Protein Phosphatase 2A (PP2A) Holoenzymes Regulate Death-associated Protein Kinase (DAPK) in Ceramide-induced Anoikis

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The tumor suppressor, death-associated protein kinase (DAPK), is a Ca\textsuperscript{2+}/calmodulin-regulated Ser/Thr kinase with an important role in regulating cytoskeletal dynamics. Autophosphorylation within the calmodulin-binding domain at Ser-308 inhibits DAPK catalytic activity. Dephosphorylation of Ser-308 by a previously unknown phosphatase enhances kinase activity and proteasome-mediated degradation of DAPK. In these studies, we identified two holoenzyme forms of protein phosphatase 2A (PP2A), AB\textsubscript{b}C and AB\textsubscript{c}C, as DAPK-interacting proteins. These phosphatase holoenzymes dephosphorylate DAPK at Ser-308 in vitro and in vivo resulting in enhanced kinase activity of DAPK. The enzymatic activity of PP2A also negatively regulates DAPK levels by enhancing proteasome-mediated degradation of the kinase. Overexpression of wild type DAPK induces cell rounding and detachment in HEK293 cells; however, this effect is not observed following expression of an inactive DAPK S308E mutant. Finally, activation of DAPK by PP2A was found to be required for ceramide-induced anoikis. Together, our results provide a mechanism by which PP2A and DAPK activities control cell adhesion and anoikis.

Death-associated protein kinase (DAPK)\textsuperscript{3} is a Ca\textsuperscript{2+}/CaM-dependent Ser/Thr kinase that regulates multiple signaling pathways, including cell apoptosis, autophagy, survival, motility, and adhesion (1–5). DAPK functions as a positive mediator of apoptosis induced by a variety of stimuli, including interferon γ, transforming growth factor β, ceramide, and the oncoproteins c-myc and p53 (for a review see Ref. 1), and as negative mediator of apoptosis induced by tumor necrosis factor α (3, 6). Forced expression of DAPK results in morphological changes, including cell rounding, shrinking, detachment, and anoikis in multiple cell types (4, 5). In animal studies, the expression level of DAPK was inversely correlated with the metastatic potential of tumors and reintroduction of DAPK into the metastatic tumor-initiated anoikis (7). DAPK has also been suggested to be a tumor suppressor, and in human cancers, DNA methylation within the promoter of DAPK is a frequent event and strongly correlates with the rates of recurrence and metastasis (1). Loss or reduced expression of DAPK underlies the cases of heritable predisposition to chronic lymphocytic leukemia and the majority of sporadic chronic lymphocytic leukemia (8). Recent studies focusing on the post-transcriptional control of DAPK have identified a complex network regulating the protein levels of DAPK. Translation repression of DAPK occurs by the interferon-γ-activated inhibitor of translation complex (9). Post-translational control of DAPK protein levels is regulated by at least two distinct E3 ubiquitin ligases, C-terminal HSC70-interacting protein E3 ubiquitin ligase (CHIP) (10) and Mind bomb 1 (Mib1) (11), which polyubiquitinate DAPK resulting in proteasomal degradation. In addition, the lysosomal protease cathepsin B (6) negatively regulates protein levels of DAPK. Finally, a small alternatively spliced form of DAPK was shown to cause decreased stability of full-length DAPK independent of the proteasome or lysosome (12).

The catalytic activity of DAPK is regulated by Ca\textsuperscript{2+}/CaM and by autophosphorylation of Ser-308, which resides within the calmodulin-binding domain (3, 13). Autophosphorylation of Ser-308 prevents calmodulin binding, which is necessary for the kinase activity of DAPK; thus, Ser-308 phosphorylation negatively regulates DAPK activity (3, 13). Despite the obvious importance associated with dephosphorylation of Ser-308, the phosphatase that dephosphorylates this site has not been extensively characterized. The unphosphorylated, active form of DAPK is rapidly ubiquitinated and degraded by the proteasome (3), thereby providing an additional mechanism to limit DAPK activity. Thus, the Ser-308 phosphatase not only controls DAPK activation but also DAPK levels. Recent evidence has suggested that a PP2A-like phosphatase may control Ser-308 phosphorylation; however, the specific holoenzyme form(s) of PP2A involved in this event is unknown (14). PP2A is a major Ser/Thr protein phosphatase that regulates numerous cellular processes, including proliferation, differen-

\[\text{The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.}\]

\[\text{The abbreviations used are: DAPK, death-associated protein kinase; PP2A, protein phosphatase 2A; Ca\textsuperscript{2+}/CaM, calcium/calmodulin; PARP, poly-ADP-ribose polymerase; z-VAD, carbobenzoxy-valyl-alanyl-aspartyl; fmk, fluoromethyl ketone; PIPES, 1,4-piperazinediethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; siRNA, short interfering RNA; shRNA, short hairpin RNA; EV, empty vector; OA, okadaic acid; HEK, human embryonic kidney; WT, wild type.}\]

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- DAPK has been shown to suppress cell adhesion and migration (3–5, 13, 19). In addition, activation of the regulatory light chain at Ser-19, to regulate cytoskeletal dynamics, is necessary for ceramide-induced cell death in multiple cell types. The activation of DAPK by ceramide is thought to be mediated by a ceramide-activated phosphatase; however, the identity of this phosphatase remains uncertain (14). Once active, DAPK phosphorylates substrates, including myosin regulatory light chain at Ser-19, to regulate cytoskeletal dynamics, cell adhesion, and migration (3–5, 13, 19). In addition, activation of DAPK has been shown to suppress β integrin-mediated cell adhesion through disrupting the association of Talin and CDC42 (4).

