Peptide Combinatorial Libraries Identify TSC2 as a Death-associated Protein Kinase (DAPK) Death Domain-binding Protein and Reveal a Stimulatory Role for DAPK in mTORC1 Signaling*

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Death-associated protein kinase (DAPK) is a multidomain enzyme that plays a central role in autophagic and apoptotic signaling, although the protein-protein interactions regulating DAPK functions are not well defined. Peptide aptamer libraries were used to identify the tumor suppressor protein tuberin (TSC2) as a novel DAPK death domain-binding protein, and we evaluated whether DAPK is a positive or negative effector of the TSC2-regulated mammalian target of rapamycin (mTORC1) signaling pathway. Binding studies using death domain miniproteins in vitro and deletion analysis in vivo determined that the death domain of DAPK is the major site for the interaction with TSC2. Recombinant DAPK phosphorylates TSC2 in vitro, and DAPK kinase activity is stimulated by growth factor signaling. Transfection of DAPK promotes phosphorylation of TSC2 in vivo, whereas short interfering RNA-mediated attenuation of DAPK reduces growth factor-stimulated phosphorylation of TSC2. DAPK-dependent phosphorylation leads to TSC1-TSC2 complex dissociation, and consequently manipulation of DAPK by transfection or short interfering RNA demonstrated that DAPK is a positive regulator of mTORC1 in response to growth factor activation. Epistatic studies suggest that DAPK functions downstream from the RAS-MEK-ERK and phosphatidylinositol 3-kinase-AKT growth factor signaling pathways. DAPK+/− mouse embryo fibroblasts have attenuated mTORC1 signaling compared with DAPK+/+ counterparts, and overexpression of DAPK in DAPK+/− MEFs stimulates mTORC1 activity. These data uncover a novel interaction between DAPK and TSC2 proteins that has revealed a positive link between growth factor stimulation of DAPK and mTORC1 signaling that may ultimately affect autophagy, cell survival, or apoptosis.

Death-associated protein kinase (DAPK2; also known as DAPK1) is a calcium/calmodulin (CaM)-regulated serine/threonine protein kinase that plays a central role in a diverse range of signal transduction pathways, including growth factor activation, apoptosis, and autophagy. DAPK is composed of several functional domains, including a kinase domain, a CaM binding domain, eight ankyrin repeats, and a death domain (1). DAPK was originally identified as a factor that regulates apoptosis in response to various death inducing signals, including interferon-γ, TNF-α, and transforming growth factor-β (1, 2). The mechanisms through which DAPK activates apoptosis include an oncogene-activated p53-dependent pathway (1), inactivation of integrin and adhesion-mediated survival signaling (3), and cytoskeletal remodeling via phosphorylation of myosin light chain 2 (4, 5). Overexpression of the death domain module by itself protects cells from death-inducing signals, demonstrating the importance of this domain for DAPK proapoptotic function (6). The death domain of DAPK regulates its proapoptotic function in part by interacting with netrin-1 receptor UNC5H2 (7), the mitogen-activated protein kinase ERK (8), and members of the tumor necrosis superfamily TNFR1 and FADD (9). Autophosphorylation of DAPK at Ser-308 interferes with CaM binding and inhibits DAPK catalytic activity (10). UNC5H2 binding to the death domain of DAPK promotes its proapoptotic activity by reducing DAPK autophosphorylation on Ser-308 and increasing its catalytic activity (7). Phosphorylation of DAPK by ERK at Ser-735 also enhances its kinase activity and death-promoting effects (8). In accordance with its proapoptotic activity, recent evidence suggests that DAPK functions as a tumor suppressor. DAPK can suppress transformation in vitro (11) and block tumor metastasis in vivo (12); furthermore, in human cancers, DAPK expression is frequently lost due to promoter hypermethylation, and the attenuation of DAPK gene expression correlates with chronic lymphocytic leukemia development (13).

DAPK also plays a role in survival pathways, reflected in its autophagy-signaling activity (14) and its ability to counter TNF-mediated apoptosis (15). Overexpression of DAPK can lead to the appearance of autophagic vesicles, and knockdown of DAPK by antisense RNA has demonstrated that DAPK is necessary for autophagy induced by interferon-γ (14). Recent studies suggest that the regulation of autophagy overlaps with that of apoptosis (16), and it was recently demonstrated by...
genetic studies that in *Caenorhabditis elegans*, starvation-induced autophagy is regulated in part through a DAPK signaling pathway and that DAPK levels are critical to drive such cell fate decisions, leading to survival or death (17). At present, little is known about how DAPK exerts its effects on cell survival, autophagy, and apoptosis at the molecular level. We had previously set up a peptide-aptamer screen to identify novel proteins that bind to the DAPK core kinase domain to define novel steps in DAPK function. A protein-protein interaction was defined between MAP1B and DAPK that can modify the extent of autophagy, thus linking DAPK function to microtubule signaling pathways during the autophagic process (18). In this report, a peptide-aptamer interaction screen was set up for the DAPK death domain, where we identify TSC2 as a novel DAPK-interacting protein. The TSC complex, formed by two proteins (TSC1 (hamartin) and TSC2 (tuberin)), is a major regulator of the mTORC1 signaling pathway (19). TSC2 contains a GTPase-activating protein domain that converts the small GTPase Rheb (Ras homolog enriched in brain) to its inactive GDP-bound form (20). mTORC1 activity is stimulated by the active GTP-bound form of Rheb; thus, the TSC complex acts to inhibit mTORC1 function (20). mTORC1 directly regulates cell growth by controlling the phosphorylation of a number of components of the translational machinery. In particular, phosphorylation and activation of 4E-BP1 and S6K1 are stimulated by serum, insulin, and growth factors in an mTORC1-dependent manner (21). The pathway that regulates autophagy also acts through mTORC1. mTORC1 is sensitive to the lipophilic macrolide antibiotic rapamycin; thus, rapamycin and its analogue CCI-779 form a complex with FKBP12 (FK506-binding protein 12), which then bind to and inactivate mTORC1, leading to an up-regulation of autophagy (22). We report that DAPK is a positive upstream regulator of mTORC1 that mediates its effects in part by binding to and regulating the phosphorylation of TSC2 in response to growth factor signaling. The novel linking of the DAPK and mTORC1 signaling pathways has implications for how DAPK exerts its effects on cell survival, autophagy, and apoptosis in a manner that may depend on regulation of DAPK signaling thresholds (17).

