Death-Associated Protein Kinase-Related Protein 1, a Novel Serine/Threonine Kinase Involved in Apoptosis

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In this study we describe the identification and structure-function analysis of a novel death-associated protein (DAP) kinase-related protein, DRP-1. DRP-1 is a 42-kDa Ca\(^{2+}\)/calmodulin (CaM)-regulated serine threonine kinase which shows high degree of homology to DAP kinase. The region of homology spans the catalytic domain and the CaM-regulatory region, whereas the remaining C-terminal part of the protein differs completely from DAP kinase and displays no homology to any known protein. The catalytic domain is also homologous to the recently identified ZIP kinase and to a lesser extent to the catalytic domains of DRAK1 and -2. Thus, DAP kinase DRP-1, ZIP kinase, and DRAK1/2 together form a novel subfamily of serine/threonine kinases. DRP-1 is localized to the cytoplasm, as shown by immunostaining and cellular fractionation assays. It binds to CaM, undergoes autophosphorylation, and phosphorylates an exogenous substrate, the myosin light chain, in a Ca\(^{2+}\)/CaM-dependent manner. The truncated protein, deleted of the CaM-regulatory domain, was converted into a constitutively active kinase. Ectopically expressed DRP-1 induced apoptosis in various types of cells. Cell killing by DRP-1 was dependent on two features: the status of the catalytic activity, and the presence of the C-terminal 40 amino acids shown to be required for self-dimerization of the kinase. Interestingly, further deletion of the CaM-regulatory region could override the indispensable role of the C-terminal tail in apoptosis and generated a "superkiller" mutant. A dominant negative fragment of DAP kinase encompassing the death domain was found to block apoptosis induced by DRP-1. Conversely, a catalytically inactive mutant of DRP-1, which functioned in a dominant negative manner, was significantly less effective in blocking cell death induced by DAP kinase. Possible functional connections between DAP kinase and DRP-1 are discussed.

Apoptosis is a genetically controlled cell death process which is important at various developmental stages as well as for cell maintenance and tissue homeostasis (16). During the last few years, many of the key players in this process, including receptors, adapter proteins, proteases, and other positive and negative regulators, have been identified (13, 33). One of the positive mediators of apoptosis, which was cloned in our laboratory, is death-associated protein (DAP) kinase (9). This protein was discovered by a functional approach to gene cloning, based on transfections of mammalian cells with antisense cDNA libraries and subsequent isolation of death-protective cDNA fragments (9, 10, 19, 20, 23). The antisense cDNA of DAP kinase protected HeLa cells from gamma interferon-induced cell death, and this property served as the basis for its selection. DAP kinase is a calcium/calmodulin (CaM)-regulated serine/threonine protein kinase (160 kDa), associated with actin microfilaments (6, 9). Its structure contains at least two additional domains that might mediate interactions with other proteins: ankyrin repeats, and a typical death domain located at the C-terminal part of the protein (9, 12). Overexpression of DAP kinase in various cell lines results in cell death, and this death-promoting effect of DAP kinase depends on at least three features: catalytic activity, presence of the death domain, and correct intracellular localization (6, 7). Several independent lines of evidence proved that DAP kinase is involved in apoptosis triggered by different external signals including gamma interferon, tumor necrosis factor alpha (TNF-\(\alpha\)), activated Fas receptors, and detachment of cells from the extracellular matrix (6, 7, 9, 15). A tumor-suppressive function was recently attributed to DAP kinase, coupling the control of apoptosis to metastasis (15).

Recent studies have implicated several serine/threonine kinases in the regulation of programmed cell death, either as death-promoting or as death-protecting proteins (1, 3). One such candidate is the c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (2, 32). In one example it was shown to mediate apoptosis induced by detachment from extracellular matrix (named anoikis) (4). In that system, the JNK pathway is activated by MEKK-1, whose kinase activity is stimulated by caspase cleavage (4). JNK may antagonize the antiapoptotic activity of Bcl-2 by phosphorylation (24, 27). Another serine/threonine kinase is RIP, which, like DAP kinase, possesses the death domain. RIP was shown to positively mediate apoptosis in cell cultures (30). However, in vivo studies performed in RIP-deficient mice documented another aspect of its function, i.e., its ability to exert antiapoptotic effects by mediating the TNF-\(\alpha\)-induced activation of NF-\(\kappa\)B (18). Other RIP members, RIP2 and RIP3, were also recently identified and shown to possess proapoptotic activity (25, 31, 34). Among the negative regulators of apoptosis is the protein kinase Akt (protein kinase B). This protein was shown to phosphorylate BAD, thereby preventing it from dimerizing with and blocking the antiapoptotic activity of BCL-X\(_L\) (8, 11). Akt was also recently shown to phosphorylate proapoptase-9, thus blocking its normal processing and activation (5).

