DAPK-1 Binding to a Linear Peptide Motif in MAP1B Stimulates Autophagy and Membrane Blebbing*

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DAPK-1 (death-activated protein kinase) has wide ranging functions in cell growth control; however, DAPK-1 interacting proteins that mediate these effects are not well defined. Peptide-protein interactions are driven in part by linear interaction motifs, and combinatorial peptide libraries were used to identify peptide interfaces for the kinase domain of DAPK-1. Peptides bound to DAPK-1core kinase domain fragments had homology to the N-terminal domain of the microtubule-associated protein MAP1B. Immunobinding assays demonstrated that DAPK-1 can bind to the full-length human MAP1B, a smaller N-terminal miniprotein containing amino acids 1–126 and the 12-amino acid polypeptides acquired by peptide selection. Amino acid starvation of cells induced a stable immune complex between MAP1B and DAPK-1, identifying a signal that forms the endogenous complex in cells. DAPK-1 and MAP1B co-expression form a synthetic lethal interaction as they cooperate to induce growth inhibition in a clonogenic assay. In cells, two co-localizing populations of DAPK-1 and MAP1B were observed using confocal microscopy; one pool co-localized with MAP1B plus tubulin, and a second pool co-localized with MAP1B plus cortical F-actin. Reduction of MAP1B protein using short interfering RNA attenuated DAPK-1-stimulated autophagy. Transfected MAP1B can synergize with DAPK-1 to stimulate membrane blebbing, whereas reduction of MAP1B using short interfering RNA attenuates DAPK-1 membrane blebbing activity. The autophagy inhibitor 3-methyladenine inhibits the DAPK-1 plus MAP1B-mediated membrane blebbing. These data highlight the utility of peptide aptamers to identify novel binding interfaces and highlight a role for MAP1B in DAPK-1-dependent signaling in autophagy and membrane blebbing.

Stress-activated signal transduction events coordinate cellular responses to environmental change. Cells have evolved a large battery of protein kinase families to direct adaptation of cells to stress, including AGC, PTK, CMGC, phosphatidylinositol 3-kinase, and calcium-calmodulin kinase family (1–3). These kinases are usually regulated post-translationally through modifications, including phosphorylation or ubiquitination to permit rapid responses to changes in environmental or diseased states (4). The primary response of kinases involves integration of signaling to nuclear transcription factors. As a result, dysregulation of gene expression by mutation of the upstream protein kinases or the downstream transcription factors can result in disease development (5).

The calcium-calmodulin kinase family of protein kinases play a specialized role in coordinating certain cellular responses to distinct stresses such as p53 responses that include the DNA damage-activated kinase network of ATM-CHK2 (6–8), the metabolic kinase axis of LKB-AMPK (9), and oncogene activation mediated by ERK-DAPK-1 (10, 11). Attenuating germ line mutations exist in CHK2, LKB, ATM, and DAPK-1 (12–15). Of these enzymes, DAPK-1 is one of the least biochemically characterized stress-responsive protein kinases. However, a number of cellular studies have shown that DAPK-1 can exhibit diverse biological functions that include the following: interferon-induced cell death (16); amino acid starvation-linked autophagy and membrane blebbing (17); integrin inhibition and cell detachment (18); ERK4-dependent apoptosis (10, 15); oncogene activation of p53 (11); induction of phosphorylation within the p53 transactivation domain (19); and genetic tumor suppressor functions (20). Furthermore, although DAPK-1 can function as a growth suppressor (21), growing evidence also indicates it can function as an anti-apoptotic factor (22–25) suggesting that DAPK-1 can respond to different input signals catalyzing distinct effects on cell growth.

It is widely accepted that caspase-dependent apoptosis is one of multiple cell death programs. Programmed cell death is classified based on morphological criteria into several categories. Apoptosis or type I cell death is characterized by cell rounding and cytoplasmic condensation, membrane blebbing, cytoskeletal collapse, chromatin condensation and fragmentation, and formation of apoptotic bodies that are phagocytosed by macro-

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Regulation of DAPK-1 and MAP1B Interactions

phages or neighboring cells. Autophagic or type II cell death is characterized by the formation of autophagic vesicles in the cytoplasm accompanied by mitochondrial dilation, enlargement of the ER and the Golgi, and nuclear condensation without ordered DNA fragmentation. Type II cell death has been observed both in whole organisms during development, in pathological situations, and during neurodegeneration such as Alzheimer and Parkinson diseases. Type I and type II cell death programs are not mutually exclusive and can coincide in vivo in certain tissues and in cell culture. DAPK-1 is capable of initiating both type I apoptotic and type II autophagic programs, depending on the cell system and specific stimulus. The extent to which DAPK-1 contributes to type I apoptotic death often depends on p53 status, but p53 does not impact on the regulation of type II cell death by DAPK-1. The two modes of action of DAPK-1 add to the notion that it is a central player in cell death activated by a diverse array of different stimuli.