- Although a PP2A-like enzyme was recently identified as the putative phosphatase that dephosphorylates Ser(P)-308 in DAPK (14), additional studies are needed to determine whether PP2A is indeed responsible for dephosphorylation of Ser(P)-308 and, if so, to identify the unique holoenzyme(s) involved. In a screen to identify potential DAPK-binding partners, we identified the PP2A regulatory Bo subunit as a candidate DAPK-binding partner. Additional experiments indicated that the highly homologous Bβ regulatory subunit also associates with DAPK. Furthermore, we determined that Bα- and Bβ-containing holoenzymes control whether the stability and activity of DAPK via their interaction with DAPK and their dephosphorylation of Ser(P)-308. Overexpression of a DAPK mutant that cannot be phosphorylated, DAPK S308A, induced cell detachment, whereas overexpression of the phosphomimetic, DAPK S308E, completely abrogated the ability of DAPK to promote loss of adhesion. Finally, we show that ceramide-treated HeLa cells required activation of DAPK by PP2A to undergo anoikis. Collectively, these data indicate a complex regulation of the kinase activity and protein turnover of DAPK by PP2A holoenzymes, AβαC and ABδC.

**MATERIALS AND METHODS**

**Reagents**—MG132, chloroquine, doxycycline, protease inhibitor mixture, phosphatase inhibitor mixture-1, N-hexanoyl-D-sphingosine (C₆-ceramide), FLAG peptide (DYKDDDK), anti-FLAG M2-agarose, and Proteosilver silver stain kit were from Sigma. Okadaic acid (OA), microcystin-LR, and microcystin-LW were from EMD (Gibbstown, NJ). Microcystin-agarose was from Millipore (Billerica, MA). FTY720 was from Alexis Biochemical (San Diego). Absolute quantitative PCR mixtures were from ABgene (Rockford, IL). FuGENE 6 transfection reagent was purchased from Roche Diagnostics. Lipofectamine 2000 siRNA transfection reagent was from Invitrogen. DAPK substrate peptide was from Tocris (Ellisville, MO). PP2A immunoprecipitation phosphatase assay kit was from Millipore (Temecula, CA). [γ-32P]ATP was from MP Biomedicals, Inc. (Irvine, CA). Z-VAD-fmk was from BD Biosciences. Recombinant adenoviruses were produced at ViraQuest Inc. (North Liberty, IA).

**Antibodies**—Antibodies to DAPK (DAPK55), Ser(P)-308 DAPK, FLAG M2, and vinculin antibodies were purchased from Sigma. Anti-DAPK (DAP-3) was from BD Biosciences. PP2A catalytic C, structural A, and regulatory Bo subunit antibodies were from Cell Signaling (Beverly, MA). The generation and characterization of affinity-purified Bo/Bβ antibody were as reported previously (20). Anti-Omni probe (D-8) and anti-PARP were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). TrueBlotTM anti-mouse secondary antibody was from eBioscience (San Diego).

**Plasmids**—The expression vector for human DAPK, pcDNA3-DAPK, was kindly provided by Dr. Adi Kimchi (Weizmann Institute of Science, Israel). Ser-308 mutant constructs were generated using site-directed mutagenesis (Stratagene) with mutated oligonucleotide primers corresponding to mutation sites. Domain expression plasmids of DAPK containing either the ankyrin repeats (amino acids 372–628), cytoskeletal (amino acids 628–1215), or death domain tail (amino acid 1216–1457) were generated by PCR and ligation into the pcDNA3.1/His expression vector. Doxycycline-inducible expression plasmids for the regulatory B subunits, Bo-, Bδ-, and Bβ- pcDNA5/TO, and empty vector (EV) (pcDNA5/TO) were described previously (16).

**Tissue Culture and Transient Transfection**—Human embryonic kidney (HEK) T-Rex cell lines harboring pcDNA5/TO (EV), pcDNA5/TO-Bo-FLAG, or pcDNA5/TO-Bδ-FLAG were generated previously (16). Expression of Bo-FLAG and Bδ-FLAG was accomplished by treating with 2 μg/ml doxycycline for 48 h at 37 °C as described previously (16). HeLa and HEK293 cells were obtained from ATCC (Manassas, VA). HEK T-Rex, HeLa, and HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Transient transfection of HeLa and HEK293 cells was carried out using equal plasmid DNA (adjusted with the corresponding empty vectors) together with FuGENE 6 transfection reagent according to the manufacturer’s guidelines. Short interfering RNAs (siRNAs) for Bo were obtained from Dharmacon (target sequence 5’-UGAUAGAUAGGAUCUCUAUAC-3’) as well as a SMARTpool for Bβ. Nontargeting siRNAs were purchased from Dharmacon and used as negative control. Lipofectamine 2000 was used for the siRNA transfection.

**Western Blotting and Immunoprecipitation**—Western blotting and immunoprecipitation were performed as described previously (21). Cell extracts were prepared in a lysis buffer containing 0.1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 m NaCl, 10 mM sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM sodium fluoride, protease inhibitor mixture, and phosphatase inhibitor mixtures, including microcystin LR, cantharidin, (−)-p-bromotetramisole, and OA (Sigma phosphatase inhibitor mixture-1), where appropriate. For immunoprecipitation, lysates were prepared in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.1% Nonidet P-40, 1% Triton X-100, 10% glycerol, 1 mM EGTA, 1 mM EDTA, 0.15 m NaCl, 10 mM sodium fluoride,
2 mM sodium vanadate and protease inhibitors). Cell lysates were clarified by centrifugation, and the supernatant was pre-cleared by incubation with Trueblot IgG (eBioscience) beads. For each immunoprecipitation, 1-ml aliquots of lysates (1 mg of protein) were incubated with 4–8 μg of DAP-3 antibody at 4 °C for 3 h. The immune complexes were then isolated by the addition of 40 μl of protein G beads and incubation for 2 h. FLAG-tagged proteins were isolated by incubating 1-ml aliquots of lysates (1 mg of protein) with 40 μl of a 50% slurry of anti-FLAG-agarose at 4 °C for 3 h. Immune complexes were washed three times with lysis buffer to reduce nonspecific binding. The immune complexes were resolved by electrophoresis and analyzed by Western blotting.

