site of the pG4.5 vector, which produced fusion proteins of the cDNA-derived peptide and the LexA transcription activation domain. Initially, several constructs encompassing the entire TNF-R1 cytoplasmic domain were tested for any potential intrinsic transcription activity in the absence of the library plasmids (Fig. 1A). One of the constructs containing amino acids 334–475 did not reveal basal transcription activity and was utilized as the bait for the screening. This bait contains the entire death domain and additional upstream 20 amino acids. Among 4 × 106 HeLa library colonies that were screened using the bait, 34 colonies were scored positive for the two selection criteria: by their ability to grow in the leucine deficient medium and to turn blue in the presence of X-gal substrate (Fig. 1B). Using dot blot hybridization, these 34 colonies were further categorized into seven distinct groups (data not shown), which we named death domain-associated proteins or DAPs. Fifteen of these 34 positive colonies (44%) correspond to DAP-1.

To estimate the molecular weight of DAP-1 protein, three clones with variable DNA length, clone 3 (1.2 kb), clone 16 (1.5 kb), and clone 7 (1.7 kb), were further analyzed. In the original yeast extracts, DAP-1 clones encoded proteins with molecular masses of approximately 25–27 kDa (Fig. 2A). Since all these clones contain 10 kDa of the LexA DNA binding domain, their actual molecular masses are about 15–17 kDa. To further confirm the protein size, DAP-1 was inserted into the CDMS mammalian expression vector with HA tag epitope attached at the 5′ end of the cDNA. These constructs were transfected into the human embryonic kidney carcinoma, 293T cell line. Transfected lysates were analyzed by Western blot analysis using monoclonal anti-HA (upper panel) or anti-GST (lower panel) antibodies. As shown, DAP-1/HA tag proteins were specifically co precipitated with the TNF-R1/GST in the glutathione affinity column.

Apoptosis Assay —The test constructs, including DAP-1/CDMS, sentrin/CDMS, FADD/CDMS, FADD-DN-CD2, or in combination, were cotransfected with pCMV-LacZ reporter plasmid at 2:1 ratio into either L929, 293T, or U2OS cell lines. All transfections were performed by calcium phosphate method in triplicates in six-well plates with total 12 µg of DNA/well. Where indicated, some transfecants were treated with 10 ng of murine TNF-α at 3 h post-transfection. Cells were harvested at 48 h after transfection and fixed with 0.02% glutaraldehyde in phosphate-buffered saline prior to incubation with the X-gal staining solution. Cells were analyzed by phase contrast microscopy. Several hundred to two thousand blue cells were counted depending upon the transfection efficiency. Wrinkled blue cells with condensed nuclei were scored as dead cells, whereas flat blue cells with intact morphology were scored as live transfected cells. The percentage of apoptotic cells was calculated as 100 × (dead cell count/total transfected cell count). All transfections were repeated in at least three independent experiments.

To test that DAP-1 is a gene product and not derived from a random sequence in the genome, Northern blot analysis was performed using total RNA derived from HeLa and a murine B cell lymphoma cell line, WEHI 231. A 1.5-kb transcript was detected by radioisotope-labeled DAP-1 clone 7 probe (Fig. 2C). This result confirms that DAP-1 is indeed derived from a mammalian gene transcript.

Sequence Analysis of DAP-1 and Comparison with Ubiquitin-homology Proteins — DNA sequencing analysis of DAP-1 cDNA reveals an open reading frame that encodes proteins of 122 amino acids (clone 3) and 142 amino acids (clones 7 and 16), respectively (Fig. 3A). By searching BLAST and EST GenBankTM data bases, we identified several sequences with homology to DAP-1. DAP-1 is identical to the PML interacting clone 1 (or PIC1), which is part of the PML-containing multinucleated cells that are frequently disrupted in acute promyelocytic leukemia (Fig. 3B) (3). Except for the additional NH2-terminal 40 amino acids, DAP-1 and PIC1 are identical to the sentrin, a Fas receptor-associated protein (4).

