A Death-associated Protein Kinase (DAPK)-interacting Protein, DIP-1, Is an E3 Ubiquitin Ligase That Promotes Tumor Necrosis Factor-induced Apoptosis and Regulates the Cellular Levels of DAPK

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Regulation of protein degradation by the ubiquitin proteasome pathway is now known to be a major pathway through which cells modulate the expression levels of critical signaling proteins (1–6). This tightly regulated, complex pathway is a key regulator of many important signaling pathways and has an important role in many cellular processes including apoptosis, and recent studies have identified many apoptosis regulatory proteins as targets for ubiquitination (7–11). In addition to being targets for degradation, some apoptosis regulatory proteins have a more active role and act as components of the ubiquitin cascade via the ubiquitin ligase activity ascribed to the RING finger domains that is part of their primary structure. Targeting proteins for degradation by the ubiquitin proteasome pathway involves the covalent linkage of ubiquitin either to the amino terminus or specific lysine residues in the target protein through the action of three enzymes. In this process ubiquitin is first activated by an E1 ubiquitin-activating enzyme, transferred to an E2 ubiquitin-conjugating enzyme, and then ligated to the target protein by an E3 ubiquitin ligase (4, 12)

Yeast Two-hybrid Screening—A mouse 11-day embryo cDNA library (Clontech) was screened using a pAS2–1 plasmid containing either the kinase domain (residues 1–290), ankyrin repeats (residues 292–672), or death domain/carboxyl-terminal “tail” (residues 1216–1442), or the region between the ankyrin repeats and death domain (residues 628–1215) for expression as “bait” to clone proteins that interact with DAPK in a yeast-two-hybrid screen. The cDNA library (pGAD10) and pAS2–1 DAPK were simultaneously transformed into Y190 yeast using the manufacturer’s protocols (Clontech). Selection was performed, β-galactosidase-positive colonies were identified, and plasmid DNA was isolated from the yeast. The cDNA insert of one of the positive clones encoding the carboxyl-terminal region of DIP-1 (bp 2155–4331) was amplified and sequenced (Seqwright; Houston, TX). A full-length cDNA having a predicted open reading frame were identified using SMART (Simple Modular Architecture Research Tool; smart.embl-heidelberg.de) (20, 21). The full-length cDNA was subcloned into pSFFlagCMV10 (Sigma) or pEGFP-C3 (Clontech) vectors such that the 110-kDa DIP-1 protein was fused in-frame to the 3XFLAG epitope (FL-DIP-1) or GFP (GFP-DIP-1) at its amino terminus. Similar constructs were made to express residues 815–998 encompassing the three RING fingers (FL-RING1–3) or GFP-RING1–3. Each construct was verified by Western blotting of COS cell lysates after transient expression using either anti-DIP-1 affinity-purified antibody or an appropriate antibody to detect the fusion protein.

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**Antibodies and Reagents**—An affinity purified, rabbit polyclonal sera against DIP-1 (residues 483–1006) was generated using standard methodology (Sigma-Genosys). Two monoclonal anti-human DAPK (clone 17 from Sigma and clone 55 from BD Biosciences) were used at dilutions of 1:250 and 1:10,000, respectively, and gave similar results. The anti-FLAG epitope monoclonal antibody (M2, Sigma) was used at a dilution of 1:1,000, anti-T7 antibody (Novagen) was used at a dilution of 1:100,000, and anti-GST antibody (Sigma) used at a dilution of 1:2,000. Antibodies against poly-ADP-ribose polymerase, ubiquitin, and cytochrome c were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Northern and Western Blotting—RNA was prepared from mouse tissues and cell lines using the Totally RNA kit (Ambion). For Northern blotting, 10 μg of total RNA per lane was fractionated on a 1.2% agarose gel, transferred to a nylon membrane, and hybridized to a 32P-labeled antisense DIP-1 riboprobe (cRNA) corresponding to bp 3123–3674. The blot was washed and exposed to X-Omat AR film with an intensifying screen for 1 week. Western blotting was performed as described previously (22). Briefly, cell extracts were prepared from cells or tissues by homogenization in a lysis buffer containing 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 mM NaCl, 10 mM sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM sodium fluoride, 0.2 mM sodium vanadate, 20 μg/ml leupeptin, 40 μg/ml aprotinin, 6 μg/ml t-1-lysylamide-2-phe- nylethyl chloromethyl ketone, 6 μg/ml N3-p-tosyl-l-lysine chloromethyl ketone, 1 μM PefablocSC (Roche Molecular Biochemicals), 10 μg/ml (4-amidinophenyl)-methanesulfonyl fluoride (APMSF). Equivalent amounts of total cellular protein or immunoprecipitates were fractionated by electrophoresis through an SDS-polyacrylamide gel and transferred to nitrocellulose. Immunoreactive proteins on Western blots were visualized using the Supersignal West Dura or West Pico detection kit. Western blots were washed and exposed to X-Omat AR film with an intensifying screen for 1 week. For immunoprecipitations, cell lysates were mixed with polyclonal anti-DIP-1 or FLAG antibodies. Immunoprecipitated proteins were found to co-immunoprecipitate with DAPK.