Microcystin-agarose Affinity Isolation—Isolation of endogenous forms of PP2A with microcystin-agarose was performed as described previously (22). Briefly, HEK293 cell lines were lysed in buffer containing (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, and protease inhibitor mixture). Soluble lysates (2 mg) were preincubated with DMSO or 5 μM microcystin-LR for 1 h at 4°C. Microcystin-agarose (25 μl of 1:1 slurry) was added to lysates and incubated overnight at 4 °C. The beads were washed six times with lysis buffer and analyzed by Western blotting.

Reverse Transcription-PCR—RNA was extracted from HEK T-Rex and HeLa cells with TRIzol reagent (Invitrogen), and 0.5 μg of RNA was used as template for reverse transcription using Superscript first strand cDNA synthesis kit (Invitrogen). The resulting cDNAs were resuspended in 20 μl of H2O. The cDNA levels of specific genes were measured by quantitative real time PCR using Absolute quantitative PCR mixtures (ABgene) and an ABI 7500 real time PCR system (Applied Biosystems). The gene-specific primers used for quantitative PCR mixtures were as follows: sense hHPRT1, 5′-CCT TGG TCA GCC AGT ATA ATC CA-3′, and antisense hHPRT1, 5′-GGT CCT TTT CAC CAGCAA GCT-3′; sense hDAPK1, 5′-CCC GGA AAA AAA TGG AAA CAA-3′, and antisense, hDAPK1 5′-TGG ACA GGA GTA ACC TGG ATA AT-3′; sense hPPP2R2A (Bo), 5′-AGC AGG GTC ACC ATT TGC-3′, and antisense hPPP2R2A (Bo), 5′-ACC TGA GAA AAA CAC CAC TGA AT-3′; and sense hPPP2R2D (Bb), 5′-TGG CAC TTA GAA ATC ACA GAT AGA A-3′, and antisense hPPP2R2D (Bb), 5′-AAC TCG GCT GCA GTG ATG A-3′. All samples were amplified in duplicate, and every experiment was repeated independently at least two times. Relative gene expression was calculated using the 2−ΔΔCt method against the internal control HPRT1 housekeeping gene as described previously (23).

Tandem Affinity Purification—Full-length human DAPK was cloned into tandem affinity purification (TAP) vector (Stratagene) using a standard PCR-based cloning strategy. HEK293 cells were seeded in 15-cm plates and then transiently transfected with DAPK-TAP (15 μg of plasmid/plate). The TAP protocol was essential as described by the manufacturer. The final eluted bound samples were concentrated and submitted to the Indiana Center for Applied Proteomics for analysis, including tryptic digestion, high performance liquid chromatography separation, and tandem mass spectrometry (MS/MS) to determine peptide sequences.

Purification of PP2A Holoenzymes—PP2A AβoC and AβoC holoenzymes were prepared as described previously (24). Briefly, HEK T-Rex cell lines harboring pcDNA5-TO (EV), Ba-FLAG-pcDNA5-TO, or Bb-FLAG-pcDNA5-TO were treated with 2 μg/ml doxycycline for 48 h to induce protein expression. Cells were lysed in buffer containing 20 mM Tris-HCl, pH 7.6, 0.1% Igepal CA-630, 150 mM NaCl, 3 mM EDTA, 3 mM EGTA, and protease inhibitor mixture. The clarified cell lysates were incubated with 20 μl of a 50% slurry of anti-FLAG-agarose for 4 h. Bound proteins were washed twice with PAN buffer (10 mM PIPES, pH 7.0, 17 μg/ml aprotinin, and 100 mM NaCl) containing 0.5% Igepal CA-630, once with PAN buffer, and once in phosphatase assay buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 0.25 mg/ml bovine serum albumin). Bound proteins were eluted by incubation for 1 h at 4 °C in 100 μl of phosphatase assay buffer containing 100 μg/ml FLAG peptide. The amount of PP2A catalytic subunit in the purified PP2A holoenzymes was determined by SDS-PAGE and silver staining using serial dilutions of bovine serum albumin as standards as described previously (24). These values were used to calculate the protein concentration of purified PP2A holoenzymes based upon stoichiometric levels of the A, B, and C subunits in each preparation. Aliquots of the purified holoenzymes were either assayed for phosphatase activity or subjected to SDS-PAGE followed by silver stain or immunoblot analysis.

Phosphatase and Kinase Assays—Protein phosphatase assays were performed essentially as described previously (16). HEK293 cells were transfected with FLAG-DAPK for 48 h, and DAPK was immunoprecipitated from clarified cell lysates (1 mg of protein) using 40 μl of anti-FLAG-agarose for 4 h in lysis buffer (0.1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 10 mM sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM sodium fluoride, protease inhibitor mixture, and phosphatase inhibitor mixtures). The FLAG-DAPK immune complexes were washed once in lysis buffer containing both protease and phosphatase inhibitors and twice in phosphatase assay buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 0.25 mg/ml bovine serum albumin) and then resuspended in 40 μl of the same buffer. The washed immune complexes were incubated with purified ABoC and ABoC holoenzymes (~100 ng); FLAG peptide eluates for EV-expressing cells were used as a control. Following a 30-min incubation (with agitation) at 37 °C, the phosphatase reactions were terminated by washing twice in phosphatase assay buffer containing 100 mM okadaic acid and subjected to immunoblot analysis using antibodies recognizing. For the experiments coupled to an in vitro kinase assay, 20% of the reaction products from the phosphatase assay were analyzed by immunoblotting, and the remaining 80% was used in a kinase assay. For the kinase assay, the beads were washed extensively in kinase assay buffer (50 mM MOPS, pH 7, 10 mM magnesium acetate, 1 mM dithiothreitol, 10 mM CaCl2, 10 μM calmodulin, and 100 mM okadaic acid). The kinase reactions were carried out as described previously (25) with minor modifications. Kinase assays (40 μl) contained kinase assay buffer, 100 μM DAPK peptide substrate (Tocris), and [γ-32P]ATP (200 cpm/pmol) diluted in 1 mM cold ATP. Incubation was carried out for 30 °C for up to 22.5 min with
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The amount of DAPK peptide substrate was analyzed as described previously. Precipitated DAPK was treated with PP2A holoenzymes in an in vitro phosphatase assay. Following SDS-PAGE, DAPK was transferred to a nitrocellulose membrane and incubated with 100 nM biotinylated CaM (Sigma) in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM CaCl₂, and 5% nonfat dry milk. Calmodulin was detected with streptavidin-conjugated horseradish peroxidase (The Jackson Laboratory).