At the biochemical level DAPK-1 is composed of several functional domains, including its kinase domain, ankyrin repeat domain, calmodulin binding domain, and death domain (26). How these domains directly affect the many biological activities of DAPK-1 is not well defined, and there are relatively few DAPK-1-binding proteins. For example, one of the few bona fide partner proteins that exists for the death domain of DAPK-1 is ERK, whose docking to a specific LXL motif mediates DAPK-1-dependent apoptosis (10). The ERK docking is attenuated by a germ line death domain mutation that abrogates ERK binding and prevents apoptosis (15). By contrast to ERK, RSK-mediated phosphorylation of DAPK-1 attenuates the apoptotic response by an undefined mechanism (27). Identifying novel DAPK-1-binding proteins and substrates will expand on our understanding of the mechanisms of the many diverse functions of DAPK-1. In this study we focus on developing a linear interaction peptide consensus for the core kinase domain of DAPK-1 using peptide-aptamers, and we characterize one interaction that occurs with the microtubule-associated protein MAP1B. The MAP1B protein has been classically studied in neuronal cells (28) where it regulates the organization of microtubules, mediates axonal migration (29, 30), and contributes to the transport of cargo proteins on microtubules (31). Like DAPK-1, MAP1B has been independently shown to play a role in cytoskeletal dynamics (for review see Ref. 21) and to influence cell fate in cultured cells and in whole tissues. Interestingly, MAP1B has been shown recently to interact with the autophagy marker protein LC3 (32), prompting us to investigate its role in the regulation of autophagic processes in relation to DAPK-1. Here, data are presented demonstrating a genetic and biochemical interaction between MAP1B and DAPK-1 that reduces cultured cell growth independently of apoptosis and stimulates membrane blebbing dependent on an active autophagic program. These data show that DAPK-1-induced cell membrane blebbing is linked to autophagy and provide a novel interaction partner whose study may shed light on the mechanisms of DAPK-1-mediated changes in cytoskeletal structures leading to an enhancement of its activity in autophagy, membrane blebbing, and associated pathologies.

EXPERIMENTAL PROCEDURES

Reagents and Plasmids—All chemicals were acquired from Sigma unless stated otherwise. Gateway recombination cloning vectors were obtained from Invitrogen. Human full-length DAPK-1 cDNA and mRNA corresponding to the DAPK-1 kinase domain (N1–274) were obtained by reverse transcription-PCR amplification from total RNA isolated from HCT116 cells. The forward primer atgacccgttggcagcgg was used with reverse primers tccaggagttcagacgctgg and tccagctggcttctgctgcctggc for the kinase domain. Forward and reverse primers were designed to contain attB1 (gggacagcaattgtagacaaacgacgctgg) and attB2 (gggaccacttggacaaagtaaactctgctgcctggc) sites, respectively, for incorporation into vector pDONR201. The following MAP1B constructs were cloned from human fetal brain total RNA (Ambion) into pDONR221 using the gateway system: ~4-kb fragments of cDNA variants 1 (V1) and 2 (V2) (equivalent to rat constructs (33)) with forward primers atgacccgttggcagcgg and atgacccgttggcagcgg. Respectively, in conjunction with the reverse primer actgatgtgtaatttttacttcg and the N terminus of variant 1 (N126) using reverse primer taaagatgtgtaatttttacttcg. The various DAPK-1 pDONR201 and MAP1B pDONR221 constructs were then recombined using the LR reaction (components Invitrogen) into the required destination vector for bacterial, insect cell, or mammalian expression for native expression or with either His, GST, V5, or GFP tags as required. Empty vector constructs contained 1 kb of nonspecific DNA preceded by two stop codons incorporated by Gateway recombination cloning. Full-length human native transcript variant 1 (NM 005909) of MAP1B was acquired from Origene. Double HA/FLAG-tagged DAPK-1 (NM 004938), kinase activity attenuated (K42A), and calmodulin domain deleted (∆CaM) constructs were a gift from Adi Kimchi (Weizmann Institute, Israel).