**Exogenous Protein Expression, Cell Lines, and Immunoprecipitation Analysis**—All transient transfections were carried out using FuGENE 6 according to the manufacturer’s directions (Roche Molecular Biochemicals). Stable HeLa cell lines expressing DIP-1, RING1–3, or RING1–5 were established by selection with Zeocin of HeLa-tet cells transfected with FLAG-tagged DIP-1 or RING1–3 cloned into pcDNA4TO vector (Invitrogen) as described previously (DAPK paper). For all experiments, HeLa-tet parental cells and HeLa cells expressing DIP-1 or RING1–3 were treated with doxycycline (2 μg/ml) for 24 h for stable expression of the transgene. Because all experimental results obtained with HeLa-DIP-1 and HeLa-RING1–3 in the basal, uninduced state were indistinguishable from the HeLa parental cell line (either in the presence or absence of tet), they are not included in the figures. Immunoprecipitations were carried out using standard protocols where cellular lysates were clarified by centrifugation and pre-cleared using protein A-Sepharose beads. The indicated proteins were immunoprecipitated by the addition of protein A beads pre-complexed with appropriate antibodies. Immunocomplexes bound to protein A beads were washed in wash buffer containing 0.1% Triton X-100, 50 mM NaCl, pH 7.4, 0.3 mM NaCl, 5 mM EDTA, 0.02% NaN3, and then resuspended in protein gel sample buffer.

**Apoposis Assays**—Apopotic cell death was determined by DNA fragmentation analysis using fluorescence-activated cell sorting (FACS). For these experiments, the indicated HeLa cell lines were transiently transfected with vectors encoding GFP-actin, GFP-DIP-1, or GFP-RING1–3. At 24 h post-transfection, cells were enzymatically detached, fixed in 5% acetic acid, 95% ethanol at −20 °C for 2 h, and stained with 50 μg/ml propidium iodide (Sigma) (23). For transient analysis, cells were co-transfected with plasmids encoding GFP-DIP-1, RING1–3, and a FLAG-tagged DAPK vector (pcDNA4TO control) together with pCMV-CDNA vector encoding GFP-actin (Clontech) at a ratio of 5:1. Expression of the exogenous transgenes was confirmed by Western blotting. A BD Biosciences FACStar plus was used to identify GFP-positive cells and simultaneously analyze their DNA content. A minimum of 50,000 cells was counted for each analysis. Where indicated, cell death was determined by measuring the number of viable, trypan blue dye-staining cells as described previously (17). The % cell death was calculated using the formula 1 − (the number of viable cells)/number of viable cells in control) × 100. Measurements of caspase activity were performed as described previously (17). Briefly, HeLa cell lines expressing DIP-1, RING1–3, or parental cells were lysed with CHAPS lysis buffer (0.1% CHAPS, 0.1% SDS, 1% sodium deoxycholate, 100 mM sodium phosphate, pH 7.4). After centrifugation, equal amounts of total cellular proteins were incubated at 37 °C, and the assay was initiated by the addition of either 200 μM Ac-IETD-p-nitroanilide (caspase-8), 200 μM Ac-DEVD-p-nitroanilide (caspase-3), or 200 μM Ac-LEHD-p-nitroanilide (caspase-9). Change in absorbance at 405 nm over time was monitored by spectrophotometry and caspase activities (pmol/min/mg of total protein) were calculated after subtraction of background using the following calibration curve. For each cell sample, the background was determined by adding the caspase specific inhibitors, Ac-IETD-CHO (caspase-8), Ac-DEVD-CHO (caspase-3), or Ac-LEHD-CHO (caspase-9) as the negative control.