Adenoviral Transduction—HeLa cells were transduced with recombinant adenovirus directing expression of either DAPK (Ad-DAPK), green fluorescent protein (Ad-GFP), a control scrambled shRNA (Ad-shScr), or an shRNA for depletion of endogenous DAPK (Ad-shDAPK) for 48 h. Cells were washed with PBS, trypsinized, and collected in 1% paraformaldehyde for analysis using a Beckman Coulter cell counter or in RIPA lysis buffer for SDS-PAGE.

Cell Detachment Assays—Cell detachment was quantified by counting only adherent cells. Briefly, cells were washed twice in PBS to remove detached cells. Adherent cells were then trypsinized and counted with a Beckman Coulter cell counter. The extent of cell detachment is defined as ([the number of control cells — the number of treated cells]/the number of control cells).

RESULTS

PP2A-Bø and PP2A-Bø Holoenzymes Associate with DAPK—To identify novel DAPK-interacting proteins in HEK293 cells, we exploited a TAP approach that consists of two specific binding and elution steps that are designed to minimize nonspecific interactions. The affinity-purified proteins were identified using liquid chromatography/tandem mass spectrometry (data not shown). Of the several proteins identified using this TAP screen, the regulatory Bø subunit of PP2A was selected for further study. To validate the TAP results, we examined the ability of DAPK to co-immunoprecipitate with Bø and the highly homologous (90% identity) Bø subunit of PP2A. Co-immunoprecipitation experiments were performed using HEK T-REX stable cell lines expressing either EV, FLAG-Bø (Bo), or FLAG-Bø (Bø) as described under “Materials and Methods.” Western analysis of the FLAG immune complexes confirmed endogenous DAPK immunoprecipitates with Bø and the closely related Bø isoform; no DAPK was detected in the control immune complexes (Fig. 1A). Additionally, both the structural (A) and catalytic (C) subunits of PP2A were detected in the FLAG-Bø subunit eluates, whereas no PP2A subunits were observed in FLAG immune complexes isolated from lysates of control cells. Similarly, Western analysis of FLAG-DAPK immune complexes revealed that endogenous Bø subunit co-immunoprecipitated with FLAG-DAPK (Fig. 1B). To test whether the endogenous proteins interact, we utilized microcystin-agarose. Microcystin is a catalytic subunit binding inhibitor (26) of both PP1 and PP2A and can be used to isolate these phosphatases and their binding partners (22, 27, 28). For these

FIGURE 1. DAPK Interacts with PP2A ABøCa and ABøCc Holoenzymes. A, HEK T-Rex stable cell lines harboring empty vector (EV), FLAG-Bo, or FLAG-Bø were lysed 48 h post-doxycline induction. Anti-FLAG immunoprecipitations (FLAG IPs) were performed from the lysates. The FLAG immunoprecipitations (IP) and a 1/10 aliquot of each cell lysate were analyzed by Western blotting. B, HeLa cells transduced with adenovirus (Ad) for expression of Ad-DAPK were lysed 48 h post-transduction, and immunoprecipitations were performed from the lysates using anti-DAP3 or control IgG and analyzed by Western blotting. C, soluble extracts from HEK293 cells were preincubated with DMSO (−) or 5 μM un conjugated microcystin-LR (+) and then incubated with microcystin (MCYST)-agarose. Bound proteins were washed and analyzed by Western blotting. WCL, whole cell lysate. D, Western analysis of FLAG immune complexes (FLAG IPs) and cell lysates from HEK293 cells transiently transfected with EV, FLAG-Bo, or FLAG-Bø for expression of Ad-DAPK (Ad-DAPK) were performed from the lysates using anti-DAP3 or control IgG and analyzed by Western blotting. E, schematic representations of the Omni-tagged DAPK fusion proteins showing the amino acid residues and kinase, calmodulin (CaM), ankyrin (Ank), cytoskeletal (Cyto), and death (DD) domains. F, plasmids encoding the DAPK fusion proteins shown in E were transiently transfected into HEK T-Rex cell lines harboring EV (T-Rex-EV), FLAG-Bo (T-Rex-Bo), or FLAG-Bø (T-Rex-Bø). FLAG immunoprecipitations (FLAG IPs) were performed from cell lysates 48 h post-doxycline treatment. Cell lysates and FLAG immune complexes were subjected to Western analysis. The migrations of the DAPK fusion proteins and IgG light chain are indicated on the right. IB, immunoblot.
experiments, HEK293 soluble extracts were preincubated with DMSO(−) or unconjugated microcystin-LR(+), and then incubated with microcystin-agarose. Bound proteins were washed and analyzed by Western blotting. These results show that endogenous DAPK was found in complexes containing endogenous PP2A (Fig. 1C). The binding of endogenous DAPK to endogenous PP2A was attenuated by pretreatment of extracts with unconjugated microcystin, indicating that binding occurred in a microcystin-dependent manner. To determine whether PP2A binds other PP2A holoenzymes, we tested its ability to interact with B′β, a member of the B′ family that shares little homology to members of the B family. HEK293 cells were transiently transfected with FLAG-Bα, -Bδ, or -B′β. Western analysis of the FLAG immune complexes confirmed endogenous DAPK immunoprecipitates with Bα and Bδ but not B′β, indicating in vivo specificity for the targeting subunits of PP2A (Fig. 1D). To determine the region of DAPK that mediates its interaction with PP2A, fragments of DAPK corresponding to its known domains (Fig. 1E) were transfected into HEK T-REX cell lines expressing either FLAG-Bα, FLAG-Bδ, or EV. Western analysis of the FLAG immune complexes revealed that only the DAPK cytoskeletal domain, encompassing residues 628–1215, was required for binding to AB2C and AB8C holoenzymes (Fig. 1F). Although there is some background contamination in the EV immunoprecipitation, the significant increase in signal with either DAPK-WT or DAPK-Cyto suggests that the interaction is real. Collectively, these results indicate the PP2A holoenzyme associates with the cytoskeletal domain of DAPK through the regulatory Bα and Bδ subunits.