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The C terminus of DAPK (amino acids 1313–1431) was cloned into the Gateway system vector pDONR221 (Invitrogen) using the following primers: Fwd (5′-GGGGACAAGTTTTCTACAAAAAGCAGGCTGAAACTGACGCTGCTGACCCG-3′) and Rev (5′-GGGGACCATTTGTAACAAGAAGCTGGTGTCACCAGGATACACAGAGCTTAAAT-3′). For expression of glutathione S-transferase fusion protein in *Escherichia coli*, pDONR221-DAPK vectors were recombined with the pDEST15 vector (Invitrogen), as recommended by the manufacturer. HA-tagged DAPK-WT, ΔCAM, and K42A vectors were a gift of Adi Kimchi (Weizmann Institute). For the generation of HA-tagged DAPK deletion mutants, stop codons were introduced, as described previously (29). FLAG-tagged TSC2 was a gift from Andrew Tee (University of Cardiff).

**Cell Culture, Transfection, and Immunoblotting**—HeLa, A375, A549, HEK293, and primary DAPK+/+ and DAPK+/-MEFs (Dr. Owen Sansom, Beatson Institute, Glasgow) were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) at 37 °C in a 5% CO2/H2O-saturated atmosphere. Cells for transient transfection were plated out 24 h before transfection at ~1.5 × 10^6 cells/100-mm dish or 5 × 10^5 cells/60-mm dish. For Lipofectamine 2000 transfection (Invitrogen), 2 μl of Lipofectamine was used for every 1 μg of DNA transfected. Cells were harvested after a further incubation of 16–18 h. Cells were lysed in ice-cold extraction buffer (75 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 5 mM NaF, 1 mM sodium vanadate, 1× protease inhibitor mixture) for 30 min and centrifuged at 13,000 rpm for 15 min to remove insoluble material. The protein content of cell extracts was measured using Bio-Rad reagent. Typically, 20–30 μg of cell extract was used for immunoblot. Samples were resolved by denaturing gel electrophoresis, typically 4–12% precast gels (Novex), and electrotransferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences), blocked in PBS, 10% nonfat milk for 30 min, and then incubated with primary antibody overnight at 4 °C in PBS, 5% nonfat milk, 0.1% Tween 20. After washing (3 × 10 min) in PBS/Tween 20, the blot was incubated with secondary antibody, either horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (Dako; 1:5000), for 1 h at room temperature in PBS, 5% nonfat milk, 0.1% Tween 20. After washing (3 × 10 min) in PBS/Tween 20, proteins were visualized by incubation with ECL reagent (Sigma). Equal protein loading was confirmed with Ponceau S staining. FLAG antibody (M2) was purchased from Sigma. HA-11 antibody (Ascites) was purchased from Covance. Tuberin/TSC2 (C-20) antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). DAPK antibodies were purchased from BD Transduction Laboratories and Santa Cruz Biotechnology (clone 55). Phospho-p70 S6 kinase (Thr-389, 1A5), p70 S6 kinase, phospho-S6 ribosomal protein (Ser-235/236, 2F9), S6 ribosomal protein (54D2), phospho-p44/42 mitogen-activated protein kinase (Thr-202/Tyr-204), p44/42 mitogen-activated protein kinase, phospho-AKT (Thr-308), AKT, Hamartin/TSC1 (1B2), and phospho-MLC (S19) antibodies were purchased from Cell Signaling Technology. Phospho-Ser/Thr antibody was from BD Transduction Laboratories. β-Actin antibody was from Sigma. Immunoblots were quantified using Scion Image (National Institutes of Health).

**Immunoprecipitation**—For immunoprecipitation of endogenous proteins, 30 μl of protein G-Sepharose beads (Amersham Biosciences) bound to DAPK or TSC2 antibody were incubated overnight at 4 °C with rotation, together with 500 μl of cell extract (~1 mg) prepared in extraction buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 5 mM NaF, 1 mM sodium vanadate, 1× protease inhibitor mixture). The bead pellets were then washed five times in extraction buffer before elution with 3× SDS loading buffer. Eluted proteins were then analyzed by denaturing gel electrophoresis and immunoblotted with appropriate antibodies. For immunoprecipitation of exogenous HA-DAPK from cells, HA-11 antibody bound to 30 μl of protein G-Sepharose beads was incubated with cell extract (~1 mg) overnight at 4 °C with constant rotation. For immunoprecipitation of exogenous TSC2, 30 μl of
FLAG-conjugated Sepharose beads (Sigma) were incubated with cell extract (−1 mg) overnight at 4 °C with constant rotation. For precipitation of endogenous DAPK using TSC-derived peptides, cell extract (−1 mg diluted to a volume of 500 µl in extraction buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 5 mM NaF, 1 mM sodium vanadate, 1× protease inhibitor mixture)) was incubated overnight at 4 °C with constant rotation, together with peptides coupled to streptavidin beads. The bead pellets were then washed five times in extraction buffer before being resuspended in 3× SDS-loading buffer and analyzed by denaturing gel electrophoresis and immunoblotting.