Recently, the isolation and characterization of three novel kinases, homologous in their catalytic domains to DAP kinase, have been reported (17, 22, 29). One protein, named ZIP (Dlk) kinase, was found to be 80% identical to DAP kinase within the kinase domain, yet it lacks the CaM-regulatory domain and the other domains and motifs characteristic of DAP kinase. ZIP kinase contains a leucine zipper domain at the C terminus and is localized to the nucleus (17, 22). The activation of ZIP...
kinase occurs by a different mechanism involving homodimerization, mediated by its leucine zipper domain. Another two novel, closely related proteins, DRAK1 and DRAK2, which share ~50% identity with the kinase domain of DAP kinase, were also recently characterized (29). Like ZIP kinase, the DRAK1 and DRAK2 proteins also lack the CaM-regulatory domain. Ectopic expression of the three wild-type kinases, but not of their catalytically inactive mutants, induced morphological changes characteristic of apoptosis (17, 29). In the case of ZIP kinase, the data on its death-inducing properties in some cells are still controversial (22).

Here we report on the cloning and biochemical and functional characterization of a novel member of the DAP kinase subfamily of serine/threonine kinases, a 42-kDa protein named DAP kinase-related protein kinase 1 (DRP-1). Unlike ZIP kinase and the DRAK proteins, DRP-1 contains a typical CaM domain resembling that of DAP kinase and by that mean appears to be the closest homologue to DAP kinase. The carboxy-terminal tail encompassing the last 40 amino acids has no homology to other known proteins and was found to be required for self-dimerization. In vitro kinase assays confirmed the ability of DRP-1 to undergo autophosphorylation and to phosphorylate an exogenous substrate, myosin light chain (MLC), in a Ca2+/CaM-dependent manner. The enzyme became constitutively active upon deletion of the CaM-regulatory domain, and the ectopically expressed DRP-1 was shown to be localized to the cytoplasm as a detergent-soluble form, with minimal association to matrix-insoluble elements. Its function was implicated in apoptosis based on the finding that it induced apoptotic cell death when overexpressed and that a catalytically inactive DRP-1 mutant reduced cell death triggered by the ectopic expression of p55 TNF receptors (TNFR). The death-promoting effects of DRP-1 depended on the functionality of the catalytic domain and on the presence of the C-terminal tail, yet further deletion of the CaM-regulatory domain abrogated the requirement for the C-terminal tail. Cell death induced by DRP-1 was blocked specifically by the death domain of DAP kinase, suggesting a possible cross talk between these two kinases.

**MATERIALS AND METHODS**

cDNA cloning and Northern blotting. A PCR fragment of 364 bp was obtained from a Agt11 human spleen cDNA library (Clontech), using primers from the deduced DRP1 sequence (1813GGGCGGGAGCTGAGG-1914ATTGCGATGCG-1066, and 1411TCCACACTCCCACCCCAGACTC-1390). To obtain the full-length deduced DRP-1 sequence (1047-GGCCGGATGAGGACCTGAGG-1066 and from the T7 promoter. This RNA was translated in reticulocyte lysate (TNT T7 Quick Coupled Transcription/Translation system; Promega) by conventional procedures, with [35S]methionine (Amersham) as a labeled precursor. The reaction product was then run on a sodium dodecyl sulfate (SDS)-12% polyacrylamide gel, followed by sodium salicylate incubation for signal amplification. The gel was dried and exposed to X-ray film.