Development of DAPK-1 Binding Ligands Using Peptide Combinatorial Libraries—A peptide phage combinatorial library of random 12-mer peptides (New England Biolabs) was used as a source of combinatorial peptides. 96-Well flat bottom plate wells (Microlite 2, Dynatech Laboratories) were coated with 1 μg/ml anti-GST mouse monoclonal antibody (Sigma) to capture purified GST-tagged DAPK-1 kinase domain. After capture and washing, wells were incubated for 1 h with 2 × 10^11 phages in PBS containing 0.1% Tween 20. The phage particles were eluted by acid or ATP incubation and neutralized with 15 μl of 1 M Tris-HCl (pH 9.1). Eluted phage particles were then amplified by infection of ER2378 cells and then polyethylene glycol-precipitated. This procedure was repeated three times with ~2 × 10^11 plaque-forming units of the first or second round amplified eluate used as input phage. In addition, the concentration of Tween 20 in the binding and wash buffers was increased stepwise, from 0.1 to 0.5%, with each successive round of biopanning to reduce nonspecific binding of the amplified phage peptide particles. The third round of polyethylene glycol-precipitated phage was titrated, and individual plaques were regrown and tested for DAPK-1 kinase domain binding activity. DNA from 10 binding phage plaques was then amplified and prepared according to the manufacturer’s protocol (New England Biolabs). The Abi Prism 377 automated DNA
sequencer was used to sequence the DNA with the -96gIII primer. Sequence data from the 12-mer peptides obtained were then analyzed for target homology using both the e-motif and/or NCBI algorithms blastp, psi-blast, and phi-blast. After phage display biopanning, the ability of purified DAPK-1 to bind isolated phage 12-mers was further assessed using synthetized biotinylated peptides (Cambridge Peptides). Peptide association was detected using horseradish peroxidase-conjugated streptavidin.

Recombinant Protein Purification and DAPK-1 Kinase Assay—Active GST-tagged DAPK-1 kinase domain 1–274 (DAPK-1-KD) pDEST20 was expressed in insect Sf9 cells using the baculovirus expression system (Invitrogen) and in Escherichia coli. After gentle lysis, GST fusion proteins were purified using glutathione-Sepharose 4B beads (Amersham Biosciences) according to the manufacturer’s instruction. His-tagged DAPK-1-KD pDEST10 were expressed in E. coli, extracted using lysis with lysozyme, and purified using nickel beads (Qiagen) according to the manufacturer’s protocol. A nonradioactive kinase assay was used to assay the activity of purified kinase. Tetrameric p53 was purified for use as a substrate in kinase reactions as described previously (34). Immunoblots for phospho-Ser20 p53 was used to assay the activity of purified kinase. Tetrameric p53 was purified for use as a substrate in kinase reactions as described previously (34). Immunoblots for phospho-Ser20 p53 was performed using monoclonal antibody DO1 or ICA9. Immunoblotting for phospho-Ser20 p53 was used to assay the activity of purified kinase. Tetrameric p53 was purified for use as a substrate in kinase reactions as described previously (34). Immunoblots for phospho-Ser20 p53 was performed using monoclonal antibody DO1 or ICA9.

Cell Culture Transfection and siRNA—A375 and HCT116 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in a humidified incubator in 10% CO₂ at 37 °C. For transfections, cells were seeded into 6-well plates at a density of 2 × 10⁵ per well 1 day prior to transfection. Transient transfections were performed using 3 μl of Lipofectamine 2000 reagent/μg of DNA in Opti-MEM (Invitrogen) according to the manufacturer’s instruction. Cells were harvested 24 h after transfection unless otherwise stated. For transient MAP1B knockdown cells were seeded at half-density (1 × 10⁵) per well. MAP1B siGENOME SMARTpool siRNA was obtained from Dharmacon. 120 pmol of siRNA oligonucleotides were transfected per 30-mm plate with 2 μl of Lipofectamine for 18–72 h. Nontargeting siCONTROL was used as a negative control in siRNA experiments.