DAPK Kinase Assays—For phosphorylation of TSC2 in vitro, HEK293 cells were transfected with FLAG-TSC2, and protein was immunoprecipitated by FLAG-conjugated Sepharose beads. The immunoprecipitates were then washed three times with extraction buffer and twice with kinase buffer (50 mM Hepes (pH 7.4), 10 mM MgCl2, 1 mM dithiothreitol, 1 mM EDTA). Immunoprecipitated TSC2 on beads was then subjected to a kinase assay using purified DAPK kinase domain (1 µg) in 40 µl of kinase buffer supplemented with 50 µM ATP and 10 µCi of [γ-32P]ATP at 30 °C for 30 min. Reactions were stopped by the addition of 3X SDS LB and resolved by denaturing gel electrophoresis followed by immunoblot or visualized using a STORM 840 scanner and software (Amersham Biosciences).

For DAPK activity, HEK293 cells were transfected with HADAPK-WT or K42A, serum-starved for 16 h, and then reconstituted with serum for 30 min, and DAPK proteins were immunoprecipitated by HA-11 antibody and protein G-Sepharose beads. The immunoprecipitates were then washed three times with extraction buffer and twice with kinase buffer (50 mM Hepes (pH 7.4), 10 mM MgCl2, 1 mM dithiothreitol, 1 mM EDTA). Immunoprecipitated DAPK on beads was then incubated with purified bacteria-derived MLC protein (1 µg) in 40 µl of kinase buffer supplemented with 50 µM ATP and 10 µCi of [γ-32P]ATP at 30 °C for 30 min. Reactions were stopped by the addition of 3X SDS LB and resolved by denaturing gel electrophoresis, followed by immunoblot. MLC protein was visualized by Ponceau staining.

Short Interfering RNA (siRNA)—For the knockdown of DAPK gene expression, cells were transfected with DAPK siRNA (Dharmacon, siGENOME SMARTpool DAPK1 human) or a nonspecific siRNA as control (Dharmacon, siGENOME nontargeting pool). After 48 h, cells were lysed in ice-cold extraction buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 5 mM NaF, 1 mM sodium vanadate, 1× protease inhibitor mixture) for 30 min and centrifuged at 13,000 rpm for 15 min to remove insoluble material. Samples were resolved by denaturing gel electrophoresis followed by immunoblotting.

Peptide Library Selection—15 µg of glutathione S-transferase-tagged DAPK death domain protein in 150 µl of coating buffer (0.1 mM NaHCO3, pH 8.6) was added to a well of a 96-well plate and incubated overnight at 4 °C. The following day, the coating solution was removed, and the well was blocked with blocking buffer (5 mg/ml BSA in NaHCO3, pH 8.6) for 1 h at 4 °C followed by six washes with 200 µl of TBST (TBS plus 0.1% (v/v) Tween 20). 10 µl of peptide aptamer library (New England Biolabs) (4 × 1010 phage-peptide fusions) was diluted in 100 µl of TBST, added to the well, and incubated at room temperature for 1 h. Nonbinding peptides were removed, and the well was washed six times with TBST. Binding peptides were then eluted with 100 µl of 0.2 M glycine-HCl, pH 2.2, containing 1 mg/ml BSA by incubating at room temperature for 10 min, and the eluate was neutralized with 15 µl of 1 M Tris-HCl, pH 9.1. A small amount of this eluate was titered, and the remainder was amplified. The peptides were amplified by infection of ER2738 E. coli cells for 4.5 h, precipitated with PEG (20% (w/v) polyethylene glycol 8000, 2.5 mM NaCl), and resuspended in 200 µl of TBS. This biopanning procedure was repeated a further three times using the amplified peptides, and a small amount of eluate from each round was titered. From the phage titers carried out for each round of biopanning, individual plaques were isolated and amplified by infection of ER2738 E. coli cells for 4.5 h. Amplified peptides were then precipitated with PEG and resuspended in 100 µl of iodine buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 4 mM NaI), and their DNA from phage was precipitated with 250 µl of ethanol. After thorough washing, the DNA was amplified and prepared for sequencing using the Big Dye terminator (Amersham Biosciences) kit. The corresponding amino acid sequences of the peptides were identified using BLAST and EMOTIF.