**Expression of Fusion Proteins in Bacteria**—Constructs for isopyrrol- 1-thio-β-galactopyranoside-induced bacterial expression of GST-tagged DIP-1 fusion proteins were obtained by PCR amplification and subcloning of DIP-1 sequences corresponding to residues 493–1006 (GST-DIP-1 493–1006) or the three RING fingers (residues 815–998, GST-DIP-1 RING1–3) into pGEX-4T-2 expression vector (Amersham Biosciences). Verification of the sequences was achieved by DNA automated sequencing. For GST fusion proteins, bacteria cells were lysed by sonication at 4 °C in PBS binding buffer (phosphate-buffered saline containing 1 mM dithiothreitol, 0.1% Triton X-100, and 10 mg/ml bovine serum albumin). After centrifugation, GST fusion proteins were purified from the cell lysate using glutathione-Sepharose 4B affinity column chromatography (Amersham Biosciences). I-Chloro-2,4-dinitrobenzene (CDNB) assay (Amerham Biosciences) was used to quantify the relative amounts of the isolated GST fusion proteins.

**In Vitro Ubiquitination Assays**—Ubiquitination assays were modified from those previously described (24). Briefly, the reactions contain bacterial-expressed ubiquitin-activating enzyme (E1, 250 nm), ubiquitin-conjugating enzyme (E2; UbC5a, 2 μM), His-ubiquitin (0.6 μM), and ATP (2 mM). Identical results were obtained using commercially available ubiquitin-activating enzyme (E1; Calbiochem) or GST-tagged ubiquitin-conjugating enzyme UbC5a (E2; Calbiochem) and amino-terminal His-tagged ubiquitin (Sigma). Where indicated, DIP-1 or RING1–3 (1 μg), DAPK immunoprecipitated from HeLa cell lines or DAPK fragments purified from bacteria (1–5 μg) were added to the reactions. After incubation at room temperature, the reactions were analyzed by Western blotting to detect the ubiquitinated proteins. For expression of E1 (250 nm) and E2 (UbC4 or UbC5a; 2 μM) were kindly provided by Dr. Tony Hunter (Salk Research Institute) and have been described before (24). Vectors for expression of E1 (250 nm) and E2 (UbC4 or UbC5a; 2 μM) were kindly provided by Dirk Bohmann (University of Rochester) and have been described before (25). For some assays, ubiquitin-conjugating enzyme (E1A) and ubiquitin-conjugating enzyme (UbC5a) (Calbiochem) and His-ubiquitin (Sigma) were used.