Immunoblotting—Cells were lysed in denaturing RIPA buffer (10 mM Tris (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 1% deoxycholic acid, 0.1% SDS, and 1 mM EDTA with 1× protease inhibitor mixture) (Roche Applied Science). Lysates were reduced in sample buffer containing 0.2 M DTT at 85 °C for 5 min, resolved in 4–12% NuPAGE gels (Invitrogen), and transferred onto nitrocellulose Hightbond-C Extra membranes (Amersham Biosciences) using high molecular weight transfer buffer (1.5 × 1TG, 1.5% SDS, 10% methanol). Membranes were then rinsed and blocked in 5% nonfat milk/PBS for 1 h at room temperature before probing with primary antibody either overnight at 4 °C or at room temperature for 2 h in PBS, 1% Tween 20 (PBS-T) containing 5% nonfat milk. This was followed by washing three times for 5 min in PBS-T. Membranes were then incubated with the appropriate secondary horseradish peroxidase-conjugated swine anti-rabbit or rabbit anti-mouse antibody (DakoCytomation) at room temperature for 45 min, followed by washing five times for 5 min in PBS-T. Secondary antibody was detected by enhanced chemiluminescence and autorography. Primary mouse monoclonal antibodies were used at the following concentrations: DAPK-1 1:250 (BD Biosciences), MAP1B 1:500 (AA6 – Sigma), anti-V5 1:2500 (Invitrogen), p53 1:1000 (DO1), and GFP 1:1000 (Abcam ab1218).

Immunoprecipitation—For co-immunoprecipitation of co-transfected proteins, HCT116 cells were co-transfected for 24 h with a total of 2 μg of plasmid DNA consisting of 1 μg of DAPK-1 and 1 μg of V5-tagged MAP1B V1/V2 construct or control vector as indicated. Cells were lysed with mammalian cell detergent lysis buffer (1% Nonidet P-40, 50 mM HEPES (pH 7.6), 5 mM DTT, 0.4 M KCl with 1× protease inhibitor mixture) (Roche Applied Science) for 20 min on ice and cleared at 13,000 rpm for 10 min. 2 μg of anti-V5 mouse monoclonal antibody (Invitrogen) was immobilized per 20 μl of protein G-agarose beads (Amerham Biosciences) and washed three times in 1 ml of PBS-T for immunoprecipitation. Immunoprecipitations were carried out on a rotor for 2 h at 4 °C in binding buffer (25 mM HEPES (pH 7.5), 15% glycerol, 0.1% Triton X-100, 1 mM EDTA, 100 mM KCl, and protease inhibitor) using 20 μl of V5 beads with 200 μg of total protein. After precipitation, the beads were sedimented, washed three times with PBS-T, and incubated for 5 min at 85 °C with sample buffer + 0.2 M DTT. Co-precipitated DAPK-1 was detected by immunoblotting using mouse monoclonal anti-DAPK-1 antibody (BD Biosciences). For immunoprecipitation of endogenous proteins after starvation, HEK293 cells were starved for 3 h with Earles’ balanced salt solution before treatment with 2 μg/ml nocodazole and 5 μM latrunculin B for 1 h to liberate cytoskeletal associated protein complexes. Cells were lysed by mixing cell pellets with IP lysis buffer (25 mM HEPES, 1 mM EDTA, 150 mM KCl, 10 mM β-glycerophosphate, 50 mM NaF, 1% Triton X-100, and a protease inhibitor mixture) (1× Calbiochem) for 30 min on a rotor at 4 °C. Immunoprecipitation was performed for 1 h at 37 °C with 2 μg/ml nocodazole and 5 μM latrunculin B.

Microtubule Polymerization Cycling—137-mm plates of confluent A375 cells were used to prepare microtubule complexes by temperature-dependent polymerization/dem polymerization cycling. Cells were harvested in 2.5 ml of homogenization buffer HB (0.1 M MES, 0.5 mM MgCl₂, 1 mM EGTA) containing phosphatase inhibitor 10 mM β-glycerophosphate and protease inhibitor mixture (Calbiochem). Cell lysis was achieved by addition of Triton X-100 to a final concentration of 0.1% for 30 min at 4 °C on a rotor, and then the lysates were cleared by centrifugation for 20 min at 13,000 × g three times. GTP was added to cleared lysates (final concentration 1 mM) to allow microtubules to form at 37 °C for 30 min. Microtubules were then pelleted by centrifugation at 13,000 × g for 15 min; the supernatant was removed, and the microtubules in the pellet were depolymerized in 1 ml of HB for 40 min at 4 °C. Microtubule polymerization and dem polymerization were per-
formed a further two cycles to obtain a purified microtubule preparation.