In vitro kinase assay. 293 cells were transfected with various FLAG or hemagglutinin (HA) epitope-tagged DRP-1 constructs. Immunoprecipitation of the various ectopically expressed DRP-1 proteins from 150 μg of total extract was performed with 20 μl of anti-FLAG M2 gel (IBI, Kodak) in 500 μl of PLB (10 mM NaH2PO4 [pH 7.5], 100 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 5 mM EDTA) supplemented with protease and phosphatase inhibitors, and incubated for 2 h at 4°C. The samples were centrifuged for 1 min at 12,000g, and the supernatants were washed twice with an ice-cold wash buffer. The precipitates were washed once with reaction buffer (50 mM HEPES [pH 7.5], 20 mM MgCl2, 0.1 μg of bovine serum albumin per ml) followed by two washes with wash buffer containing 15 μM of either γ-32P-ATP (3 pmol), 50 μM ATP, and 5 μg of BSA (Sigma). In addition, either 1 μM bovine CaM (Sigma) plus 0.5 mM CaCl2 or 3 mM EGTA was added as indicated. Protein sample buffer was added to terminate the reaction, and after boiling, the proteins were analyzed on an SDS–10% polyacrylamide gel. The gel was stained with Coomassie blue, dehydrated, and the nucleic acid dye Oligreen (dilution of 1:200; Jackson Immunoresearch Laboratories) and the nucleic acid dye Oligreen (dilution of 1:5,000; Molecular Probes) used for nuclear staining were then applied. The gels were exposed to Hyperfilm-MP (Amersham) for 2 days and then dried. For further analysis, bands were excised from the gel, and the proteins were extracted from soluble fractions and equivalent volumes of insoluble fractions were loaded into SDS–10% polyacrylamide gels and resolved by electrophoresis (PAGE). Western blot analysis was then performed with monoclonal anti-FLAG antibodies (dilution of 1:200; IBI, Kodak).

Cell lines, transfactions, and apoptotic assays. All cell lines were grown in Dulbecco modified Eagle medium (Biological Industries) supplemented with 10% fetal calf serum (Bio-Lab). For transient transfection, 105 cells were seeded per well in a six-well plate a day before transfection. Transfections were done by the calcium phosphate method or by using SuperFect transfection reagent (Qiagen). For cell death assays by overexpression, a mixture containing 1.5 μg of cell death-inducing plasmid (pCDNA3 expressing the different DRP-1 constructs or mutants) and 1 μg of plasmid expressing either ZIP kinase or DAPk [see below (30)] was used. Nuclear staining of 293 cells transfected by the DRP-1 ΔT5 mutant was done 60 h after transfection, using Hoechst dye (2 μg/ml; Molecular Probes). For the cell death protection assays in Fig. 8B, the mixture consisted of 1.2 μg of DRP-1, 0.5 μg of a plasmid to be tested for cell death prevention (expressing the DAP kinase death domain [DAPk-DD]), DN [dominant negative] FADD, or luciferase as a negative control), and 0.5 μg of plasmid pEGFP-N1. For cell death protection assays in Fig. 8C, the mixture consisted of 1.5 μg of cell death-inducing plasmid (pCDNA3 expressing either ZIP kinase or DAPk) and 0.5 μg of plasmid pEGFP-N1 (expressing either ZIP kinase or DAPk) was used. Nuclear staining of 293 cells transfected by the DRP-1 ΔT5 mutant was done 60 h after transfection, using Hoechst dye (2 μg/ml; Molecular Probes). For the cell death protection assays in Fig. 8D, the mixture consisted of 1.2 μg of DRP-1, 0.5 μg of a plasmid to be tested for cell death protection prevention (expressing the DAP kinase death domain [DAPk-DD]), DN [dominant negative] FADD, or luciferase as a negative control), and 0.5 μg of plasmid pEGFP-N1. For cell death protection from p55 TNFR shown in Fig. 5C, the mixture consisted of 0.1 μg of p55 TNFR, 1.6 μg of a plasmid to be tested for cell death protection (expressing DRP-1 ΔK42A or luciferase as a control), and 0.5 μg of plasmid pEGFP-N1. Cells were counted and photographed 24 h after transfection. For each transfection, at least three fields, each consisting of at least 100 green fluorescent protein (GFP)-positive cells, were scored for apoptotic cells according to their morphology. When indicated, cell lysates were prepared from the transient transfection at 24 h for protein analysis. Transfections of rat embryonic fibroblasts (REFs) and fluorescence-activated cell sorting (FACS) analysis of transfected fibroblasts for DNA content distribution were performed as previously described in detail (21).