**RESULTS**

**Identification of a Component of a DAPK Protein Complex**—To confirm our prediction that DAPK associates in vivo with other cellular proteins, DAPK was immunoprecipitated under non-denaturing conditions from COS cells cultured in the presence of 35S-labeled methionine and cysteine. Several proteins were found to co-immunoprecipitate with DAPK whose molecular masses range in size from 40 to 240 kDa. This result suggested that DAPK might exist in cells as a large multi-protein complex (Fig. 1A). To identify these proteins, the entire cDNA encoding DAPK was divided into regions that encompassed the kinase domain, ankyrin repeats, death domain/”tail,” or the region between the ankyrin repeats and death domain, and each of these four regions was subcloned into pcAS-2-1 to be expressed as bait in a yeast-two-hybrid interaction screen. One of the positive clones, encoding a 110-kDa protein now called DAPK interacting protein-1 (DIP-1), identified by interaction with the ankyrin repeats in DAPK, has been cloned, expressed, and characterized. The sequence of the predicted open reading frame from a 3.5-kilobase cDNA clone encoding DIP-1 that was obtained by screening a mouse bladder cDNA library is shown in Fig. 1B. The sequence of DIP-1 includes several motifs that were identified using SMART (simple modular architecture research tool) (Fig. 1C) (26, 27). These motifs include a zinc finger (ZnF; residues 79–124), 9 ankyrin repeats (ANK; residues 430–729), three RING fingers (RING1, 819–853; RING2, 866–900; RING3, 963–995), and an α-helical coiled-coil located between RING2 and RING3 (COIL; residues 934–962). Data base searches for similar protein motifs suggested that the RING fingers present in DIP-1 had the highest...
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To investigate the functional role of DIP-1 in cells undergoing TNF-induced apoptosis, tetracycline-inducible HeLa cell lines expressing full-length DIP-1 or the carboxyl-terminal region of DIP-1 that encompasses all three RING fingers (residues 815–998; RING1–3) were generated and characterized as described previously (17). Overexpression of DIP-1 or RING1–3 enhanced the sensitivity of HeLa cells to TNF-induced apoptosis (Fig. 4A). In these experiments the dose-response curve of the cell lines expressing DIP-1 or RING1–3 is left-shifted, consistent with the enhanced sensitivity of these cell lines to TNF, and the TNF dose at which 50% apoptosis occurs decreased from 15 ng/ml to less than 1 ng/ml. Paralleling the enhanced sensitivity of these cell lines to TNF, and the TNF dose at which 50% apoptosis occurs decreased from 15 ng/ml to less than 1 ng/ml. Paralleling the enhanced sensitivity of these cell lines to TNF, and the TNF dose at which 50% apoptosis occurs decreased from 15 ng/ml to less than 1 ng/ml. Paralleling the enhanced sensitivity of these cell lines to TNF, and the TNF dose at which 50% apoptosis occurs decreased from 15 ng/ml to less than 1 ng/ml. Paralleling the enhanced sensitivity of these cell lines to TNF, and the TNF dose at which 50% apoptosis occurs decreased from 15 ng/ml to less than 1 ng/ml.

Transient expression of DIP-1 or RING1–3 in HeLa cell lines expressing DAPK–α, DAPK–β, or the parental HeLa cells revealed that either protein could effectively antagonize the anti-apoptotic activities of DAPK (17). For these experiments plasmids encoding GFP fusion proteins (GFP-DIP-1, GFP-RING1–3, or GFP-actin) were transfected into the parental HeLa cell line or HeLa cell lines with tetracycline-inducible expression of DAPK–α or DAPK–β (17). Fig. 5 shows the results.

Expression of DIP-1 Promotes TNF-induced Apoptosis and Antagonizes the Survival Function of DAPK—To investigate the functional role of DIP-1 in cells undergoing TNF-induced apoptosis, tetracycline-inducible HeLa cell lines expressing full-length DIP-1 or the carboxyl-terminal region of DIP-1 that encompasses all three RING fingers (residues 815–998; RING1–3) were generated and characterized as described previously (17). Overexpression of DIP-1 or RING1–3 enhanced the sensitivity of HeLa cells to TNF-induced apoptosis (Fig.
DOX (or not (H9251)) or not (H11001) 3 or in parental HeLa cells. Cells expressing DIP-1 or RING1–3 are increased in response to 10–8 nM TNF (10 ng/ml) and cyclohexamide (10 μg/ml) for 4 h. This resulted in enhanced release of cytochrome c (Fig. 5B). Additional support for a pro-apoptotic function of DIP-1 comes from the direct determination of caspase activity in cell lysates of HeLa cell lines expressing DIP-1 or RING1–3. Using caspase-specific colorimetric peptides, the activities of caspase-3, caspase-8, and caspase-9 were determined. A statistically significant increase in both caspase-3 and caspase-9 activities was found. The levels of caspase-3 activity increased 1.5- and 1.6-fold, and the levels of caspase-9 activity increased 1.5- and 2.1-fold after 4 h of TNF treatment in cells expressing DIP-1 or RING1–3, respectively, over parental cells, whereas overexpression of DIP-1 or RING1–3 had no effect on TNF-induced caspase-8 activation.