DAP-1 sequence is identical to the translated peptide sequence of SMT3C in the human EST data base and also has 55% identity (75% homology) to SMT3A and SMT3B EST sequences. DAP-1 reveals 52% identity (73% homology) to the Smt3p protein in Saccharomyces cerevisiae (Fig. 3B). The yeast Smt3p gene product has been implicated in the modulation of chromatin stability during mitosis by regulating a centromere-binding protein, MIF2 (1, 2). Furthermore, DAP-1, PIC1 and SMTs, share a significant degree of homology to ubiquitin and...
other ubiquitin-homology proteins (see “Discussion”), but potential ubiquitin-like activities are yet to be determined.

**Direct in Vivo Interaction of DAP-1 and TNF-R1 in Mammalian Cells**—In addition to an association in yeast, we also demonstrate that DAP-1 can directly interact with TNF-R1 in mammalian cells. DNA fragment encoding the cytoplasmic domain of TNF-R1 was subcloned into the mammalian expression vector, pEBG-GST, which contains the glutathione S-transferase (or GST) coding motif at the 5′ end of the cloning site. After cotransfection of TNF-R1/pEBG-GST and DAP-1/CDM8-HA into 293T cells, the cell lysates were applied to glutathione-agarose beads, to which TNF-R1-GST fusion protein and its amide gel electrophoresis gel and blotted with anti-HA antibody. As shown in Fig. 4, DAP-1 protein can coprecipitate with TNF-R1-GST fusion protein. The interaction was specific to TNF-R1 cytoplasmic domain because DAP-1 protein did not coprecipitated with other GST fusion proteins containing irrelevant peptide motifs (data not shown). These data confirm an interaction of DAP-1 with TNF-R1 cytoplasmic domain in mammalian cells under physiological condition.

**Overexpression of DAP-1 Suppresses NF-kB/Rel Activity**—To investigate the influence of DAP-1 protein on TNF-R1-mediated NF-κB/Rel activation, we transfected DAP-1/CDM8, NFκB-luciferase reporter, and the pBMN-LacZ reporter construct (34) and pBMN-LacZ reporter plasmid into 293T cells in triplicates at two different DNA concentrations. Transfected cell lysates were analyzed by luciferase assay. Transient transfection efficiencies among each sample were normalized by measuring β-galactosidase activity.

![Graph](image)

**Fig. 5. DAP-1 suppresses NF-κB/Rel activity in 293T cells.** Various DAP-1/CDM8 plasmids were cotransfected with IgB-luciferase construct (34) and pBMN-LacZ reporter plasmid into 293T cells in triplicates at two different DNA concentrations. Transfected cell lysates were analyzed by luciferase assay. Transient transfection efficiencies among each sample were normalized by measuring β-galactosidase activity.

![Graph](image)

**Fig. 6. DAP-1 induces apoptosis in L929 cells.** DAP-1/CDM8 plasmids and pCMV-LacZ reporter plasmid were transfected at 2:1 ratio in triplicate into the TNF-sensitive cell line, L929. Half of the transfected cells were treated with TNF-α (10 ng/ml) for 14 h prior to fixation and incubation in the X-gal solution. Blue cells, indicative of plasmid DNA incorporation, were visualized by phase contrast microscopy. Dead cells were determined by counting the blue cells with dark and condensed nuclei (A) and the total number of transfected viable cells assessed by counting blue cells on each transfection plate (B).

We have attempted to address if DAP-1 has any effect on TNF-induced NF-κB activity by transfecting DAP-1 into 293T cells that were subsequently treated with TNF-α. As TNF-α induced nearly a 100-fold increase in NF-κB activity, this activity was not diminished by transient transfection of DAP-1 (data not shown). However, this is a technically challenging experiment given the limitation of transfection efficiency (less than 30%) but high inducibility of NF-κB by TNF in nearly all 293T cells. Thus, the portion of cells that contain κB-luciferase construct, but not the DAP-1 cDNA, would contribute to the surging luciferase activity which is not affected by DAP-1.