**PP2A Negatively Regulates the Cellular Levels of DAPK**—As our previous studies have suggested that the dephosphorylated form of DAPK is ubiquitinated and degraded (3, 10, 11), we examined the effects of a PP2A inhibitor (OA) and a PP2A activator (FTY720) on the cellular levels of DAPK. HeLa cells were treated with low concentrations (0.5–5 nm) of OA to inhibit PP2A activity and analyzed by Western blotting. This concentration (0.5–5 nm) of OA is relatively specific for inhibiting PP2A (IC50 ~ 1 nm) while not affecting the other OA-sensitive phosphatases (e.g., PP1) (29–33). Addition of OA to HeLa cells slightly enhanced the cellular levels of endogenous DAPK (Fig. 2A). Likewise, treatment of HeLa cells with the cell-permeable phosphatase inhibitor microcystin-LW also resulted in increased DAPK protein levels (supplemental Fig. S1). A recent study by Neviani et al. (34) identified a novel activator of PP2A, FTY720. To determine whether enhanced activation of PP2A altered endogenous DAPK protein levels, we treated HeLa cells with increasing amounts of FTY720 in the presence or absence of OA. FTY720 reduced the cellular levels of endogenous DAPK in the absence of OA, but this effect could be reversed by co-treating cells with 1 nm OA (Fig. 2B). The maximum concentration of FTY720 that we were able to use in these experiments was 5 μM as rapid cell detachment and cell death occurred at higher dosages.

To determine whether directly targeting PP2A to DAPK would alter its cellular levels, HEK293 cells were transiently transfected with the targeting subunits Bα, Bδ, or B′β. With forced expression of the Bα or Bδ regulatory subunits, we observed a decrease in the endogenous protein levels of DAPK (Fig. 2C). No reduction in endogenous DAPK protein levels was observed in control cells (EV) or cells overexpressing B′β, a result consistent with our co-immunoprecipitation studies (Fig. 1D). These findings further validate the in vivo specificity of the Bα and Bδ subunits in targeting PP2A to DAPK. Finally, siRNA-mediated knockdown of Bα or Bδ separately or in combination (Bα/δ) was performed to determine the effects on endogenous DAPK protein levels. As an antibody that detects endogenous Bδ is unavailable, it is difficult to detect decreases in expression of Bδ in response to siRNA treatment (16). Alternatively, quantitative reverse transcription-PCR was used to determine the relative mRNA levels of Bα and Bδ in cells treated with specific siRNAs for each isoform. This experiment confirmed that each respective siRNA resulted in >75% decline in their mRNA expression (supplemental Fig. S2). Consistent with our pharmacological and overexpression data, siRNA-mediated depletion of Bα or Bδ separately or in combination (Bα/δ) resulted in an increase in endogenous protein levels of DAPK (Fig. 2D). As the effects of siRNA-mediated knockdown of Bα and Bδ on DAPK levels were indistinguishable, we focused on Bα in subsequent experiments.

Previous studies have shown activation of DAPK (via dephosphorylation of p-S308) leads to enhanced degradation by the proteasome (3, 10, 11) and lysosome (6). To distinguish between proteasomal and lysosomal degradation, we induced the expression of Bα or Bδ in HEK-T-Rex cells and then treated the cells with 10 μM MG132 or 100 μM chloroquine for 6 h. Cells treated with proteasomal inhibitor MG132 could rescue the Bα- or Bδ-induced decrease in DAPK protein levels, whereas the lysosomal inhibitor could not (Fig. 2E). No change in the steady state level of DAPK1 mRNA was observed when overexpressing Bα or Bδ either in absence or presence of MG132 or chloroquine, indicating that the observed PP2A-induced decrease in DAPK occurs at the post-translational level and not at the transcriptional level (Fig. 2F). Together, these results indicate that the association of PP2A with DAPK negatively regulates the cellular levels of DAPK via a proteasome-dependent process.