**Immunofluorescence and Cell Morphology Assessment**—A375 cell cytoskeletons were visualized by partial cytoplasm extraction using fixative supplemented with detergent (3% paraformaldehyde and 0.01% Triton in PBS) for 10 min at 37°C before simultaneous blocking and membrane permeabilization in antibody dilution buffer: rat anti-HA 1:50 (3F10, Roche Applied Science), rabbit anti-tubulin 1:100 (ab6046, Abcam), and mouse anti-MAP1B 1:200 (mouse monoclonal AA6, Sigma). After washing with PBS, the following highly cross-adsorbed fluorescent Alexa dye-conjugated secondary antibodies (Invitrogen) were diluted 1:100 and bound for 45 min at room temperature as follows: Alexa488 anti-rat, Alexa405 anti-tubulin, and Alexa568 anti-mouse. F-actin was stained with Alexa633-conjugated phalloidin. Cells were visualized using a Leica SP5 confocal microscope using the ×63 objective lens.

**Quantification of HA-DAPK-1 and Endogenous MAP1B Co-localization with Cortical Actin**—After transfection for 24 h with HA-DAPK-1, A375 cells were fixed with 3% formaldehyde and 0.2% glutaraldehyde in PBS for 10 min at 37°C before simultaneous blocking and membrane permeabilization in antibody dilution buffer for 30 min. Primary antibodies were used at the following concentrations: MAP1B (AA6) 1:200 and HA (rat monoclonal 3F10, Roche Applied Science) 1:50 in antibody dilution buffer for 1 h at room temperature. Fluorescent highly cross-adsorbed Alexa dye-conjugated Alexa488 anti-rat and Alexa568 anti-mouse secondary antibodies were diluted 1:100 in antibody dilution buffer and incubated at room temperature for 45 min. F-actin was stained with Alexa633-conjugated phalloidin. Cells were visualized using a Leica SP5 confocal microscope using the ×63 objective lens.

**Autophagy Assays**—Autophagy was detected in a HEK293 cell line stably expressing the autophagy marker GFP-LC3 (36, 37). HEK293-LC3 cells were a gift from Sharon Tooze (Cancer Research UK, London). For assay of autophagy after MAP1B modification was detected by Western blotting.

**Cell Viability Determination by Trypan Blue Exclusion**—Cells were transfected for 24 h before harvesting by trypsinization. Cells were diluted to ~1 × 10⁶ cells/ml in PBS before addition of 1:1 to 0.4% trypan blue solution. 20 μl of cells were then loaded into the hemocytometer before immediately counting the total number of cells and the number of trypan blue-positive cells to calculate percentage cell viability.

**Apoptosis Assays Using FACS**—HCT116 cells were co-transfected with the indicated amounts of full-length native DAPK-1 and full-length native MAP1B expression vectors for the indicated times. Cells were co-transfected with the cell surface marker CD-20 (at 1:5 DNA ratio) to allow efficient selection of the transfected population using anti-CD20 fluorescein isothiocyanate-conjugated antibody (Caltag Biotech). After trans-
Regulation of DAPK-1 and MAP1B Interactions