**Overexpression of DAP-1 Induces Apoptosis**—To investigate whether DAP-1 can modulate apoptosis function of TNF-R1, the DAP-1/CDM8 and pCMV-LacZ plasmids (ratio 2:1) were cotransfected into the mouse fibroblast cell line, L929, which is extremely sensitive to TNF-α-induced apoptosis. Half of the transfectants were treated with TNF-α 36 h postelectroporation. Dead cells were determined by counting the blue cells with dark and condensed nuclei under microscope after incubating in the X-gal buffer. DAP-1 alone induced 25–30% of cell death in L929 cells (Fig. 6A). Treatment of DAP-1-transfected cells with TNF-α only slightly enhances apoptosis to 35–40% (Fig. 6A). Since there is no significant synergy between TNF-α and DAP-1, it suggests that DAP-1 and TNF-R1 may converge at common cell death pathways. As the blue nuclei condensed cells represent only a fraction of cells that are dying at the time of analysis, we also counted the total transfected viable cells to better estimate the effect of DAP-1 on cell death during the transfection process. By counting the total blue cells, DAP-1-transfected plates had 50–70% reduced cell number as compared with the control transfectants (Fig. 6B). TNF-α treatment alone also resulted in similar reduction in total cell number.

TNF induces the activation of caspases which eventually leads to programmed cell death. We therefore tested if DAP-1-
mediated cell death is also due to the activation of caspase activity. DAP-1-transfected L929 cells were treated with either the pan-caspase inhibitor, Z-VAD peptide, or solvent (Me$_2$SO) at 12 h posttransfection (35). While DAP-1 alone induced 47% cell death, treatment of these transfectants with the Z-VAD peptide reduced the cell death to 22% (Fig. 7A). This experiment thus indicated that DAP-1-induced cell death involves the activation of caspases.

Previously, it was shown that sentrin, a Fas receptor-associated protein, protects BJAB cells from Fas-mediated apoptosis (4). Since DAP-1 and sentrin share 99% identity except for the additional 40 amino acids in the NH$_2$ terminus of DAP-1, we compared these two proteins in our transfection assay system. As clearly demonstrated in Fig. 7A, sentrin alone also induced significant percentage of cell death (53%) in the TNF-sensitive L929 cell line. Further comparison was performed in a TNF-resistant U2OS cell line. Consistent with our previous observation, both DAP-1 and sentrin enhanced the percentage of apoptotic cells in U2OS cells (Fig. 7B). Together, these repeated experiments on several independent cell lines allowed us to conclude that both DAP-1 and sentrin induced programmed cell death that is mediated through the activation of caspases.

It has been demonstrated that TNF-R1 associates with the death effector, TRADD and FADD, leading to the activation of FLICE and downstream caspases (5–7). Since our data indicated that DAP-1 interacts with TNF-R1 and that both pathways result in the activation of caspases, we tested whether DAP-1-induced apoptosis converges with TNF-R1 signals at the death effectors, TRADD or FADD. To address this issue, we cotransfected DAP-1 with the dominant negative FADD (FADD-DN), which has been shown to block FADD mediated cell death into L929 cells. As FADD-DN mutant clearly blocked FADD-induced cell death (reduction from 50% to 24%), it also prevented DAP-1 induced apoptosis (reduction from 39% to 16%) (Fig. 8). FADD-DN alone did not cause cell death. Collectively, these transfection experiments strongly suggest that DAP-1 converges with TNF receptor signaling pathway and that it induces downstream caspase activities through the activation of FADD death effector.
FIG. 8. FADD dominant negative mutant blocked the apoptosis induced by DAP-1. Various expression plasmids that contain DAP-1, sentrin, FADD, or FADD dominant negative mutant (FAD-DN) were cotransfected into L929 cells, either alone or in combination, along with pCMV-LacZ reporter plasmid. The amount of total DNA in all transfectants was brought to the same level with CDMS vector. Percentage of apoptotic cells was determined by the ratio of blue cells with condensed nuclei over total blue cells.