Coimmunoprecipitation assays. 293 cells grown in 90-mm-diameter plates (105 cells/plate) were cotransfected with 5 μg of FLAG-tagged or HA-tagged DRP-1 and 20 μg of HA-tagged or FLAG-tagged RFX1ΔSmal (28) (deleted at amino acids 903 to 913), respectively, or with DRP-1-HA and DRP-1FLAG (5 μg of each). Immunoprecipitation of DRP-1 or RFX1ΔSmal from 1 mg of total extract was done with anti-FLAG M2 gel or Protein-G PLUS-agarose (Santa Cruz Biotechnology) conjugated to anti-HA antibodies. Detection of bound protein by Western blot analysis using antibodies (dilution of 1:10,000; BA040) or anti-FLAG antibodies. For the deletion mutant study, 5 μg of FLAG-tagged full-length DRP-1 was cotransfected with 5 μg of HA-tagged DRP-1 deletion mutants. Immunoprecipitation of DRP-1 from 1 mg of total extract was performed with anti-FLAG M2 gel. Detection of coimmunoprecipitated proteins (mutant or full-length DRP-1) was done with anti-HA antibodies.
CaM overlay assay. Transfections with the indicated HA-tagged proteins into 293 cells, preparation of cell lysates, immunoprecipitation, and immunoblotting were performed as previously described in detail (6). The overlay assay was performed as previously detailed (6). The membranes were preincubated for 1 h in CaM binding buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1 mM CaCl₂) containing 5% nonfat dry milk powder. Recombinant [³⁵S]-labeled CaM was added, and the membrane was subjected to gentle shaking at room temperature for 16 h, washed three times (5 min each) in CaM binding buffer, dried, and exposed to a phosphorimager. The various proteins were detected with anti-HA antibodies in a standard Western blot procedure.

FIG. 1. Sequence of the DRP-1 cDNA clone and alignments to related kinases. (A) Nucleotide and deduced amino acid sequences of human DRP-1. Initiation (ATG) and stop (TAA) codons are boxed. A polyadenylation signal (AATAAA) is underlined. The kinase domain and the CaM-regulatory regions are in bold and underlined by dashes, respectively. (B) Multiple sequence alignment of the serine/threonine kinase domains of the related proteins DAP kinase, ZIP kinase, DRP-1, and DRAK1 and 2. Alignment was done as described by Hanks and Quinn (14). Identical amino acids are boxed; homologous amino acids according to PAM250 matrix are shown in grey. (C) Phylogenetic rooted neighbor-joining tree of the 16 catalytic domains belonging to proteins closely related to DAP kinase. Numbers shown are bootstrap values. Confidence values lower than 50% are considered unreliable. CaMKIIα was used as a representative of other CaM kinases and was outgrouped to root the tree. smMLCK and skMLCK, smooth muscle and skeletal MLCK, respectively. (D) Multiple sequence alignment of the CaM-regulatory regions of DAP kinase, DRP-1, smMLCK, CaMKIIα, CaMKI, and CaMKIV. Alignment was done manually, keeping the conserved (boxed) regions aligned to each other. The corresponding region of ZIP kinase which does not contain homology to DAP kinase and DRP-1 CaM-regulatory regions is given at the bottom.
with refinements, using the ClustalX program. The phylogenetic tree is based on the neighbor-joining method (paupsearch program; Genetics Computer Group package version 9).

Nucleotide sequence accession numbers. The nucleotide sequence reported in this paper has been submitted to the GenBank/EBI data bank (accession no. AF052941). The murine DRP-1 is also deposited at the GenBank/EBI Data Bank (accession no. AF052942).

RESULTS

Cloning of DRP-1. To identify proteins that share homologous sequences with DAP kinase, we searched expressed sequence tag (EST) databases with the BLASTN program. Two ESTs from human and murine origin showed remarkable amino acid homology to the catalytic domains of DAP kinase and the recently identified protein ZIP kinase (79.5 and 80.2% identity, respectively). We performed a second EST search using the 5′ and the 3′ ends of the human EST and identified a few more overlapping ESTs. A putative novel cDNA sequence was generated and used to design primers for cloning the full-length cDNA. PCR performed on human spleen cDNA library amplified a 364-bp fragment that was further used to screen the same library. The full-length cDNA was then isolated, subcloned into BlueScript vector, and sequenced. The isolated cDNA is 1,742 bp long, coding for a protein which comprises 360 amino acids. The deduced amino acid sequence predicted a serine/threonine kinase domain with all of the 12 characterized subdomains present (14) (Fig. 1A).

