Functional Role of Death-associated Protein 3 (DAP3) in Anoikis*

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Detachment of adherent epithelial cells from the extracellular matrix induces anoikis, known as anoikis. Integrin stimulation protects cells from anoikis, but the responsible mechanisms are not well known. Here, we demonstrated that a pro-apoptotic GTP-binding protein, DAP3 (death-associated protein 3), is critical for induction of anoikis. Down-regulation of DAP3 expression by antisense oligonucleotides inhibited anoikis. Conversely, overexpression of DAP3 augmented cell death and caspase activation induced by cell detachment. Furthermore, the association of DAP3 with FADD and the activation of caspase-8 were induced by cell detachment. We also showed that DAP3 is phosphorylated by kinase Akt (PKB), and active Akt can nullify apoptosis induction by DAP3. Mutation of a consensus Akt phosphorylation site in DAP3 renders it resistant to suppression by active Akt in cells. Integrin ligation stimulates Akt activation and phosphorylation of DAP3 in intact cells, as well as suppresses the ability of DAP3 overexpression to augment anoikis. Involvement of DAP3 in anoikis signaling demonstrates a novel role for this GTP-binding protein in apoptosis induction caused by cell detachment.

The process of apoptosis is critical for the development and maintenance of all multicellular metazoans. Dysregulated apoptosis is germane to many human diseases, including cancer, immunodeficiency, and neurodegeneration. At least two major pathways for apoptosis induction have been identified, commonly known as the “extrinsic” and “intrinsic” pathways (reviewed in Refs. 1 and 2). The extrinsic pathway is triggered by cytokines of the tumor necrosis factor (TNF) family and in- volves the adapter protein FADD (Fas-associated death domain), which recruits pro-caspases-8 and -10 to death receptor complexes, resulting in activation of these cell death proteases and initiation of the apoptosis process. The intrinsic pathway, in contrast, is triggered by myriad stimuli that impinge on mitochondria, causing these organelles to release apoptogenic proteins into the cytosol, such as cytochrome c, which binds the caspase-9 activator Apaf-1, initiating the apoptosis mechanism.

Death-associated protein 3 (DAP3) was identified as a pro-apoptotic protein during a functional screening based on tumor cell transfection with an antisense cDNA expression library and screening for rescue from cytokine-induced apoptosis (reviewed in Refs. 3 and 4). It was subsequently shown that DAP3 antisense protects tumor cells from death induced by certain cytokines, including TNFα, Fas ligand, and TNF-related apoptosis-inducing ligand (5). DAP3 is a protein of 46 kDa that carries a “P-loop” motif capable of binding GTP (6, 7). Mutagenesis studies suggest that GTP binding is critical for the pro-apoptotic function of DAP3 (7). Two intracellular pools of DAP3 have been identified, including a mitochondrial pool of DAP3 molecules, which undergoes proteolytic processing upon import into these organelles (8), and a cytosolic pool (9). Functional mapping of the apoptosis pathway pertinent to DAP3 by use of antisense and dominant negative mutants indicates that DAP3 is involved in the so-called extrinsic cell death pathway activated typically by TNFα, Fas ligand, TNF-related apoptosis-inducing ligand, and related TNF family death ligands and receptors (5, 7). However, it has also been shown recently that DAP3 is involved in the process of mitochondrial fragmentation during cell death and mitochondrial maintenance (10).

Anoikis is apoptosis that results from cell detachment from extracellular matrix, and it has been linked to the extrinsic pathway, typically involving FADD and caspase-8 (reviewed in Refs. 11 and 12). We provide evidence here that DAP3 is at least partially required for anoikis and that cell detachment induces DAP3 interaction with FADD, correlating with caspase-8 activation. Furthermore, we present evidence that DAP3 is a substrate of the kinase Akt (PKB), with Akt opposing the pro-apoptotic action of DAP3. The findings provide novel insights into the mechanisms responsible for anoikis regulation and thus may have relevance to pathophysiological situations where cell detachment from matrix is involved, such as tumor metastasis and wound healing.