DAP-1 Induces Apoptosis

DAP-1- and sentrin-induced apoptosis in TNF-α- and Fas-mediated cell death signaling pathways. The other relevant phenomenon was our observation that the yeast clones expressing the DAP-1 protein had consistently slower growth rate than other yeast colonies (data not shown). Furthermore, our attempts to obtain stable cell lines expressing constitutive DAP-1 protein were unsuccessful, presumably due to the toxicity of DAP-1 on the host cells. The growth arrest nature of this protein in yeast (smt3) and mammalian cells may be related to its apoptosis-inducing activity as observed here by transient transfection assays. Future studies will be necessary to discern the molecular mechanism of DAP-1- and sentrin-induced apoptosis in TNF-α- and Fas-mediated cell death signaling pathways.

A Potential Role of DAP-1 in Cell Cycle Control—The other

2 M.-L. Liou, unpublished observation.

DISCUSSION

TNF-α-mediated receptor trimerization recruits a variety of intracellular proteins that are crucial for signal transduction. Among these are death domain-containing proteins and TRAF, which mediate apoptosis or activation of NF-κB/Rel, respectively. Using yeast two-hybrid system, we identified a protein, DAP-1, which specifically interacts with the TNF-R1. Sequence analysis of DAP-1 indicated that it is a member of the expanding ubiquitin-homology protein family. Our studies demonstrated that DAP-1 can suppress NF-κB/Rel activity. Furthermore, we provided evidence that overexpression of DAP-1 can lead to programmed cell death, which can be blocked by caspase inhibitor and the dominant negative FADD protein.

DAP-1 Suppresses NF-κB/Rel Activity—Our data indicated that one of the unique features of DAP-1 is its ability to suppress the constitutive NF-κB/Rel activity in 293T cells. Recently, the mechanism of NF-κB/Rel activation by TNF-R1 has been characterized. It was shown that the association of TRAF or RIP with NF-κB-inducing kinase activates the IKKα and IKKβ (36–38). The IKKα/β heterodimer phosphorylates the IκBs in the NF-κB/IκB complexes, leading to the specific ubiquitination and degradation of IκBs by ubiquitin-proteosome pathways. Degradation of the IκBs is necessary for NF-κB/Rel activation and translocation to the nucleus.

Several possibilities may explain the nature of NF-κB/Rel suppression by DAP-1. For instance, DAP-1 may interfere with TRAF- or RIP-mediated NF-κB/Rel activation. Alternatively, since DAP-1 is a UbH protein, it may modulate NF-κB/IκB complex activity by competing for common ubiquitin substrates. One distinct feature of the UbH proteins is that, unlike the ubiquitin-targeted proteinolysis, some UbH proteins can conjugate to other proteins without causing substrate degradation. The diglycine residues at position 141 and 142 in DAP-1 protein is that, unlike the ubiquitin-targeted proteinolysis, some UbH proteins can conjugate to other proteins without causing substrate degradation. The diglycine residues at position 141 and 142 in DAP-1 protein can potentially serve as the conjugation site for protein substrates, similar to its yeast homologue, Smt3p (2). It will be of interest to test whether NF-κB/Rel or IκB are the potential protein substrates of DAP-1.

Association of DAP-1 with Apoptosis—Our studies demonstrated that expression of DAP-1 alone induced apoptosis in several cell lines, regardless of their TNF sensitivity. It is clearly a programmed cell death, rather than necrosis or non-specific toxicity, because the cell death induced by DAP-1 can be blocked by the caspase peptide inhibitor as well as by the dominant negative FADD molecule. This raises an interesting point, which suggests that DAP-1-induced cell death is mediated through the interaction or recruitment of FADD death effector, which also associates with the TNF-R1. Given the unique ubiquitin-like conjugation activity of DAP1 (see below), DAP-1 may facilitate the formation of the death-inducing signaling complex by serving as the docking site for assembly of intracellular death domain containing proteins.