**DIP-1 Is an E3 Ligase in Vitro and Is Ubiquitinated in Vivo**—Previous studies show that the RING fingers present in proteins such as IAP-1, IAP-2, and XIAP can function as E3 ubiquitin-protein ligases (24, 28–30). The finding that the carboxyl terminus of DIP-1 contains three similar RING finger motifs led us to examine the possibility that the RING fingers in DIP-1 function as E3 ligases. For these in vitro ubiquitination assays (24), purified ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2 (Ubc5a), His-ubiquitin, and ATP were added to affinity-purified GST or GST-RING1–3 immobilized on glutathione-Sepharose beads. After incubation, the reactions were washed, separated by SDS-PAGE, and analyzed by Western blotting to detect ubiquitinated proteins using anti-His antibody. Fig. 6 shows that the higher molecular mass RING1–3 protein is detectable only after incubation in the presence of His-ubiquitin but does not occur in reactions containing GST fusion protein alone, suggesting that RING1–3 can act as an E3 ligase in vitro and undergo autoubiquitination. To determine whether DIP-1 or RING1–3 is found in vivo as the ubiquitinated protein, HeLa cells expressing stable levels of these proteins were transfected with a plasmid encoding His-ubiquitin. At 24 h after transfection, the cells were treated with the proteasome inhibitor MG132 for 4 h before lysis. Talon affinity purification was used to fractionate His-ubiquitin-tagged proteins. Western blotting with anti-FLAG antibody was used to detect the FLAG-tagged RING1–3 (FL-RING1–3) or DIP-1 (FL-DIP-1). The detection of higher mass species of RING1–3 or DIP-1 only in the presence of His-tagged ubiquitin suggests that both DIP-1 and RING1–3 are ubiquitinated in cells.

**DAPK Is a Target for Ubiquitination by DIP-1.**—The finding that the RING fingers at the carboxyl terminus of DIP-1 can function as an E-3 ligase in vitro and antagonize the anti-apoptotic effects of DAPK-β in TNF-induced apoptosis lead us to consider whether DAPK is a target for ubiquitination by its binding protein, DIP-1. We first determined if DAPK could be ubiquitinated by DIP-1 in an in vitro ubiquitination assay. For these experiments, DAPK was immunoprecipitated from HeLa cells using anti-OMNI-tag antibody to immunoprecipitate recombinant DAPK. A control immunoprecipitation using protein A-Sepharose complexed with non-immune IgG was performed in parallel. The washed immune complexes were added to an in vitro ubiquitination reaction in the presence or absence of recombinant DIP-1(492–1006) purified from bacteria as indicated in Fig. 7A. After incubation for 90 min, the immune complexes were washed to remove unconjugated ubiquitin and then analyzed by Western blotting. Inspection of these Western blots revealed that the major species of immunoreactive DAPK appears to migrate in a position that potentially corresponds to mono-ubiquitinated DAPK (mass increase of ~8–10 kDa). However, a higher molecular mass "smear" of immunoreactive DAPK is also detectable representing polyubiquitinated DAPK.

![Fig. 4. Expression of DIP-1 promotes TNF-induced apoptosis.](image-url)