MATERIALS AND METHODS

Plasmids and Antisense Oligodeoxynucleotides—The DAP3 mutants DAP3(T237A) and DAP3(T237E) were generated by polymerase chain reaction mutagenesis from a human DAP3 cDNA (a gift from A. Kimchi) and subcloned into pCDNA3-FLAG plasmid. The sequence of threonine 237 (ACA) was mutated to the sequence of alanine (GCA) for DAP3(T237A) or glutamate (GAA) for DAP3(T237E). DAP3(T237A) cDNA was cloned into pET21d-N vector for bacterial expression. Anti-sense (AS) oligodeoxynucleotides (ODNs) for human DAP3 were generated with the sequences: AS1, 5′-CATCATCATTGACGCTGA-3′ and AS2, 5′-TTCGACATCTCCCAGC-3′. Control sense oligodeoxynucleotides were synthesized with the sequence 5′-ATGACGCTGAGGAAT-3′. These oligodeoxynucleotides were transfected into HEK293 cells as reported previously (7).

Anoikis Assays—Anoikis assays were performed using established procedures (13). For cell viability assays or caspase-8 assays, cells were detached and maintained in suspension as described above in HEMA-coated culture plates. After 48 h, the cells were harvested, and the percentage of dead cells was assayed by trypan blue staining. Caspase-8
activity of the cell lysates was assayed by hydrolysis of the fluorogenic substrate Ac-IETD-AFC (Calbiochem, Inc.) as described in the next paragraph.

Caspase-8 Activity Assays—Caspase-8 activity was measured in the cell lysates as described by Miyazaki and Reed (7) at 37°C using a fluorometric plate reader (LS50B, PerkinElmer Life Sciences) in the kinetic mode, with excitation and emission wavelengths of 400 and 505 nm, respectively. Activity was measured by the release of 7-amino-4-trifluoromethyl-coumarin (AFC) (relative fluorescence units) from the synthetic substrate Ac-IETD-AFC (BD Biosciences) after 30 min of incubation.

Immunoprecipitation Assays—HEK293 cells (1 × 10⁶) in 10-cm plates were transiently transfected with 10 ng (total) of plasmid DNA. The cells were cultured in attached or detached (in suspension culture) conditions as described under “Anoikis Assays.” After 24 or 48 h, the cells were suspended in lysis buffer containing 0.1% Nonidet P-40, 20 mM Tris-HCl, pH 7.4, 2 mM MgCl₂, 1 mM EGTA, 130 mM NaCl, and protease inhibitors (Roche Applied Science). After preclearing with 10 μl of protein A-agarose or protein G-agarose, immunoprecipitations were performed using 10 μl of anti-FLAG antibody M2-conjugated-agarose (Sigma) or each antibody at 4°C for 4 h. After extensive washing by lysis buffer, the immune complexes were analyzed by SDS-PAGE or immunoblotting using the indicated antibodies followed by HRP-conjugated antibodies and detection using an ECL system (Amersham Biosciences).

In Vitro Kinase Assays—Plasmid of pCMV6-myrAkt-HA, pCMV6-Akt(E40K), or pCMV6-dnAkt was for the expression of each Akt mutant, Akt-myr (myristoylated), Akt-E40K, or Akt-DN (dominant negative), respectively (14). HEK293 T-cells (10⁵) were transiently transfected with 25 μg of plasmid DNA, normalizing the total DNA using the pCDNA3 (control) plasmid. After 24 h, the cells were lysed in 1.5 ml of 20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 1 mM Na₃VO₄, 1 mM EDTA, and protease inhibitors. After normalizing for protein concentration, the lysates were precleared with protein G-Sepharose and preimmune serum for 1 h and then incubated at 4°C with 0.5 μg of rat high affinity monoclonal antibody to hemagglutinin (HA) (Roche Applied Science) followed by the addition of 10 μl of protein G-Sepharose (Amersham Biosciences) for 1 h. The immunoprecipitates were washed three times in lysis solution and twice in kinase solution (20 mM Hepes, pH 7.2, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM dithiothreitol, and 3 μM ATP). In vitro kinase reactions were performed using these immunoprecipitates with γ-[³²P]ATP and purified His₆-tagged DAP3 (7) or His₆-tagged DAP3(T293A) substrate proteins. His₆-tagged proteins were expressed with NHE-terminal His₆ tags from pET-P4 in BL21 cells and affinity-purified with nickel-nitrilotriacetic acid spin column (Qiagen).