Protein-Protein Interactions Using ELISA—For the DAPK-TSC2 peptide ELISA, a 96-well microtiter plate (Corning Inc.) was coated with appropriate amounts of purified DAPK death domain protein diluted in 0.1 mM Na2HCO3, pH 8.0, and incubated overnight at 4 °C. Each well was washed six times with PBS containing 0.1% Tween 20 (PBS-T), followed by incubation for 1 h at room temperature with gentle agitation in PBS-T supplemented with 3% BSA. The wells were washed six times with PBS-T prior to incubation with 5 µg/ml Biotin-labeled peptides (Upstate Biotechnology, Inc., Lake Placid, NY) diluted in PBS-T, 3% BSA for 1 h at room temperature. After 1 h of incubation, the plate was washed again six times with PBS-T and incubated with streptavidin peroxidase horseradish peroxidase conjugate (S5512; Sigma) for 1 h at room temperature, followed by further washing and ECL. The results were quantified using Fluoroskan Ascent FL equipment (Labsystems) and analyzed with Ascent Software version 2.4.1 (Labsystems). For DAPK-ERK ELISA, a 96-well microtiter plate (Corning Inc.) was coated with purified DAPK death domain protein diluted in 0.1 mM Na2HCO3, pH 8.0, and incubated overnight at 4 °C. Each well was washed six times with PBS-T, followed by incubation for 1 h at room temperature with gentle agitation in PBS-T supplemented with 3% BSA. The wells were washed six times with PBS-T prior to incubation with appropriate amounts of purified ERK protein (Upstate Biotechnology) diluted in PBS-T, 3% BSA for 1 h at room temperature. After 1 h of incubation, the plate was washed again six times with PBS-T and incubated with antibody specific to ERK for 1 h at room temperature, followed by further washing and ECL. The results were quantified using Fluoroskan Ascent FL equipment (Labsystems) and analyzed with Ascent Software version 2.4.1 (Labsystems).
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Gel Filtration—For gel filtration, a Superdex 200 10/300 GL (Tricorn) high performance column was equilibrated with buffer (PBS, 5% glycerol, 1 mM benzamidine prefiltrated with a Millipore filter (0.22 μm)). HeLa cell extract (5 mg) was prepared in buffer containing 25 mM HEPES (pH 7.5), 0.15 M KCl, and 5% glycerol. Lysates were injected and eluted into fractions of 1 ml each. The protein eluted in each fraction was then concentrated by trichloroacetic acid precipitation before being processed through four rounds of selection, the sequence of representative peptides was enriched and analyzed using BLAST, and e-motif identified a homology to LBX1, VGL-3, and TSC2. E, unfractionated DAPK death domain protein was incubated with a DAPK antibody bound to protein G beads. Immunoprecipitation with a nonspecific antibody bound to protein G beads served as a control. Immunoblotting of precipitates with an antibody specific for DAPK revealed the existence of a DAPK-TSC2 complex in cells (lane 3 that was not present in the control (lane 2). The level of input DAPK protein is shown (lane 1). The level of input TSC2 protein is shown (lane 1).

RESULTS

TSC2 Is a DAPK-Binding Protein—Dynamic regulation of protein-protein interactions in signal transduction processes is driven in part through linear interaction motifs or docking motifs interacting in synergy (23). As such, approaches identifying bioreactive linear peptide binding motifs will assist in expanding the "interactome" of a target signaling protein. A common germ line mutation in the DAPK death domain (N1347S) disrupts its structure and oligomerization (24); thus, the death domain module encoded by the N1347 wild-type allele was cloned, purified, and expressed in a bacterial expression system for presentation to a 12-mer peptide combinatorial library. Peptides acquired after four rounds of selection had significant homology to the homeodomain-containing transcription factor LBX1 and the transcription co-factor VGL-3 (Fig. 1A) that are involved in myogenic differentiation. In addition, a high affinity peptide that was repeatedly acquired showed homology with known proteins in the data base (Fig. 1A). Interestingly, two peptides acquired showed significant homology with the TSC2 tumor suppressor protein (Fig. 1A). Using an ELISA-based assay, DAPK death domain protein binding to synthetic peptides was analyzed. A titration of increasing amounts of peptide-Δ1 or the corresponding peptide derived from human TSC2 with a fixed amount of death domain miniprotein bound in a dose-dependent manner (Fig. 1B); therefore, we determined whether DAPK and TSC2 can form a protein complex in vivo. First, gel filtration of HeLa (cervical cancer cell) extracts revealed that DAPK and TSC2 co-elute in a large molecular weight complex (Fig. 1C). Further, endogenous TSC2 and DAPK can form stable protein complexes in lysates from HeLa cells, since the immunoprecipitation of TSC2 enriched DAPK protein (Fig. 1D), and conversely the immunoprecipitation of DAPK pulled out TSC2 protein (Fig. 1E). Together, these data indicate that TSC2 and DAPK can form a stable protein complex in cells.
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A. 

![Diagram showing kinase, CaM, Ankyrin, Cyto, and DD domains of DAPK with peptides 1, 2, 3, 4, and 5 labeled.]

**FIGURE 2.** The death domain is the major binding site for TSC2. A, in order to identify which regions of DAPK were necessary for the interaction with TSC2, a panel of HA-tagged DAPK deletion mutant proteins were prepared. B, the panel of HA-DAPK deletion mutant proteins were overexpressed in HeLa cells along with full-length FLAG-TSC2. For immunoprecipitation (IP) of DAPK, cell extract was incubated with antibody specific to HA bound to protein G beads. Immunoblotting of precipitates with an antibody specific for TSC2 revealed that the death domain is the major site of TSC2 binding (lane 5 versus lane 4). C, HeLa cell extracts were incubated for 16 h with control peptide, TSC2 peptide, or peptide 1 coupled to streptavidin beads. Beads were then washed prior to elution, and the amount of bound DAPK protein was assayed by immunoblot.

**TSC2 Binds to the Death Domain of DAPK**—We next evaluated whether the death domain of DAPK was required for TSC2 complex formation. The transfection into cells of a DAPK deletion series (Fig. 2A) along with TSC2 demonstrated that deletion of the death domain attenuated the binding of TSC2 protein (Fig. 2B). These data are consistent with the binding of the minimal TSC2 peptide to the death domain miniprotein (Fig. 1B) and indicate that the death domain of DAPK contains the major determinant for the binding of TSC2 in vivó. Furthermore, the phage peptide-δ1 and the peptide derived from the sequence of human TSC2 (amino acids 578–589) were able to pullout DAPK protein from HeLa cell lysates (Fig. 2C), indicating that the central region of TSC2, which is largely undefined functionally, is involved in mediating the interaction with DAPK.