A, dose-response curves representing the levels of apoptosis in response to TNF treatment for 8 h. HeLa Tet-ON cell lines expressing either vector only (HeLa), full-length DIP-1 (HeLa-DIP-1), or the three RING fingers of DIP-1 (residues 815–998; RING 1–3) were treated for 24 h with doxycycline (DOX) to induce stable expression of DIP-1 or RING1–3 before TNF treatment. Percent apoptosis was quantified by determining the number of viable, non-apoptotic TNF-treated or control cells expressing DIP-1 or RING1–3 before TNF treatment. Percent apoptosis was quantified by determining the number of viable, non-apoptotic TNF-treated or control cells expressing DIP-1 or RING1–3 or in parental HeLa cells. C, the temporal appearance of the 89-kDa poly-ADP-ribose polymerase (PARP) cleavage fragment is accelerated in HeLa cells expressing DIP-1 or RING1–3 compared with parental HeLa cells.
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Fig. 5. Expression of DIP-1 or DIP-1-RING antagonizes the survival function of DAPK-β.A. HeLa cell lines overexpressing either DAPK-α or the more potent anti-apoptotic form of DAPK, DAPK-β, were transiently transfected with GFP-DIP-1 or GFP-RING1-3. At 24 h post-transfection, the cells were treated with TNF (10 ng/ml) and cyclohexamide (10 μg/ml) for 4 h, then sorted to identify GFP-positive cells and analyzed for DNA content using propidium iodide (PI) staining. The gated region indicates sub-G1 levels of DNA (fragmented DNA). In the parental HeLa cells (left panels), expression of GFP-DIP-1 or GFP-RING1-3 enhances cell death compared with the GFP-only control. The right panels show that the death-promoting effect of DIP-1 or DIP-1-RING1-3 antagonizes the anti-apoptotic function of DAPK-β. B, HeLa cell lines expressing DIP-1 or RING1-3 have enhanced TNF-induced release of cytochrome c (Cyt c) from the mitochondria to the cytosol. WB, Western blot; CHX, cyclohexamide. C. HeLa cell lines expressing DIP-1 or RING1-3 have increased levels of caspase-3 and caspase-9 but not caspase-8. pNA, p-nitroanilide.

Fig. 6. DIP-1 is an E3 ligase in vitro and is ubiquitinated in vivo. A, Western blot (WB) to detect ubiquitinated RING1-3 after an in vitro ubiquitination assay. Recombinant RING1-3 was added to purified ubiquitin-activating enzyme (E1A) and ubiquitin-conjugating enzyme (Ube2a) and incubated in the presence of ATP and His-ubiquitin (Ub). Anti-His antibody was used to detect polyubiquitinated RING1-3. B, Western blotting to detect FLAG-tagged RING1-3 (FL-RING1-3) or DIP-1 (FL-DIP-1) after treatment of cells with the proteasome inhibitor MG132. His-tagged ubiquitinated proteins were affinity-purified on Talon metal ion affinity beads, and anti-FLAG antibody was used to detect polyubiquitinated RING1-3 or DIP-1.

(noted proteins were immunoprecipitated using anti-ubiquitin antibody. The immunoprecipitates were analyzed by Western blotting to detect endogenous DAPK. These results show that polyubiquitinated DAPK is detectable only when DIP-1-(492–1006) is added to the DIP-1-depleted lysate.

To show that DAPK is an in vivo substrate for ubiquitination by DIP-1, HeLa cells were co-transfected with vectors for expression of hemagglutinin-ubiquitin and either FL-DIP-1 or FL-RING1-3. At 24 h, ubiquitinated proteins were immunoprecipitated using anti-ubiquitin antibody, and the immunoprecipitates were analyzed by Western blotting using anti-DAPK antibody to detect the endogenous DAPK. The results of this experiment show that increasing the expression of DIP-1 or RING1-3 results in a parallel increase in the relative level of polyubiquitinated DAPK (Fig. 7C). Evidence showing that the cellular levels of DAPK are regulated by ubiquitin proteasome degradation was obtained by examining the expression levels of the endogenous DAPK in HeLa, HeLa-DIP-1, or HeLa-RING1-3 cell lines transiently expressing exogenous ubiquitin. The Western blot shown in Fig. 7D revealed either a modest or large decrease, respectively, in DAPK in HeLa cell lines expressing either DIP-1 or RING1-3. The greater decrease in expression of DAPK in HeLa-RING1-3 cells transfected with ubiquitin is likely to be due to the higher level of overexpression of RING1-3 compared with DIP-1, which is shown in the second panel in Fig. 7D. Control experiments using the HeLa-DIP or HeLa-RING1-3 cell lines in the uninduced configuration did not reveal any significant differences in the relative expression levels of DAPK. Consistent with our previous suggestion that DAPK is an anti-apoptotic factor, we also observed in HeLa-RING1-3 cells transfected with ubiquitin that the
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FIG. 7. DAPK is a substrate for ubiquitination by DIP-1. A, in vitro ubiquitination of DAPK by DIP-1. DAPK was immunoprecipitated (IP) from HeLa cells and incubated with recombinant DIP-1 (0.83 μM) in the presence of E1, E2, ATP, and His-ubiquitin. B, HeLa cell lines expressing DIP-1 or RING1-3 were transfected with a vector for expression of ubiquitin. Ubiquitinated proteins were immunoprecipitated from the in vitro ubiquitination reactions using anti-ubiquitin antibody. After Western blotting (WB) anti-DAPK antibody was used to detect DAPK. Increased expression of DIP-1 or RING1-3 enhances the appearance of polyubiquitinated DAPK (Ub₃-DAPK). CE, cell extract; C, HeLa cells were immunodepleted of endogenous DIP-1, and recombinant DIP-1-(492–1006) was added back to the cell lysate that also contained exogenous ATP, ubiquitin, and MG-132 (10 μM). After 30 min of incubation, ubiquitinated proteins were immunoprecipitated and analyzed by Western blotting using anti-DAPK antibody. Significant levels of polyubiquitinated DAPK (Ub₃-DAPK) appear only in reactions using native cell lysate (first lane) or lysate to which DIP-1 was added back (third lane). D, levels of endogenous DAPK were examined by Western blotting in extracts from the indicated HeLa cell lines after transient expression of ubiquitin. In HeLa cells expressing DIP-1, a modest decrease in the level of DAPK is observed, whereas in HeLa cells expressing RING1-3, a significant decrease in DAPK expression is detected. The Western blot in the upper panel was probed with anti-DAPK antibody and then successively with anti-DIP-1, anti-poly-ADP-ribose polymerase (PARP), and anti-vinculin antibody. Vinculin is used as a loading control to show equivalent amounts of cellular proteins in each lane.