Antibody Production—A rabbit antisera to DAP3 phosphopeptides was raised against a purified peptide, CRVNR(A/T/DAVGIVY, in which threonine (T) was phosphorylated. The first cysteine (C) residue was bound to the maleimide-activated carrier proteins (Pierce) through its sulfhydryl group. The antisera was purified using a phosphopeptide-conjugated column.

RESULTS

DAP3 Is Required for Anoikis—To explore the requirement of DAP3 for anoikis, we used antisense oligonucleotide-based gene silencing. Two different AS-ODNs targeting the human DAP3 mRNA, AS1 and AS2, were effective at reducing levels of endogenous DAP3 protein when transfected into HEK293 cells, whereas various control ODNs (including a sense sequence) did not (Fig. 1A). We then tested the effects of transfected ODNs on cell death induced by cell detachment from matrix. AS-ODNs reduced cell death by approximately half compared with control untreated cells or sense ODN-treated cells (Fig. 1B). Thus, expression of endogenous DAP3 appears to be required, at least in part, for cell death induction by cell detachment.

Cell Detachment Promotes DAP3 Interaction with FADD—Previously we showed that DAP3 associates with FADD (7). We therefore explored the effects of cell detachment on the interaction of DAP3 with FADD using co-immunoprecipitation assays. In addition, we compared control-transfected cells with cells transfected with plasmids encoding constitutively active mutants of the protein kinase Akt because of its previously documented ability to suppress anoikis (15).

In attached cells, little endogenous DAP3 associated with endogenous FADD. In contrast, when cells were suspended, association of DAP3 and FADD increased, as determined by co-immunoprecipitation assay (Fig. 2A). In cells expressing the active forms of Akt, namely myristoylated Akt or Akt-E40K, less FADD was recovered with immunoprecipitated DAP3, suggesting that Akt suppresses the interaction of these proteins. Levels of FADD and DAP3 were not altered by either cell detachment or Akt activity, excluding differences in protein expression as a trivial explanation for these findings (Fig. 2A).

As expected by a previous report (16), cell detachment correlated with elevations in caspase-8 protease activity, as measured in cell lysates by a fluorogenic substrate, Ac-IETD-AFC (Fig. 2B). Akt suppressed caspase-8 activation induced by cell detachment, consistent with prior reports (16).

Akt Phosphorylates DAP3 and Regulates the Pro-apoptotic Activity of DAP3—The ability of Akt to suppress DAP3 association with FADD in suspended cells prompted us to explore the possibility that DAP3 might be a direct or indirect substrate of this protein kinase. Akt phosphorylates proteins on serine or threonine residues within the sequence motif RXRXX(S/T) (17). Interestingly, we found that DAP3 contains a site that con-

Fig. 1. Antisense-mediated gene silencing demonstrates a role for DAP3 in anoikis. A, HEK293 cells (1 × 10⁶) were transfected with two different DAP3-targeting antisense (AS) or a control sense ODN. After 48 h, the transfected cells were lysed, and immunoprecipitations (IP) were performed using anti-DAP3 (top panel) or anti-Akt (bottom panel) antibodies followed by SDS-PAGE and/or immunoblot analysis with anti-DAP3 or anti-Akt antibody and ECL-based detection. B, HEK293 cells (1 × 10⁶) were transfected with AS or sense ODN. After 48 h, the transfected cells were cultured in HEMA-coated dishes for 48 h. The percentage of dead cells was assayed by trypan blue staining (mean ± S.D., n = 3).
forms to the consensus Akt phosphorylation motif at Thr237 (RVRNAT).