**Regulation of DAPK Phosphorylation by PP2A Holoenzymes**—Previous studies from this laboratory have revealed that the dephosphorylation of DAPK at Ser-308 results in its activation and subsequent degradation by the ubiquitin–proteasome system (3), thus indicating that dephosphorylation of Ser-308 controls not only the activity of the kinase but also its cellular protein levels. A recent study by Gozuacik et al. (14) implicated a “PP2A-like” enzyme in the control of Ser(P)-308 dephosphorylation. Although these investigators demonstrated that the AC core dimer of PP2A could dephosphorylate Ser(P)-308, no evidence was presented addressing whether or not PP2A could be targeted to DAPK to facilitate this process. To determine whether PP2A targeted to DAPK by either the Bα or Bδ subunits could dephosphorylate DAPK at Ser-308, we immunopurified ABaC and AB8C holoenzymes, using a method described previously (16, 24), and incubated them with Ser-308-phosphorylated DAPK. The relative purity of the isolated PP2A holoenzymes was examined by SDS-PAGE and silver staining as well as by Western analysis (Fig. 3, B and C). Our analyses confirmed that the purified complexes contain the
FIGURE 2. PP2A negatively regulates DAPK protein levels. A, HeLa cells were treated with increasing amounts (0–5 nM) of PP2A inhibitor, okadaic acid (OA) for 24 h, lysed, and analyzed by Western blotting to detect expression of DAPK and vinculin (Vinc). The expression of DAPK relative to vinculin is indicated below the blot. n = 3. B, HeLa cells were treated with increasing amounts (0–5 μM) of PP2A activator, FTY720, for 48 h in the presence or absence of OA (1 nM), and analyzed as above. C, HEK293 cells transiently transfected with pcDNA5/TO (EV), Bα-FLAG/pcDNA5/TO (Bα), Bδ-FLAG/pcDNA5/TO (Bδ), or Bβ-FLAG/pcDNA5/TO (Bβ). Quantification of DAPK to total vinculin is shown (*, p < 0.05). Cells were lysed 48 h post-transfection and analyzed as described above to determine the expression levels of DAPK. D, HEK cells transfected with siRNA for scrambled control (siScr), siBα, siBδ, or siBα/δ for 72 h. Cells were lysed and analyzed by Western blotting. Quantification of DAPK to total vinculin is shown (*, p < 0.05). ns, nonspecific. E, HEK T-Rex cells stably transfected with pcDNA5/TO (EV), Bα-FLAG/pcDNA5/TO (Bα), or Bδ-FLAG/pcDNA5/TO (Bδ) after 48 h of doxycycline induction were treated either with vehicle, chloroquine (100 μM) or MG132 (10 μM) for 6 h and subjected to analysis as described above. F, parallel samples of cells treated as described for E above were analyzed using reverse transcription-PCR to determine the steady state mRNA level of DAPK relative to a control housekeeping gene HPRT1 in HEK T-Rex cells. Tet, tetracycline.
three PP2A subunits (A, B, and C) with only minor amounts of contaminating proteins. To determine whether the purified holoenzymes were active, they were tested for phosphatase activity toward a generic phosphopeptide in the presence or absence of OA. Bound proteins were washed, eluted with FLAG peptide resolved by SDS-PAGE, and subjected to silver stain analysis or immunoblot. After washing to remove the phosphatase inhibitors, isolated PP2A holoenzymes were assessed for activity in the absence or presence of OA using a generic phosphopeptide as substrate. For 48 h of transfection, the FLAG-DAPK proteins were isolated using anti-FLAG-agarose. The immunoprecipitates were incubated with purified active PP2A AB\(_2\)C (B\(_\delta\)) or mock (EV) holoenzyme in an in vitro phosphatase reaction in the presence or absence of okadaic acid. Quantification of S308-DAPK to total DAPK is shown and representative of repeat experiments.

PP2A Activates DAPK—Given that previous studies have shown that dephosphorylation of Ser-308 results in increased kinase activity of DAPK (3, 10, 13), we next sought to determine whether AB\(_\alpha\)- and AB\(_\delta\)C-catalyzed dephosphorylation of DAPK would increase DAPK activity. DAPK immune complexes were dephosphorylated using the purified AB\(_\alpha\)C and AB\(_\delta\)C holoenzymes, and kinase activity was subsequently measured using a synthetic DAPK substrate peptide (Fig. 4A). As shown in Fig. 4B, dephosphorylation of DAPK by AB\(_\alpha\)/6C increased kinase activity. Consistent with this enhanced kinase activity, the PP2A-treated DAPK (dephosphorylated, activated) was able to bind Ca\(^{2+}\)/CaM with higher affinity (Fig. 4C). These findings demonstrate that DAPK is activated by both PP2A holoenzymes (AB\(_\alpha\)C and AB\(_\delta\)C). To determine whether PP2A influenced DAPK phosphorylation of one of its known substrates, the regulatory light chain of myosin II, we performed Western analysis of HeLa cells with altered B\(_\alpha\) or B\(_\delta\) levels using a phospho-Ser-19 myosin II regulatory light chain antibody. Our results did not reveal any significant changes in Ser-19 myosin II regulatory light chain phosphorylation in cells overexpressing or lacking the PP2A B subunits (supplemental Fig. S3), despite the fact that these manipulations of the B subunit levels impacted DAPK levels and activity (supplemental Fig. S3).

DAPK Autophosphorylation Prevents DAPK-induced Cell Detachment—DAPK has been reported to suppress \(\beta\)-integrin signaling and cell adhesion through disruption of Talin and CDC42 (4, 5). To determine whether dephosphorylation of
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Ser(P)-308 and activation of DAPK influence cell detachment. HEK293 cells were transiently transfected with wild type (WT) DAPK or Ser-308-mutant forms of DAPK (S308A and S308E). The S308A DAPK mutant represents an active, dephosphorylated form of DAPK, whereas mutation of Ser-308 to Asp results in an inactive DAPK due to the presence of the phosphomimetic Glu residue (13). Cell rounding and detachment were visualized by differential interference contrast microscopy (Fig. 5A). The transfected HEK293 cells were analyzed to determine the relative levels of cell detachment. The relative levels of DAPK expression in the transfected cells were examined using immunoblotting (Fig. 5B). Expression of WT-DAPK resulted in 40% cell detachment, although expression of the active form of DAPK (S308A) increased this to 60% overall cell detachment, a result consistent with previous studies showing increased kinase activity of this mutant (Fig. 4C) (13). In contrast, expression of the inactive form of DAPK (S308E) completely attenuated the ability of DAPK to induce cell detachment; no significant cell detachment was observed when compared with empty vector transfected cells. These data are consistent with previous studies highlighting a role for DAPK in inhibiting cell adhesion (4, 5).