**DAKP Regulates Phosphorylation of TSC2**—Having identified a physical interaction between DAPK and TSC2, we next wanted to determine whether DAPK could directly phosphorylate TSC2 in vitrò. As shown in Fig. 3A, recombinant DAPK kinase domain miniprotein could phosphorylate TSC2 in an immunocomplex kinase assay. No phosphorylation was observed without the addition of purified DAPK kinase domain (Fig. 3A). TSC2 can thus be a direct substrate of DAPK in vitrò. Serum-induced activation of DAPK catalytic activity has been demonstrated previously (4, 8). HEK293 cells expressing HA-DAKP-WT or kinase-dead K42A mutant were serum-starved and then restimulated with serum, and DAPK catalytic activity was assessed by its ability to phosphorylate recombinant MLC (myosin light chain 2), which is a confirmed substrate of DAPK (4, 5). In agreement with these previous reports, immunocomplex kinase assays using DAPK immunoprecipitated from cell extract confirmed that DAPK activity is stimulated by serum (Fig. 3B). Next, we used a phospho-Ser/Thr antibody to assess whether TSC2 phosphorylation status changes in vitrò after DAPK manipulation by transfection or siRNA knockdown. First, HEK293 cells were transfected with TSC2 along with DAPK, as indicated, and the cells were serum-starved and assayed for TSC2 phosphorylation. Expression of DAPK-WT or a constitutively active mutant, DAPK-ΔCAM, robustly increased TSC2 phosphorylation compared with vector control (Fig. 3C). Expression of DAPK-(1–1313) stimulated TSC2 phosphorylation but to a lesser extent than DAPK-WT or DAPK-ΔCAM (Fig. 3C). In contrast, expression of DAPK-K42A did not stimulate TSC2 phosphorylation. These results indicate that DAPK can signal to TSC2 and that kinase activity and death domain binding are important for transmitting the signal. In order to evaluate DAPK contribution to TSC2 phosphorylation in response to growth factor signaling, HEK293 cells were transfected with control siRNA or siRNA to DAPK, serum-starved, and assayed for TSC2 phosphorylation with the phospho-Ser/Thr antibody. Within 30 min of serum stimulation, endogenous TSC2 is robustly phosphorylated, whereas inhibition of DAPK expression with siRNA reduces the level of serum-stimulated TSC2 phosphorylation (Fig. 3D; quantified in Fig. 3E). Taken altogether, these data indicate that DAPK can mediate phosphorylation of TSC2 in response to growth factor signaling.

**DAKP Dissociates the TSC1-TSC2 Complex**—TSC2 exists in complex with TSC1 in vitrò. The TSC complex is a negative regulator of the mTORC1 signaling pathway (19). TSC2 repression of mTORC1 must be relieved during conditions of mitogenic sufficiency. To accomplish this, TSC2 is phosphorylated and inactivated by kinases, including AKT and ERK, resulting in TSC complex dissociation (25, 26, 27). Therefore, we next tested whether TSC2 is regulated by DAPK as defined by changes in TSC1-TSC2 complex formation. HEK293 cells were serum-starved to promote TSC1-TSC2 complex formation. Incubation of immunoprecipitates with purified DAPK kinase domain miniprotein resulted in disruption of the TSC1-TSC2 complex (Fig. 4A; quantified in Fig. 4B). Consistent with the in vitrò interaction, we also found that DAPK overexpression in serum-starved HEK293 cells could cause disruption of the TSC1-TSC2 complex in vivó (Fig. 4C; quantified in Fig. 4D). Taken together with our previous results, these data suggest that DAPK-mediated phosphorylation of TSC2 dissociates the TSC1-TSC2 complex and points to a role for DAPK as a positive effector of mTORC1 signaling.
DAPK Is a Positive Regulator of mTORC1 Signaling—Activation of the mTORC1 pathway by growth factors controls protein synthesis at the level of translation initiation and ribosome biogenesis. The major targets of mTORC1 signaling are components of the translation apparatus, including the ribosomal protein S6 and initiation factors important for the recruitment of the ribosome to the mRNA (21). The kinases that are responsible for the main in vivo phosphorylation of S6 on sites including Ser-235/236, S6K1 and S6K2, are direct targets of mTORC1, which activates these kinases through phosphorylation at specific Ser/Thr residues. Phosphorylation of Thr-389, however, most closely correlates with S6K activity in vivo (21).

To assess the impact of DAPK on the mTORC1 signaling pathway, HEK293 cells were transfected with DAPK or vector control. Transfected cells were serum-starved and assayed for S6K phosphorylation at Thr-389 and S6 phosphorylation at Ser-235/236. Expression of DAPK robustly increased S6K and S6 phosphorylation (Fig. 5A). Although DAPK-stimulated S6K and S6 phosphorylation was sensitive to rapamycin treatment (Fig. 5A), a small proportion of S6 phosphorylation remained that was insensitive to rapamycin (Fig. 5A, long exp). These results are consistent with a previous report demonstrating that DAPK directly phosphorylates S6 on Ser-235/236 in vitro (28) and indicate that DAPK may also regulate S6 phosphorylation directly in vivo. Serum contains a multitude of factors that can stimulate both the RAS-MEK-ERK and PI3K-AKT signaling pathways. To gain further insight into the role of DAPK in mTORC1 signaling, cells were stimulated with EGF, which should preferentially activate the RAS-MEK-ERK pathway, or with insulin, which should preferentially activate the PI3K-AKT pathway. First, serum-starved HEK293 cells were stimulated with EGF for 30 min, and phosphorylation of S6K at Thr-389 and S6 at Ser-235/236 was analyzed using phospho-specific antibodies. Within 30 min of EGF stimulation, S6K and S6 were robustly phosphorylated at these sites (Fig. 5B). In contrast, in cells where DAPK expression was inhibited with siRNA, phosphorylation of S6K remained largely unaffected; however, phosphorylation of S6 was inhibited (Fig. 5B) but to a lesser extent than observed in response to EGF stimulation. Together, these data indicate that DAPK is a positive regulator of growth factor signaling to mTORC1 and suggest that DAPK plays a more prominent role in the RAS-MEK-ERK signaling pathway than the PI3K-AKT signaling pathway.