To determine whether DAP3 is directly phosphorylated by Akt, active Akt was immunoprecipitated from HEK293T cells transfected with plasmids encoding Akt-myr or Akt-E40K, and in vitro kinase (IVK) assays were performed with γ[32P]ATP and recombinant purified His6-DAP3 as a candidate substrate (Fig. 3A). Incubation of immune complexes containing active forms of the kinase, Akt-myr or Akt-E40K, phosphorylated DAP3 in vitro. In contrast, His6-DAP3 was not phosphorylated in vitro when using immune complexes containing a kinase-dead mutant of Akt, previously shown to have dominant negative (DN) effects on endogenous Akt (Fig. 3A). By comparison, a variety of control kinases did not cause in vitro phosphorylation of DAP3, including JNK and Ask1 (data not shown). Thus, Akt or an Akt-associated kinase can directly phosphorylate DAP3 in vitro.

Next, to determine whether the predicted phosphorylation site in DAP3 is required for phosphorylation by Akt, we compared His6-tagged wild-type DAP3 with mutant DAP3, DAP3(T237A), and DAP3(T237E), which were produced in transfected HEK293 cells (1 × 10⁶ cells). After 24 h, the transfected cells were cultured in HEMA-coated dishes for 24 h (detached). The cells were recovered and lysed for immunoprecipitation (IP) with anti-DAP3 or anti-FADD antibody followed by SDS-PAGE and/or immunoblot analysis with anti-FADD or anti-DAP3 antibody. Alternatively, the lysates were normalized for protein content and loaded directly in gels for detection of HA-tagged Akt mutants by anti-HA antibody (bottom panel). B, control plasmid or plasmids (10 μg each sample) encoding for Akt mutants were transfected into HEK293 cells (1 × 10⁶ cells). After 24 h, the transfected cells were cultured in HEMA-coated dishes for 24 h. The cells were recovered and lysed, and caspase-8 activity was analyzed based on cleavage of Ac-IETD-AFC (100 μM final concentration) after normalization for total protein content. Data are expressed as relative fluorescence units (RFU) (mean ± S.D., n = 3).

Fig. 2. Detachment induces DAP3 association with FADD, which is blocked by Akt. A, plasmids (10 μg each sample) encoding Akt mutants were transfected into HEK293 cells (1 × 10⁶). After 24 h, the transfected cells were cultured in HEMA-coated dishes for 24 h (detached). The cells were recovered and lysed for immunoprecipitation (IP) with anti-DAP3 or anti-FADD antibody followed by SDS-PAGE and/or immunoblot analysis with anti-FADD or anti-DAP3 antibody. Alternatively, the lysates were normalized for protein content and loaded directly in gels for detection of HA-tagged Akt mutants by anti-HA antibody (bottom panel). B, control plasmid or plasmids (10 μg each sample) encoding for Akt mutants were transfected into HEK293 cells (1 × 10⁶ cells). After 24 h, the transfected cells were cultured in HEMA-coated dishes for 24 h. The cells were recovered and lysed, and caspase-8 activity was analyzed based on cleavage of Ac-IETD-AFC (100 μM final concentration) after normalization for total protein content. Data are expressed as relative fluorescence units (RFU) (mean ± S.D., n = 3).
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Using this validated phospho-specific antibody, we then evaluated the in vivo phosphorylation status of DAP3 in cells before and after detachment. As shown in Fig. 4E, cell detachment was associated with reduced phosphorylation of endogenous DAP3. The addition of the integrin ligand vitronectin to the detached cells increased DAP3 phosphorylation. Blotting of each sample with an antibody that reacts with DAP3 irrespec-
tive of phosphorylation confirmed the loading of equivalent amounts of DAP3 protein for all samples.