Our data, however, differ from the previous observation made by Okura et al. (4) in that sentrin was shown to protect a B cell lymphoma cell line from Fas-induced apoptosis as well as TNF-induced cell death in L929 cells. There are several issues that this report fails to resolve. First, given the percentage of cell survival conferred by sentrin (55%) as compared with vector alone at 25 ng/ml concentration of anti-Fas, it would indicate a nearly 30–50% transfection efficiency of the BJAB lymphocytic cell line using electroporation. To our knowledge and based on our experience with B lymphocytes, it has been extremely difficult to achieve even more than 2–5% transfection efficiency in B cell lines using most existing transfection methods.

Alternatively, the discrete activities of DAP-1 and sentrin could be due to the NH2-terminal 40 amino acids that are present in DAP-1 and PIC1, but absent in sentrin. In our repeated experiments, three independent DAP-1 constructs reproducibly induced cell death. Furthermore, a side by side comparison of sentrin and DAP-1 using the same expression plasmid in several cell lines strongly supported the similar apoptosis-inducing nature of both proteins.

In the case of human neutrophils, DAP-1 has been shown to mediate TNF death signal (41). Our data, however, differ from the previous observation made by Okura et al. (4) in that sentrin was shown to protect a B cell lymphoma cell line from Fas-induced apoptosis as well as TNF-induced cell death in L929 cells. There are several issues that this report fails to resolve. First, given the percentage of cell survival conferred by sentrin (55%) as compared with vector alone at 25 ng/ml concentration of anti-Fas, it would indicate a nearly 30–50% transfection efficiency of the BJAB lymphocytic cell line using electroporation. To our knowledge and based on our experience with B lymphocytes, it has been extremely difficult to achieve even more than 2–5% transfection efficiency in B cell lines using most existing transfection methods.

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The other relevant phenomenon was our observation that the yeast clones expressing the DAP-1 protein had consistently slower growth rate than other yeast colonies (data not shown). Furthermore, our attempts to obtain stable cell lines expressing constitutive DAP-1 protein were unsuccessful, presumably due to the toxicity of DAP-1 on the host cells. The growth arrest nature of this protein in yeast (smt3) and mammalian cells may be related to its apoptosis-inducing activity as observed here by transient transfection assays. Future studies will be necessary to discern the molecular mechanism of DAP-1- and sentrin-induced apoptosis in TNF-α- and Fas-mediated cell death signaling pathways.

A Potential Role of DAP-1 in Cell Cycle Control—The other
intriguing possibility is that the apoptosis caused by DAP-1 overexpression may be an indirect effect of DAP-1 on cell cycle checkpoint control. The supporting data came from the observation that its yeast homologue, Smt3p, is involved in the regulation of MIF2 centromere binding protein (1). Thus, a potential function of DAP-1 may be to modulate mitosis by influencing chromosome stability during mitosis. It has been demonstrated that ubiquitin-proteasome systems are involved in several major cell cycle transitions (39–41). For example, the anaphase-promoting complex is a ubiquitin ligase complex that is necessary for the degradation of proteins that inhibit separation of chromosomes during mitosis. Interruption of the anaphase-promoting complex function by a mutant E2 protein inhibits the destruction of cyclins, arrests cells in M phase, and inhibits the onset of anaphase (42). Since the DAP-1 homologue regulates the centromere-binding protein, it is possible that DAP-1 may be involved in the modulation of anaphase-promoting complex function. Future studies on the effect of DAP-1 on cell cycle checkpoint control will need to be established.

Recent studies by Johnson et al. provided the first biochemical evidence that Smt3p could be attached to other proteins posttranslationally through the functionally critical diglycine residues, Gly97 and Gly98 (2). These diglycine residues are crucial for the ability of Smt3p to conjugate with its protein substrates and to complement the lethality of a smt3 mutant strain. In several of our Western blot analyses, we also observed similar high molecular mass bands (about 95 kDa) that were present in samples transfected with DAP-1 but not in control samples (Fig. 2, A and B). This observation may suggest similar protein conjugation properties of the mammalian DAP-1 protein. The protein substrates of Smt3p and DAP-1 are yet to be determined.

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