DAPK Regulates Growth Factor-induced mTORC1 Activation Downstream of ERK and AKT—TSC2 is directly phosphorylated by AKT and by ERK, resulting in the functional inactivation of the TSC1-TSC2 complex and mTORC1 activation (25, 26). To determine the position of DAPK within the growth factor signaling pathway, serum-starved HEK293 cells were
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**FIGURE 4.** DAPK disrupts the TSC1-TSC2 complex in vitro and in vivo. A, HEK293 cells were serum-starved for 16 h, and cell lysates were immunoprecipitated with TSC2 antibody. Immunoprecipitates (IP) were incubated in kinase buffer with or without purified active DAPK kinase domain miniprotein, assayed for TSC2, and co-precipitated TSC1 by immunoblot. The level of TSC2, TSC1, and actin in the lysate is shown. B, quantification of TSC1 bound to TSC2 in A. C, HEK293 cells were transfected with DAPK or control vector as indicated and then serum-starved for 16 h. Cell lysates were immunoprecipitated with TSC2 antibody and assayed for TSC2 and co-precipitated TSC1 by immunoblot. The level of TSC2, TSC1, DAPK, and actin in the lysate is shown. D, quantification of TSC1 bound to TSC2 in Fig. 4C.

stimulated with EGF or insulin for 30 min, and activating phosphorylations of ERK at Thr-202/Tyr-204 by the mitogen-activated protein kinase kinase (MEK) and AKT at Thr-308 by PDK1 were analyzed using phosphospecific antibodies. Within 30 min of EGF stimulation, ERK was phosphorylated at Thr-202/Tyr-204 (Fig. 5D), and inhibition of DAPK expression with siRNA did not affect the phosphorylation (Fig. 5D). Similarly, within 30 min of insulin stimulation, AKT was phosphorylated at Thr-308 (Fig. 5E), and inhibition of DAPK expression with siRNA did not affect the phosphorylation (Fig. 5E). The observation that EGF and insulin-stimulated ERK and AKT phosphorylation remain unaffected suggests that DAPK functions downstream from ERK and AKT in the growth factor-mTORC1 signaling pathways.

**dapk1 Gene Expression Correlates with mTORC1 Activity**

In human cancers, DAPK expression is frequently attenuated due to promoter hypermethylation (30). To demonstrate the importance of DAPK for mTORC1 activity in a physiological setting, cells with markedly different DAPK expression levels due to hypermethylation of the *dapk1* gene were screened for basal S6K activity. Strikingly, A375 (human melanoma) cells that have low basal DAPK protein also have low basal S6K Thr-389 phosphorylation (Fig. 6A) in comparison with A549 (human lung epithelial carcinoma) cells, which have high basal DAPK protein and high basal S6K Thr-389 phosphorylation (Fig. 6A). Treatment of A549 cells with rapamycin prevented the detection of S6K Thr-389 (Fig. 6A), indicating an mTORC1 dependence for S6K phosphorylation in this cell line. Both A375 and A549 cells have similar levels of TSC2 protein (Fig. 6A), and we determined by real time PCR that the differences in DAPK levels were due to mRNA expression (Fig. 6B). Importantly, transfection of DAPK into A375 cells stimulated endogenous S6K phosphorylation at Thr-389 (Fig. 6C). Furthermore, A375 cells stably expressing DAPK-WT or DAPK-ΔCAM exhibited S6K phosphorylation at Thr-389 (Fig. 6D), in contrast to cells stably expressing DAPK-K42A or DAPK-(1–1313), where no S6K phosphorylation was detectable (Fig. 6D). Interestingly DAPK-WT and DAPK-ΔCAM were expressed to lower levels than DAPK-K42A and DAPK-(1–1313) in the stable cell lines (Fig. 6D), suggesting that sustained, high level expression of active DAPK is not well tolerated by the cells.

To substantiate the physiological relevance of our findings, we then took advantage of DAPK MEFs. Comparison of S6K and S6 phosphorylation in DAPK<sup>+/+</sup> and DAPK<sup>−/−</sup> MEFs revealed lower mTORC1 activity in the DAPK<sup>−/−</sup> MEFs (Fig. 7A; quantified in Fig. 7B). Importantly, overexpression of DAPK in DAPK<sup>−/−</sup> MEFs partially restored S6K and S6 phosphorylation (Fig. 7C; quantified in Fig. 7D), supporting our earlier observations that DAPK plays a positive role in the mTORC1 signaling pathway.