Sequence alignment indicates that the catalytic domain of DRP-1 is 80% identical to that of DAP kinase and ZIP kinase yet less identical (50%) to the newly identified DRAK proteins (Fig. 1B). We performed a multiple sequence alignment of 18 proteins that show high scores of homology to the DAP kinase catalytic domain (not shown). This alignment classifies a novel protein subfamily composed of DAP kinase, ZIP kinase, DRP-1, and the Caenorhabditis elegans DAP kinase (cDAPk). The DRAK proteins form another closely related group. Local high-homology segments unique to this subfamily are the SRRGV loop located between αB and αC and two amino acids (PR or PH) appearing in β8. Phylogenetic analyses, based on the multiple sequence alignment of the catalytic domains and
Intrinsic kinase activity of DRP-1. To test whether DRP-1 functions as a kinase, as predicted from the amino acid sequence, we performed an in vitro kinase assay using MLC as an exogenous substrate. This substrate was chosen since it is phosphorylated by MLC kinase (MLCK), DRP-1 has the highest kinase activity in the presence or absence of Ca$^{2+}$/CaM as described in Materials and Methods. The proteins were resolved by SDS-PAGE on an 11% gel and blotted to a nitrocellulose membrane. Top, autophosphorylation of DRP-1 (42 kDa) and MLC phosphorylation (17 kDa), respectively, after exposure to X-ray film; bottom, DRP-1 proteins after incubation of the same blot with anti-FLAG epitope; Fig. 2D, compare lanes 1 to 2). The other, higher bands, which reacted with the anti-DRP-1 antibodies, turned out to be nonspecific, since only the 42-kDa protein could be immunoprecipitated by the antibodies (Fig. 2E, lane 1). Cellular localization of ectopically expressed DRP-1. To determine the cellular localization of the exogenous DRP-1, we expressed the FLAG-tagged DRP-1 cDNA in COS-7 cells. Immunoblot analysis showed that DRP-1 is expressed in these cells (not shown). For the immunostaining procedure, nontransfected and DRP-1-transfected COS-7 cells were fixed and reacted both with Oligreen for nuclear staining and with anti-FLAG antibodies for DRP-1 staining. Specific DRP-1 staining was detected in the cytoplasm of these cells (Fig. 3A). We then performed a gentle cell extraction with nonionic detergent (0.5% Triton X-100) that removes lipids and soluble proteins, leaving intact the detergent-insoluble matrix composed of the nucleus, the cytoskeleton framework, and cytoskeleton-associated proteins. In contrast to the ectopically expressed DAP kinase, which is exclusively localized to the cytoskeleton and hence found only in detergent-insoluble fractions (6) (Fig. 3B), DRP-1 was preferentially eluted from the detergent-soluble fraction, with only a small amount remaining in the insoluble fraction (Fig. 3B). Thus, we conclude that ectopically expressed DRP-1 is a soluble, cytoplasmic protein with minor association with insoluble matrix components.
mixture increased the amount of phosphorylated MLC, suggesting that the enzyme is regulated by binding to CaM (Fig. 4A and C). Indeed, the full-length DRP-1 could bind directly CaM, as assessed by incubating membranes containing the immunoprecipitated protein with labeled CaM (Fig. 4B). Truncation of the last 73 amino acids, a stretch which includes the 33 amino acids of the CaM-regulatory domain (Δ73 mutant), abolished CaM binding (Fig. 4B) and converted the enzyme to a constitutively active form, fully functional in the absence of externally added Ca²⁺/CaM and in the presence of EGTA (Fig. 4C). This gain of function in the catalytic activity is in accordance with the assumption that, like DAP kinase, DRP-1 is negatively regulated by the autoinhibitory CaM-binding domain and that this inhibition is removed by the binding of Ca²⁺/CaM. A catalytically inactive mutant of DRP-1 (DRP-1 K42A), did not phosphorylate MLC and failed to undergo autophosphorylation even though higher amounts of DRP-1 protein were present (Fig. 4A). Thus, DRP-1 functions in vitro as a kinase that is capable of phosphorylating itself and an external substrate; the latter property is stimulated by the addition of Ca²⁺ and CaM.