The high conservation of the complex primary structure of DAPK and the presence of several protein-protein interaction motifs including several ankyrin repeat motifs and a death domain suggested that in addition to substrate binding and activation by calcium/calmodulin, this Ser/Thr kinase might be associated with other cellular proteins. A yeast two-hybrid interaction screen has identified a new protein called DIP-1, which binds to the ankyrin repeat region of DAPK, and co-immunoprecipitation studies have confirmed that DIP-1 and DAPK are associated in cells. Although understanding the structural components and the regulation of DAPK and DIP-1 interactions will be critical to understanding their collaborative function in apoptosis regulation, the similarity between the RING fingers in DIP-1 and those present in IAP-1 and IAP-2 led us to focus on examining the functional properties of the carboxyl-terminal region containing these motifs in DIP-1.

The primary sequence of DIP-1 has several interesting structural motifs including a B-box-type zinc finger, a series of nine ankyrin repeats, and a carboxyl-terminal region that contains three putative RING finger domains with an α-helical coiled-coil structure that separates RING2 from RING3. Classical RING finger domains are defined by a specific pattern of cysteine and histidine residues that are involved in the binding of zinc, which is important for the folding of the domain and its activities. Many RING domain proteins also have B-box zinc fingers and coiled-coil motifs arranged in a conserved order, and these motifs may function as additional sites of protein interactions (31, 32). This has led to the suggestion that collectively these motifs function as a molecular scaffold to mediate the organization of large protein signaling complexes, and the identification of DIP-1 as a component of proteins that are associated in a complex with DAPK supports this proposal.

One specific function that has been ascribed to the RING finger domain is its ability to act as an E3 ligase in the ubiquitin proteasome pathway (11, 24, 29, 33–35). Consistent with this, we have determined that DIP-1 can autoubiquitinate in vitro in the presence of ubiquitin-activating E1 and ubiquitin-conjugating E2 enzymes, suggesting that DIP-1 is a member of the “single subunit” class of RING domain E3 ligase (1). Although we do not know if the autoubiquitination is inter- or intramolecular, the demonstration that DIP-1 is found as a polyubiquitinated protein in vivo suggests this may serve as a mechanism to down-regulate DIP-1 expression.