**DISCUSSION**

DAPK is a large protein that consists of several modular domains that enable it to mediate diverse and apparently conflicting outcomes, such as cell survival, apoptosis, and autophagy. As such, DAPK will probably have a large set of upstream regulators that controls its specific activity, and it will in turn have a set of downstream target proteins that mediates its effects on cell responses. However, the sets of DAPK-interacting proteins that drive these distinct biological outcomes are only beginning to be defined. Some of these functional domains include the kinase domain, which forms an interaction site with MAP1B (18), a calmodulin-domain whose phosphorylation negatively regulates kinase domain function (10), an ankyrin-repeat domain that forms interactions with LAR protein phosphatase (31) and the E3 ubiquitin ligase DIP-1 (32), and the C-terminal death domain that forms docking sites for a growing number of signaling enzymes. These protein-protein interactions can all regulate distinct biological outcomes of DAPK, including autophagy, apoptosis, and survival. Molecular studies have shown that autophagy can comprise a predominant outcome of signaling events after DAPK up-regulation (14, 17). At present, the only known interaction partner of DAPK that contributes to autophagy is microtubule-associated interaction with MAP1B (18). Together, these data highlight the need to identify additional key regulators of other functional domains of DAPK that can alter its specific activity in these various biological outcomes linked to autophagy, apoptosis, or survival. The death domain has one direct binding protein defined so far; ERK interacts with an LXL docking site in the death domain to regulate DAPK proapoptotic functions (8). In this report using peptide combinatorial libraries to search for such “core” mod-
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FIGURE 5. DAPK is a positive upstream regulator of mTORC1. A, HEK293 cells were transfected with control vector or DAPK and serum-starved for 16 h. Quiescent cells were left untreated or treated with rapamycin (100 nm) for 30 min. Cell lysates were assayed for phosphorylated S6K Thr-389, total S6K, ribosomal protein S6 phosphorylation at Ser-235/236, total S6, and the levels of transfected DAPK and actin. B, HEK293 cells were transfected with either DAPK siRNA (lane 3) or nonspecific control short interfering RNA (lanes 1 and 2), as indicated. After 36 h of transfection, cells were serum-starved for a further 16 h. Cells were then left in serum-free medium as control or incubated in the presence of EGF (25 ng/ml) for 30 min. Extracts were prepared and immunoblotted for endogenous DAPK, phosphorylated S6K Thr-389, total S6K, phosphorylated S6 Ser-235/236, total S6, and actin. C, HEK293 cells were treated with either DAPK siRNA (lane 3) or nonspecific control short interfering RNA (lanes 1 and 2), as indicated. After 36 h of transfection; cells were serum-starved for a further 16 h. Cells were then left in serum-free medium as control or incubated in the presence of insulin (100 nm) for 30 min. Extracts were prepared and immunoblotted for phosphorylated S6K Thr-389, phosphorylated S6K Ser-235/236, total S6, phosphorylated S6 Ser-235/236, total S6, and actin. D, HEK293 cells were treated with either DAPK siRNA (lane 3) or nonspecific control short interfering RNA (lanes 1 and 2), as indicated. After 36 h of transfection, cells were serum-starved for a further 16 h. Cells were then left in serum-free medium as control or incubated in the presence of EGF (25 ng/ml) for 30 min. Extracts were prepared and immunoblotted for endogenous DAPK, phosphorylated S6K Thr-389, total S6K, phosphorylated S6 Ser-235/236, total S6, and actin. E, HEK293 cells were treated with either DAPK siRNA (lane 3) or nonspecific control short interfering RNA (lanes 1 and 2), as indicated. After 36 h of transfection, cells were serum-starved for a further 16 h. Cells were then left in serum-free medium as control or incubated in the presence of insulin (100 nm) for 30 min. Extracts were prepared and immunoblotted for phosphorylated S6K Thr-389, phosphorylated S6K Ser-235/236, total S6, phosphorylated S6 Ser-235/236, total S6, and actin. F, HEK293 cells were treated with either DAPK siRNA (lane 3) or nonspecific control short interfering RNA (lanes 1 and 2), as indicated. After 36 h of transfection, cells were serum-starved for a further 16 h. Cells were then left in serum-free medium as control or incubated in the presence of insulin (100 nm) for 30 min. Extracts were prepared and immunoblotted for endogenous DAPK, phosphorylated AKT Thr-308, total AKT, and actin, as indicated.

The death domain of DAPK is one of the key domains central to its signaling function (15). The death domain of DAPK was identified originally in a genetic screen to be one of four functional domains required for DAPK to exert its growth-suppressive activity (6). The death domain is a signaling module contained in proapoptotic proteins, including Fas, TNFR, and phospho-acceptor site(s) regulated by DAPK and that DAPK activity is important for optimal growth factor-stimulated TSC2 phosphorylation. We have shown that TSC2 phosphorylation is stimulated by DAPK and that DAPK activity is important for optimal growth factor-stimulated TSC2 phosphorylation. We have shown that TSC2 is a direct substrate of DAPK in vitro; however, at present, we are unable to say whether TSC2 is a direct substrate of DAPK in vivo and cannot rule out the possibility that DAPK regulates TSC2 phosphorylation indirectly via the activity of other protein kinases. TSC2 is a large, highly phosphorylated protein, and experiments to identify the major phospho-acceptor site(s) regulated by DAPK in vivo are currently under way. Importantly, we have demonstrated in vitro and in vivo that DAPK can reduce the stability of the TSC1-TSC2 complex. Taken altogether, these data indicate that DAPK can mediate an inactivating phosphorylation of TSC2 and reduce its bioactivity after growth factor signaling. Consistent with these results, manipulation of DAPK by transfection or
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siRNA demonstrates that DAPK acts as a positive mediator of the mTORC1 translation pathway in response to growth factor stimulation.

It is unlikely that the effect that DAPK has on growth factor-induced mTORC1 activation can be explained solely by DAPK regulation of TSC2 phosphorylation. Interestingly, other kinases that regulate mTORC1 activity, such as AKT and AMPK, phosphorylate TSC2 as well as components of the mTORC1 complex directly. For example, insulin stimulates AKT via the class I PI3K pathway, and once active AKT is known to have two stimulatory effects on mTORC1, it phosphorylates TSC2, inhibiting its Rheb-GTPase-activating protein activity (24), and it phosphorylates PRAS40, which induces RSK kinase phosphorylates the same sites on S6; however, they concluded that this was an inhibitory phosphorylation reducing S6 activity and protein translation in vivo. In contrast, Roux et al. (36) demonstrated that RSK kinase phosphorylates the same sites on S6; however, they concluded that this was an activating phosphorylation that stimulates S6 activity and promotes assembly of the translation preinitiation complex in cells. Our results are in agreement with the latter study and point toward a role for DAPK in activating S6 and protein translation. Further studies are required to clarify the role of DAPK in the regulation of S6 activity and protein translation in vivo; in particular, the interplay between DAPK and RSK signaling to S6 needs to be addressed.