Ceramide Induces Caspase-independent Cell Detachment Leading to Anoikis—Ceramide is a potent activator of some phosphatases, including PP2A and PP1 (35, 36). Ceramide has also been shown to activate DAPK via dephosphorylation at Ser-308, and DAPK is required for ceramide-induced apoptosis in multiple cell types (3, 17, 18). Because ceramide is known to activate both PP2A and DAPK, we sought to understand how ceramide might induce apoptosis. A control experiment in which HeLa cells were treated with C<sub>6</sub>-ceramide (50 μM) for up to 24 h showed that ceramide induces caspase-dependent apoptosis, as evidenced by the progressive cleavage of the caspase substrate, poly-ADP-ribose polymerase (PARP), within 8 h of ceramide treatment (Fig. 6A). As expected, addition of Z-VAD-fmk (100 μM), a cell-permeable, pan-caspase inhibitor, blocked ceramide-induced PARP cleavage (Fig. 6A). In parallel experiments, both floating and adherent cells were counted after ceramide treatment to quantify cell detachment. After 8 h of ceramide treatment, HeLa cells began to detach from the tissue culture plates with nearly 60% of the cells floating after 16 h (Fig. 6B). Addition of Z-VAD-fmk did not reduce the number of floating cells, suggesting that cell detachment induced by ceramide treatment is independent of caspase activation. To determine whether the cells treated with ceramide are able to reattach, ceramide-detached HeLa cells treated either in the presence or absence of Z-VAD-fmk were collected and washed to remove ceramide and then reseeded onto dishes in medium containing the inactive ceramide analog, dihydroceramide. Following an 8-h incubation period, both re-attached and floating cells were counted. As shown in Fig. 6C, more than 75% of the floating cells were viable and could reattach when collected after 8 h of ceramide treatment. However, after more than 8 h of ceramide treatment, the floating HeLa cells progressively lost their ability to reattach, and by 24 h of ceramide treatment, the percentage of cells capable of reattaching had decreased to 2%. Although inclusion of Z-VAD-fmk did not prevent ceramide-treated HeLa cells from detaching (Fig. 6B), it signif-
significantly increased the proportion of detached cells that were capable of reattachment after 24 h of ceramide treatment from 2 to 35% (Fig. 6C). These results suggest that prolonged cell detachment in the presence of ceramide induces an irreversible loss of cellular adherence, probably due to induction of anoikis.

Ceramide-treated HeLa cells do not exhibit typical morphological characteristics of classical apoptosis, such as membrane blebbing, cytoplasmic condensation, or nuclear fragmentation prior to their detachment from tissue culture plates (data not shown) (37). To determine whether ceramide induces caspase activation prior to cell detachment, detached or adherent cells were collected separately at the indicated times after ceramide treatment and then analyzed by Western blotting to detect caspase activity by monitoring the cleavage of PARP (Fig. 6D). Surprisingly, the adherent population of HeLa cells, even in the presence of prolonged ceramide treatment (24 h), had nearly undetectable levels of PARP cleavage. In contrast, within the population of detached cells, there was a progressive increase in the levels of PARP cleavage, which begins within 8 h of ceramide treatment (Fig. 6D). Detached cells collected after 16 h of ceramide treatment were found to be mostly apoptotic with less than 25% of the cells capable of reattachment (data not shown). The finding that caspase-dependent apoptosis occurs only in HeLa cells that have lost cellular adhesion contacts due to ceramide treatment suggests that ceramide-treated cells are not committed to apoptosis prior to detachment from the tissue culture plate, and the observed apoptosis may be induced by the loss of cell adhesion (anoikis).

**FIGURE 7.** DAPK and PP2A are required for ceramide-induced anoikis. A, levels of total DAPK and DAPK phosphorylated at Ser-308 (Ser(P)-308-DAPK) were measured by Western blotting after immunoprecipitation (IP) of endogenous DAPK in HeLa cells treated with 50 μM ceramide for 0–24 h. B, quantification of detached HeLa cells following treatment with ceramide in the presence or absence of Z-VAD. The percentage of cell detachment was determined as described under "Materials and Methods." C, quantification of ceramide-detached HeLa cells that can reattach following washing and culturing in culture medium containing the inactive ceramide analog, dihydroceramide. D, Western blotting to detect full-length (116 kDa) and cleaved (85 kDa) PARP in total cell lysates from attached or detached (floating) HeLa cells collected after ceramide treatment for the indicated times.
DAPK and PP2A Are Required for Ceramide-induced Anoikis in Human Cervical Carcinoma Cells—Ceramide can inhibit β integrin-mediated cell adhesion in HeLa cells to promote cell detachment and anoikis by an unknown mechanism (37). Interestingly, forced expression of DAPK in HEK293T cells also caused inactivation of β-integrins and resulted in cell detachment and anoikis (5). This finding, together with our knowledge that ceramide activates both PP2A and DAPK (3, 13, 18), prompted us to look at the role both proteins play in ceramide-induced anoikis.

We first confirmed that treatment of cells with ceramide caused dephosphorylation of Ser(P)-308 in DAPK. As shown in Fig. 7A, treatment of HeLa cells with 50 μM C₆-ceramide induced a time-dependent dephosphorylation of DAPK as well as a decrease in its cellular levels. Next, HeLa cells were transfected with recombinant adenovirus directing expression of either DAPK (Ad-DAPK), green fluorescent protein (Ad-GFP), a control scrambled shRNA (Ad-shScr), or an shRNA for depletion of endogenous DAPK (Ad-shDAPK). At 48 h post-infection, the relative expression levels of DAPK in the infected cells were analyzed by Western blotting, which clearly indicated overexpression and effective depletion of DAPK using the appropriate viruses. In parallel, at 48 h post-transduction ceramide was added, and the infected cells were incubated for an additional 16 h before analysis to determine the levels of cell detachment in the infected cells. As shown in Fig. 7C, ceramide induced 65–68% cell detachment in the control cell populations expressing either Ad-GFP or Ad-shScr. In contrast, HeLa cells infected with Ad-DAPK significantly increased cell detachment to 78% (p < 0.05), whereas cells with reduced expression of DAPK (Ad-shDAPK) had significantly reduced cell detachment to 51% (p < 0.05).