**DRP-1 induces apoptosis in a variety of cell lines.** The high homology to DAP kinase in the kinase and CaM-binding regions prompted us to check whether DRP-1 is involved in apoptosis. The wild-type DRP-1 and the catalytic inactive mutant of DRP-1 (DRP-1 K42A), each cloned in pCDNA3 vec-
tor, were transfected into 293 cells. To quantitate the number of apoptotic cells, we cotransfected these constructs with a vector expressing GFP. The latter was used as a marker to visualize the transfected cells and to assess the apoptotic frequency among the transfectants according to morphological alterations. Apoptotic cells were scored after 24 h. Overexpression of DRP-1 resulted in apoptotic cell death (50 to 60%), compared to the basal level of apoptotic cells caused by transfection of a nonrelevant (luciferase) gene (Fig. 5A and C). The first and very prominent morphological changes occurred at the membrane level, since a major fraction of the GFP-positive green cells showed cytoplasmic blebbing (26) (Fig. 5A-3). In addition, some of the transfected cells detached from the plate. DNA staining by Hoechst was used to monitor the status of the nucleus in the DRP-1-transfected cells, at 60 h posttransfection. Most of the GFP-positive cells displayed condensed nuclei; some of the nuclei appeared fragmented (Fig. 5B). In these experiments, the activated DAP kinase mutant lacking the autoinhibitory CaM-regulatory region (DAPkΔCaM) yielded apoptotic values of 70 to 80% (Fig. 5C). In contrast, when these cells were transfected with the kinase-inactive mutant of DRP-1 (DRP-1 K42A; Fig. 5A-4 and C), no apoptosis was observed. Western blot analysis of transfected cells with anti-FLAG antibodies confirmed the expression of both the exogenous wild-type and K42A mutant versions of DRP-1 (Fig. 5D). Similar results were observed in human SV-80 fibroblasts (not shown).

The effect of ectopically expressed DRP-1 on the DNA content of primary REFs was also assessed, as previously described in detail (21). REFs were cotransfected with DRP-1 and a membrane-bound form of GFP and after 48 h subjected to FACS analysis of their DNA content. A fraction of cells displaying a sub-G1 DNA content (27%), indicative of cells containing fragmented DNA, appeared exclusively in the DRP-1-transfected cells, not in cells transfected with a control vector (7%) or with the DRP-1 K42A mutant (9%). No effect on cell cycle distribution of the viable cells was found (not shown).

Deletion of the C-terminal tail of DRP-1 abolishes its apoptotic activity, while further truncation of the CaM-regulatory region strongly enhances the apoptotic effect. To further understand the mode of DRP-1 action in apoptosis, we generated constructs containing C-terminal truncations of DRP-1 tagged by HA (Fig. 6A). DRP-1Δ40 lacks the most C-terminal part of DRP-1, which displays no homology to any known protein. DRP-1Δ73 lacks, in addition to that, the CaM-regulatory region of DRP-1, and DRP-1Δ85 contains only the catalytic domain. The wild-type DRP-1 and the various truncation mutants of DRP-1 were transfected into 293 cells at comparable amounts (see the legend to Fig. 6). Induction of apoptotic cell death was assessed by scoring GFP-positive cells. Overexpression of the wild-type DRP-1 in these experiments resulted in apoptosis (25%), while DRP-1Δ40 had no effect in these assays. On the other hand, further truncations of the CaM-regulatory region yielded mutants (Δ73 and Δ85) which acted...
as “superkillers” (~90% apoptosis) (Fig. 6B and C). Western blot analysis of transfected cells with anti-HA antibodies confirmed the expression of all DRP-1 forms (Fig. 6D). These experiments support the finding that the apoptotic effect of DRP-1 is dependent on its kinase activity, since as shown in Fig. 4, removal of the autoinhibitory CaM-regulatory region generates a constitutively active kinase. In addition, these experiments revealed the existence of a positive module in the C-terminal region of DRP-1, which is necessary for its pro-apoptotic effect, provided that the CaM-regulatory region is still present. In the absence of the CaM-regulatory region, the C-terminal tail becomes dispensable.