DISCUSSION

By manipulating the levels of DAP3 expression using anti-
sense and gene transfer methods, we uncovered a role for this pro-apoptotic protein in anoikis. Reducing endogenous DAP3
expression using antisense ODNs decreased apoptosis caused by cell detachment, whereas overexpressing DAP3 increased the percentage of cells undergoing apoptosis/time following detachment. Anoikis is thought to play a variety of important roles in normal development, ensuring that cells survive only when they reach their correct positions in the body, as well as in disease scenarios, such as tumor metastasis and wound healing at epithelial surfaces (reviewed in Refs. 20–22). Thus, a role for DAP3 in anoikis places this protein into a biological context of relevance to normal development and disease.

Previously, the DAP3 protein has been shown to have dual roles in normal mitochondrial physiology and cell death regulation, not unlike other apoptosis-relevant proteins such as cytochrome c, which also displays dual functions in normal cell physiology and cell death. An intramitochondrial pool of DAP3 apparently operates as a subunit of the mitochondrial ribosome, presumably involved in the translation of mitochondria genome-derived transcripts (23–25), whereas an extramitochondrial pool of DAP3 participates in apoptosis signaling within the extrinsic pathway (5–7). Unlike cytochrome c, however, there is no evidence thus far that DAP3 must be released from mitochondria to perform its cell death function (10). Rather, incomplete import of DAP3 into mitochondria seems to generate an extramitochondrial pool of this pro-apoptotic protein, the amount of which varies among cell types and cell lines (9).

A role for DAP3 in extrinsic pathway signaling was first demonstrated by antisense, gene transfer, and dominant negative experiments, where DAP3 was shown to modulate apoptosis induction by TNFα, TNF-related apoptosis-inducing ligand, and Fas ligand (5). Subsequent studies showed the physical interaction of DAP3 with FADD and other TNF family death receptor components, suggesting that this GTP-binding protein may participate in the assembly of so-called “death-inducing signaling complexes” that trigger caspase-8 activation (7). Anoikis has been shown to be dependent on the extrinsic pathway participants FADD and caspase-8 but not on components of the intrinsic pathway (16, 26). Thus, a role for DAP3 in anoikis is consistent with evidence that this pro-apoptotic protein participates in the extrinsic pathway.
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Akt is recognized for its role in anoikis suppression and anchorage-independent survival of transformed cells (reviewed in Refs. 11, 12, 28–32). The mechanism(s) by which Akt suppresses anoikis, however, has(have) been unclear, particularly given the dearth of targets of this kinase in the extrinsic pathway. We observed that the activity of Akt and phosphorylation of DAP3 modulate in concert in response to changes in cell attachment/detachment and in response to integrin ligands. Furthermore, gene transfer-mediated increases in Akt activity correlated with increased in vitro phosphorylation of DAP3, whereas Akt-DN reduced basal phosphorylation of DAP3. Finally, Akt-containing immune complexes were capable of inducing phosphorylation of recombinant DAP3 in vitro, suggesting that Akt or a kinase tightly associated with Akt directly phosphorylates DAP3. In this regard, DAP3 contains a sequence motif typical of Akt substrates, and mutation of the candidate phospho-acceptor site within this motif abolished phosphorylation of DAP3 in vitro by Akt and correlated with the reduced sensitivity of DAP3 to Akt-mediated apoptosis suppression. Conversely, a T237E phospho-mimic displayed reduced pro-apoptotic activity in transfection experiments, implying an important role for this motif in regulating DAP3 function. Taken together, these observations suggest that Akt-mediated phosphorylation of DAP3 on threonine 237 represents a potential mechanism by which Akt suppresses anoikis. Future studies will determine the range of cell types and pathophysiological settings in which this Akt-mediated mechanism for anoikis suppression prevail.

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