The determination that DAPK is also found in vivo as a polyubiquitinated protein also suggests that cellular levels of DAPK are regulated by the ubiquitin-proteasome system; the association of DIP-1 with DAPK provided the basis for proposing that DAPK is a target for DIP-1-mediated ubiquitination, and four experimental approaches were utilized to confirm this proposal. First, an in vitro ubiquitination assay using purified RING1–3 as an E3 ligase in the presence of purified E1 and E2 showed that DAPK can be polyubiquitinated by DIP-1. An add-back experiment using purified DIP-1-(492–1006) to supplement DIP-1-immunodepleted cell lysates restored the appearance of polyubiquitinated DAPK. Western blotting to examine the in vivo ubiquitination levels of DAPK in cell lines that overexpress either DIP-1 or RING1–3 showed enhanced ubiquitination of DAPK in these cells compared with the parental HeLa cells. Finally, the endogenous levels of DAPK are diminished significantly in HeLa cell lines overexpressing DIP-1 or RING1–3, suggesting that the endogenous levels of DAPK may be regulated by DIP. Together these findings support the proposal that the apoptosis regulatory protein kinase

including condensed chromatin and membrane blebbing (data not shown).

**DISCUSSION**

The decreased expression of endogenous DAPK correlated with enhanced poly-ADP-ribose polymerase cleavage (Fig. 1D, third panel) and morphological changes consistent with apoptosis

*Figure 7: DAPK is a substrate for ubiquitination by DIP-1.* A, in vitro ubiquitination of DAPK by DIP-1. DAPK was immunoprecipitated (IP) from HeLa cells and incubated with recombinant DIP-1 (0.83 μM) in the presence of E1, E2, ATP, and His-ubiquitin. B, HeLa cell lines expressing DIP-1 or RING1-3 were transfected with a vector for expression of ubiquitin. Ubiquitinated proteins were immunoprecipitated from the in vitro ubiquitination reactions using anti-ubiquitin antibody. After Western blotting (WB) anti-DAPK antibody was used to detect DAPK. Increased expression of DIP-1 or RING1-3 enhances the appearance of polyubiquitinated DAPK (Ub₃-DAPK). CE, cell extract; C, HeLa cells were immunodepleted of endogenous DIP-1, and recombinant DIP-1-(492–1006) was added back to the cell lysate that also contained exogenous ATP, ubiquitin, and MG-132 (10 μM). After 30 min of incubation, ubiquitinated proteins were immunoprecipitated and analyzed by Western blotting using anti-DAPK antibody. Significant levels of polyubiquitinated DAPK (Ub₃-DAPK) appear only in reactions using native cell lysate (first lane) or lysate to which DIP-1 was added back (third lane). D, levels of endogenous DAPK were examined by Western blotting in extracts from the indicated HeLa cell lines after transient expression of ubiquitin. In HeLa cells expressing DIP-1, a modest decrease in the level of DAPK is observed, whereas in HeLa cells expressing RING1-3, a significant decrease in DAPK expression is detected. The Western blot in the upper panel was probed with anti-DAPK antibody and then successively with anti-DIP-1, anti-poly-ADP-ribose polymerase (PARP), and anti-vinculin antibody. Vinculin is used as a loading control to show equivalent amounts of cellular proteins in each lane.
DAPK is a target for ubiquitination and proteasome degradation by the E3 ligase activity of one of its binding proteins, DIP-1. Although additional work will be needed to determine whether DIP-1 has other ubiquitination targets, the ability of DIP-1 to deplete cellular levels of DAPK by targeting it for proteasomal degradation suggests a mechanism by which DIP-1 could antagonize the anti-apoptotic effects of DAPK to promote TNF-induced apoptosis. Paralleling our previous findings with DAPK (17), we also find that DIP-1 enhances caspase-3 and caspase-9 activities while having little effect on TNF-induced caspase-8 activity. These results suggest that DAPK activities may be regulated through proteasomal degradation. Finally, we show that expression of DIP-1 can antagonize the anti-apoptotic activity of DAPK to promote caspase-dependent apoptosis. This result is consistent with our previous determination that DAPK is an important anti-apoptotic survival factor in cells (17).

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A Death-associated Protein Kinase (DAPK)-interacting Protein, DIP-1, Is an E3 Ubiquitin Ligase That Promotes Tumor Necrosis Factor-induced Apoptosis and Regulates the Cellular Levels of DAPK

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