Based on our findings showing that PP2A ABαC and ABβC are DAPK-Ser-308 phosphatases, we next tested whether or not the regulatory Bα subunit of PP2A is required for ceramide-mediated activation of DAPK and anoikis. For these experiments, HeLa cells were transiently transfected with Bα-targeted siRNA (siBα) for depletion of endogenous Bα or a control, scrambled siRNA (siScr). At 48 h the siRNA-transfected cells were treated with ceramide for 8–24 h as indicated (Fig. 7D). Cells were then analyzed for levels of DAPK and DAPK phosphorylation at Ser-308 (SerP-308-DAPK) as well as the relative levels of cell detachment (Fig. 7E). Depletion of Bα subunit significantly reduced dephosphorylation of DAPK at Ser-308 and prevented ceramide-induced degradation of DAPK (Fig. 7D). Consistent with reduced levels of active, dephosphorylated DAPK, cell detachment was also significantly reduced from 62% in siScr control treated cells to 41% (p < 0.05) in cells treated with siBα (Fig. 7E). These findings support the idea that ceramide induces the activation of the PP2A ABαC holoenzyme to regulate the activities and cellular levels of DAPK to promote cell detachment and anoikis.

DISCUSSION

Previous studies have established that the activities of DAPK can be regulated by Ca²⁺/CaM, autophosphorylation of Ser-308 within its calmodulin-binding domain, and ubiquitin-mediated degradation (3, 10, 13). Activation of DAPK catalytic activity is initiated by dephosphorylation of Ser-308 by a PP2A-like phosphatase (14) and binding of Ca²⁺/CaM. Following dephosphorylation and activation, DAPK protein levels are decreased by ubiquitin-mediated proteasomal degradation, a mechanism for terminating its activities (3, 10, 11). This paradigm suggests that a network of protein modifications and protein-protein interactions act to regulate the activities of this Ser/Thr protein kinase. In this study, we extend these findings and identify a physical interaction between DAPK and PP2A ABαC and ABβC holoenzymes. Pharmacological inhibition of PP2A, as well as siRNA-mediated depletion of the Bα or Bβ regulatory subunits of PP2A, results in increased cellular levels of DAPK. Consistent with these observations, pharmacological activation of PP2A or overexpression of Bα or Bβ regulatory subunits led to decreased cellular levels of DAPK. Moreover, treating cells with MG132 to inhibit proteasomal degradation rescued PP2A-induced degradation of DAPK, whereas treatment with chloroquine to inhibit lysosomal proteases had no effect on the cellular levels of DAPK. Our results also show that the phosphorylation status of DAPK-Ser-308 is crucial for adhesion of cells to tissue culture plates. Expression of wild type or the DAPK-S308A mutant, which is partially active DAPK (13), rapidly caused apoptotic cell morphological changes, whereas expression of an inactive, phosphomimetic form of DAPK (S308E) had no effect. The mechanism by which DAPK modulates cell attachment/detachment does not appear to involve myosin regulatory light chain phosphorylation, as we were unable to detect a change in phosphorylation of this DAPK substrate following manipulation of the regulatory Bα and Bβ subunits of PP2A. Thus, it appears that DAPK may regulate attachment/de-
tachment via another mechanism, such as through phosphorylation of other substrates associated with the cytoskeleton or by modulating integrin functions (4, 38).

Studies examining the mechanism by which ceramide induces apoptosis have focused on the ability of this compound to activate ceramide-activated protein phosphatases. The ceramide-activated protein phosphatases, principally PP2A but also PP1, are thought to induce apoptosis by dephosphorylating specific target proteins that include the mitochondrial apoptosis regulators Bcl-2 and Bax (36, 39, 40). However, the potential significance of the dephosphorylation of protein kinase DAPK in the regulation of ceramide-induced apoptosis signaling has not been considered. In this study, we have shown that the ceramide-induced dephosphorylation and activation of DAPK by PP2A ABαC and ABβC holoenzymes controls the balance between cell adhesion and detachment-induced anoikis. Our data reveal that there is a unique relationship between dephosphorylation of Ser-308 to enhance DAPK catalytic activity and cell detachment, suggesting that in response to ceramide treatment activation of DAPK correlates with loss of cell adhesion. Down-regulation of either endogenous DAPK or the PP2A regulatory Bα subunit blocked ceramide-induced cell detachment and anoikis, thus providing physiological evidence for an important role for PP2A in the activation of DAPK. Importantly, we also found that caspase activation by ceramide does not occur until after the ceramide-treated cells have detached from the substratum, which is known to lead to anoikis (41). These results suggest a novel pathway by which ceramide can lead to anoikis in adherent cells by inducing a DAPK-dependent cell detachment from the substratum.

In summary, our data not only provide new evidence to extend previous studies (4, 5) showing DAPK plays a role in anoikis but also provide a physiological stimulus and signaling pathway through which DAPK is activated. Based on the results of this study, we propose the following model describing how DAPK regulates ceramide-induced apoptosis. As illustrated in Fig. 8, activation of PP2A by ceramide results in dephosphorylation of the autophosphorylation site, Ser-308, within the calmodulin-binding region of DAPK as well as promoting increases in cytoplasmic calcium (42, 43). Dephosphorylation of DAPK-Ser-308 serves to enhance calcium/calmodulin binding and activation of DAPK (3, 13, 21). Active DAPK can phosphorylate its substrates as well as inactivate β-integrin signaling (3, 4). These events promote cell detachment from the substratum and subsequent apoptosis (anoikis). Active DAPK is also targeted by two distinct E3 ubiquitin ligases (CHIP and Mib1) for proteasomal degradation, which serves to reduce cellular levels of DAPK and terminate phosphorylation of substrates (3, 10, 11). Future studies aimed at identifying the DAPK substrate(s) controlling anoikis will aid in our understanding of the complex mechanisms governing cell adhesion and the ability of DAPK to induce indirect apoptosis through the anoikis pathway.

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