**The C-terminal part of DRP-1 functions as a homodimerization domain.** Western analysis performed on proteins extracted from 293 cells transfected with FLAG-tagged DRP-1 revealed, in some cases, an additional band of approximately 85 kDa (not shown). This observation led us to test whether DRP-1 can undergo homodimerization. To this end, we cotransfected two constructs expressing DRP-1 fused to either a FLAG or an HA tag into 293 cells and performed classical pull-down experiments with each of the two epitopes. FLAG-tagged DRP-1 was shown to bind specifically HA-tagged DRP-1 in both immunoprecipitation directions (Fig. 7A, lanes 3 in both IP panels). No binding of DRP-1-HA to FLAG beads or to the irrelevant cytoplasmic protein RFXΔSmaI (28) could be observed (Fig. 7A, IP anti-FLAG panel, lane 2 or lanes 1 and 2, respectively). Also, we could not detect nonspecific binding of DRP-1-FLAG to HA beads or to RFXΔSmaI protein (Fig. 7A, IP anti-HA panel, lane 1 or lanes 1 and 2, respectively). Western analysis confirmed the expression of all proteins in these cell extracts (Fig. 7A, Western panels).

Next we tried to map the domain which may be required for the homodimerization of DRP-1. To this end, DRP-1-FLAG was expressed in tandem with the various deletion mutants of DRP-1 tagged by HA. We could detect strong binding of DRP-1-FLAG to wild-type DRP-1-HA, whereas binding to DRP-1Δ40, Δ73, and Δ85 was minimal or undetectable (Fig. 7B, upper IP panel, compare lane 1 to lanes 2 to 4). Western analysis confirmed the expression of wild-type DRP-1-HA and all other DRP-1-HA deletion mutants in these transfections (Fig. 7B, Western panel). The lower IP panel depicts the presence of wild-type DRP-1-FLAG in all these immunoprecipitates. Thus, we concluded that a region spanning the C-terminal 40 amino acids of DRP-1 is required for its homodimerization. This homodimerization is probably required for the apoptotic effect of DRP-1, since DRP-1Δ40 lost the ability to induce apoptosis in 293 cells (Fig. 6B and C).

**DAP kinase death domain protects from DRP-1-induced apoptosis.** The sequence homology between DRP-1 and DAP kinase within the catalytic domain, the common regulation by Ca2+/calmodulin, and the finding that both proteins induced apoptosis upon overexpression raised the possibility that they...
function along a common apoptotic pathway. To assess a possible functional cross talk between the two kinases, dominant negative mutants derived from each of the two kinases were used. We first tested whether a fragment of DAP kinase encompassing the death domain (DAPk DD) affected DRP-1-induced cell death. This fragment of DAP kinase was previously shown to act as a specific dominant negative mutant, negating the effects of the full-length protein when ectopically expressed (7). Interestingly, we found by cotransfection experiments that DAPk DD protected 293 cells from cell death induced by DRP-1 (Fig. 8B). A control transfection including DRP-1 and a nonrelevant luciferase DNA excluded the possibility that inhibition is simply due to larger amount of DNA used in the transfection. Moreover, the effect of DAPk DD was specific, since the death domain of FADD (also named DN FADD) failed to manifest a similar effect (Fig. 8B). Western blot analysis of transfected cells using anti-FLAG antibodies confirmed the expression of the exogenous DRP-1 in all transfections (Fig. 8B).

In the reciprocal experiment, the catalytically inactive DRP-1 K42A mutant (FLAG tagged) was assayed in cotransfection experiments, assuming that it will function in a dominant negative manner. To test this possibility, the mutant was first cotransfected with the wild-type DRP-1 (HA tagged) and was found to confer protection against the apoptotic effects of the wild-type protein without affecting its expression levels (Fig. 8C). The latter observation attributed to this mutant a moderate yet significant neutralizing function against wild-type DRP-1. Nevertheless, when cells were killed by DAP kinase, the extent of protection which was conveyed by DRP-1 K42A
was significantly less despite the fact that the direct target, i.e., the endogenous DRP-1, is expressed at lower levels than the recombinant DRP-1 in the previous experiments. In contrast, the same mutant was effective in protecting 293 cells from cell death induced by another stimulus—the ectopically expressed p55 TNFR (Fig. 8C; the death domain of FADD, which is a potent blocker of TNF signaling at the receptor level, served as a positive control). Together, the cotransfection experiments suggest that in certain genetic constellations, the death-promoting effects of DRP-1 may depend on active DAP kinase whereas a major yet not exclusive molecular arm emanating from DAP kinase is refractory to DRP-1 inactivation.