In addition to the signaling pathways mentioned above, DAPK has also been linked previously to the control of protein translation by directly phosphorylating ribosomal protein S6 on Ser-235/236 (28). In agreement with this study, we show that DAPK can stimulate phosphorylation of S6 in the presence of rapamycin, suggesting that DAPK can mediate phosphorylation of S6 in an mTORC1-S6K-dependent and -independent manner. Schumacher et al. (28) demonstrated that DAPK phosphorylates S6 directly on Ser-235/236 and concluded that this is an inhibitory phosphorylation reducing S6 activity and protein translation in vitro. In contrast, Roux et al. (36) demonstrated that RSK kinase phosphorylates the same sites on S6; however, they concluded that this was an activating phosphorylation that stimulates S6 activity and promotes assembly of the translation preinitiation complex in cells. Our results are in agreement with the latter study and point toward a role for DAPK in activating S6 and protein translation. Further studies are required to clarify the role of DAPK in the regulation of S6 activity and protein translation in vivo; in particular, the interplay between DAPK and RSK signaling to S6 needs to be addressed.

DAPK functions in a wide range of biological pathways, including TNF-regulated cell death, stress-induced apoptosis, and autophagy; however, the molecular mechanisms are just

FIGURE 6. *dapk1* gene expression correlates with mTORC1 activity. A, phosphorylation of endogenous S6K as a readout of mTORC1 activity was monitored in A375 cells (low DAPK expression) and compared with A549 cells (high DAPK expression). A375 and A549 cells were left untreated (lanes 1 and 2) or exposed to 100 nM mTORC1 inhibitor rapamycin. For immunoblotting, cell extracts were prepared and immunoblotted for DAPK with HA-antibody and phosphorylation of endogenous S6K Thr-389, total S6K, and actin. B, real-time PCR analysis reveals that A375 cells have low levels of DAPK mRNA compared with A549 cells, which have high levels of DAPK mRNA expression. C, A375 cells (low DAPK; low S6K phosphorylation) were transfected with DAPK or pcDNA as control. For immunoblotting, cell extracts were prepared and immunoblotted for DAPK with HA-antibody, phosphorylation of endogenous S6K, total S6K, and actin. D, lysates from A375 stable cell lines were prepared and immunoblotted for DAPK with HA-antibody and phosphorylation of endogenous S6K Thr-389, total S6K, and actin.

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DAPK functions in a wide range of biological pathways, including TNF-regulated cell death, stress-induced apoptosis, and autophagy; however, the molecular mechanisms are just
beginning to be defined. Genetic studies in *C. elegans* show that DAPK levels (i.e. specific activity) regulate the extent of starvation-induced autophagy (17). Thus, in response to starvation conditions, high or low levels of DAPK lead to autophagic induced death, whereas intermediate levels lead to autophagy and survival. The finding that DAPK is a positive regulator of mTORC1 signaling and a positive regulator of autophagy at first seems counterintuitive. Overexpression of DAPK can lead to the appearance of autophagic vesicles, and knockdown of DAPK by antisense RNA has demonstrated that DAPK is necessary for autophagy induced by interferon-γ treatment in human cell lines (14), whereas mTORC1 is an inhibitor of autophagy, as defined by rapamycin and its analogues (29). Therefore, we would predict that DAPK activity should be activated by starvation and that its activity would be inversely correlated with that of mTORC1. However, in mammalian cells, although DAPK is reported to be necessary for interferon-γ-induced autophagy, it is not thought to be involved in starvation- or rapamycin-induced autophagy (30). We previously reported that DAPK interacts with MAP1B to promote autophagy in response to amino acid deprivation (18). Future studies will determine whether MAP1B is the key factor that switches DAPK activity toward autophagy induced by certain stresses, such as interferon-γ. In agreement with our observations, recent studies suggest that DAPK has a role in maintaining cellular homeostasis and promoting cell survival under normal cell growth conditions (37–40). Moreover, we and others have demonstrated previously that TNFα treatment results in DAPK degradation and sensitization of cells to apoptosis (29, 41), whereas siRNA-mediated knockdown of DAPK enhanced TNFα-induced apoptosis (29, 41). These studies confirm a role for DAPK as a survival factor in the TNFα signaling pathway.

Other signaling pathways can in turn modify these core activities of DAPK. For example, RAS activation of ERK-RSK pathway can attenuate the proapoptotic function of DAPK. RSK interacts with DAPK *in vitro* and *in vivo* and catalyzes the phosphorylation of DAPK on Ser-289, suppressing its apoptotic activity (42). RSK has also been shown to interact with and phosphorylate TSC2, inhibiting its tumor suppressor function, resulting in increased mTORC1 signaling to S6K1 (43). We can speculate that the death domain forms a focal point in the control of DAPK signal transduction pathways, and factors that direct the death domain to interact with the TSC2, ERK, or RSK protein complexes will determine its biological function.

In summary, we have described a novel link between the DAPK and mTORC1 signaling pathways. The data in this paper highlighting that TSC2 and DAPK form a stable protein complex nucleated by the death domain of DAPK have led us to determine that regulation of this protein-protein interaction effects mTORC1 signaling. Our observations also reveal a complex regulation of DAPK activity by growth factor signaling pathways mediated by ERK and RSK. The interplay between ERK-RSK signaling to DAPK and TSC2 may explain how the specific activity of DAPK can be modulated to control the balance between proapoptotic and prosurvival pathways.

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