**DISCUSSION**

In this study we describe the cloning and characterization of a novel serine/threonine kinase with remarkable homology to the catalytic and CaM-regulatory domains of DAP kinase. This kinase, named DRP-1, is a 42-kDa cytoplasmic protein which when ectopically expressed exhibits minor associations with insoluble matrix elements. Another protein, ZIP kinase, which by virtue of its sequence homology to the kinase domain of DAP kinase is also a member of the DAP kinase-related protein subfamily, was recently identified (17, 22). Unlike DAP kinase and DRP-1, ZIP kinase is a nuclear protein which, instead of being regulated by a CaM-binding domain, is activated only by homodimerization via its leucine zipper motifs (17). ZIP kinase-induced cell death is controlled by its ability to undergo homodimerization. To this group of kinases, another two less homologous nuclear proteins, DRAK1 and DRAK2, were recently added (29). Together they form a novel subfamily of serine/threonine kinases, as is evident from multiple sequence and phylogenetic analyses.

To check the cellular functions of DRP-1, we overexpressed wild-type DRP-1 in various cell lines and found that it induced apoptosis as measured by various parameters. Unlike the wild-type DRP-1, a kinase-inactive mutant of DRP-1 (DRP-1 K42A) did not induce apoptosis, although it was expressed at a similar level in the transfected cells. In vitro kinase assays confirmed that DRP-1 K42A is indeed unable to phosphorylate the MLC substrate. Such dependence on the catalytic activity for apoptotic function is apparent also in the other members of DAP kinase-related proteins (17, 29). In addition, a truncated form of DRP-1 which lacks the CaM-regulatory region displayed a constitutively active kinase and induced very high levels of apoptosis, thus further confirming the dependence of apoptosis on the overall catalytic activity.

The deletion mutant study presented here confirms the existence of yet another module responsible for apoptotic induction, which is located at the C-terminal part of DRP-1. This part of DRP-1 is also essential for its dimerization. Thus, we can conclude that homodimerization is a requirement for the functionality of this kinase in apoptosis, although this property can be completely overridden by a further deletion of the CaM-regulatory region. It is presently not clear how the dimerization influences the death-promoting effects of DRP-1 and whether self-dimerization has an impact on the catalytic activity. Another challenging question is why the C-terminal tail is functionally required only when the CaM-regulatory domain is present. So far, the conventional conditions of in vitro phosphorylation assays have not resolved the issue (not shown), and it is clear that some fine-tuning of the biochemical assessments is required.

The high homology in the kinase domains of DAP kinase and DRP-1 and the finding that they are both localized to the cytoplasm (in either soluble or insoluble form) imply that they may use the same or closely related substrates. The phosphorylation sites for these kinases on the substrate may be either different or identical. Thus, these kinases may cooperate to induce apoptosis in the same cell type or, alternatively, may function independently in different cell types, tissues, or organs in response to different stimuli or in different time frames. Another possibility is that these kinases act sequentially along the same signaling pathway to induce apoptosis. Here we provide the first observations that support the assumption that these kinases may be functionally linked to each other in some constellations. This is illustrated by the ability of a dominant negative form of DAP kinase (DPk DD) to block apoptosis induced by DRP-1. Also the finding that both, DAP kinase (7) and DRP-1 (Fig. 8C) mediate killing by TNF is consistent with this scenario. These results indicate the need for a long-term study to establish whether direct or indirect interactions exist between DAP kinase and DRP-1. It should be mentioned that in the reciprocal approach, the effect of the dominant negative DRP-1 on DAP kinase was much less pronounced. A simple interpretation of these data would be to place DRP-1 upstream to DAP kinase; however, a definitive conclusion still awaits detailed biochemical data on the nature of the cross talk between these two kinases. Finally, it will be of interest to study whether DRP-1, like DAP kinase, acts as a tumor suppressor gene, subjected to loss of function in human tumors.

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The first two authors contributed equally to this work.

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**ADDENDUM IN PROOF**

After the submission of the manuscript, a work describing some initial characteristics of human and mouse DAP-1 homologues was published (T. Kawai, F. Nomura, K. Hoshino, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, and S. Akira, Oncogene 18:3471–3480, 1999).

**REFERENCES**