FIGURE 1. Purification of active DAPK-1 kinase domain. a, DAPK-1 core domain purification. Sf9 cells were infected with baculovirus encoding DAPK-1 core kinase domain, and the indicated fractions were separated by SDS-PAGE and stained with Coomassie Blue. Lysates from induced cells (lanes 1 and 2) and noninduced cells (lane 3) were applied to a glutathione column for affinity purification. Protein is depicted in the flow-through (FT) (lane 9), in the wash (lanes 6–8), and in the glutathione eluates (lanes 4 and 5). b, kinase activity in the E1 and E2 eluates. DAPK-1 core eluates are increasing amounts of eluates E1 and E2, respectively) were titrated in a kinase assay using p53 as a substrate and evaluated using phospho-specific antibodies to Ser20 in the p53 activation domain and to total p53 using DO-1 monoclonal antibody. c, specific activity of calcium-calmodulin and DAPK-1 conserved kinase superfamily members DAPK-1 and CK2 in a p53 kinase assay. kinase reactions were assembled in buffer containing pS9 and the indicated kinase and (source: lane 1, no kinase; lane 2, His-tagged DAPK-1 (E. coli); lane 3, GST-tagged DAPK-1 (E. coli); lane 4, GST-tagged DAPK-1 (Sf9 cells); and lane 5, His-tagged CHK2 (Sf9 cells). Reaction products were analyzed for phosphorylation using phospho-specific antibodies to phospho-Ser20 and to total p53 using DO-1 monoclonal antibody. d, stability of DAPK-1 core domain in binding to p53. DAPK-1 binding to p53 was evaluated using a two-site ELISA. Panel i, DAPK-1 titration (ng) and evaluation of binding to p53 (in the solid phase); panel ii, p53 titration (ng) and evaluation of binding to DAPK-1 (in the solid phase) without and with BOX-V docking site peptides that reflect the calcium-calmodulin kinase interaction site in the core domain of pS9; panel iii, p53 titration (ng) and evaluation of binding to GST-DAPK-1 (in the solid phase). The protein-protein complex was detected with anti-DAPK-1 IgG or anti-p53 IgG (DO-1 or ICA-9), as indicated, and detected using peroxidase-labeled anti-mouse or anti-rabbit IgG followed by ECL quantitation using a Fluoroscan plate reader. d, diagrams to the left of panels i–iii reflect the order of protein complex formation from protein in the solid phase through to the horseradish peroxidase-20 antibody detection system. The binding activity in the ordinate (RLU, relative light units) is plotted as a function of increasing amounts of protein.

RESULTS

Characterization and Purification of Active DAPK-1 Kinase Domain—Enrichment of peptide aptamers that bind with a high affinity to DAPK-1 requires that the conformational integrity of the enzyme is intact, so the enzyme was purified and tested for both kinase activity and peptide binding capacity. The DAPK-1 core kinase domain (amino acids 1–274) was subcloned into the Gateway destination vector, and the gene was transferred into E. coli and Sf9 insect cell expression vectors. A representative purification is shown in Fig. 1a, where GST-DAPK-1core protein from lysates derived from Sf9 cells infected with GSTDAPK-1-1core virus (Fig. 1a, lanes 1–3) was purified from a glutathione column (Fig. 1a, lanes 4 and 5). A p53 kinase assay was set up that measures Ser20 site phosphorylation of recombinant human p53 by DAPK-1, as p53 has significant homology to the DAPK-1 consensus phosphorylation site (19). A titration of GST-DAPK-1core E1 and E2 fractions demonstrate that both fractions stimulate Ser20 site phosphorylation of p53 (Fig. 1b, lanes 2–5 and 7–10). The background band seen without kinase

fection adherent cells were thoroughly resuspended by trypsinization and pooled with floating cells, washed, stained with fluorescein isothiocyanate-conjugated anti-CD20 antibody, and fixed with 50% ethanol supplemented with 10% fetal calf serum overnight. After anti-CD20 staining and fixation, cells were treated with RNase A and simultaneously stained with propidium iodide for 30 min at 37 °C to stain cell nuclei. Nuclei at each stage of the cell cycle were visualized using a FACSCalibur flow cytometer (BD Biosciences) as per the manufacturer’s instructions. Phosphatidylinositol-positive cells were visualized using a FACSCalibur flow cytometer (BD Biosciences) on the FL4 channel.

Annexin V Assay—Cells were transfected with the indicated amounts of DNA for the indicated times. Following transfection, adherent cells were thoroughly resuspended by trypsinization and pooled with floating cells before washing and staining with allophycocyanin-conjugated annexin V and propidium iodide (Bender Medsystems) as per the manufacturer’s instructions. Phosphatidylinositol-positive cells were visualized using a FACSCalibur flow cytometer (BD Biosciences) on the FL4 channel.
**Regulation of DAPK-1 and MAP1B Interactions**

**Results**

**A -- ATP Elution**

| Phage ATP 1,2 | TLPSPALLTVH |
|---------------|-------------|
| Sph1         | TLPSPALLTVH |
| Sph2         | TLPSPALLTVH |
| Sph3         | TLPSPALLTVH |
| Sph4         | TLPSPALLTVH |

**B -- Acid Elution**

| Phage Acid 1 | LPEEMEKTIEY |
|---------------|-------------|
| Phage Acid 2 | LPEEMEKTIEY |
| Phage Acid 3 | LPEEMEKTIEY |
| Phage Acid 4 | LPEEMEKTIEY |
| Phage Acid 5 | LPEEMEKTIEY |
| Phage Acid 6 | LPEEMEKTIEY |

**C -- Biotin-φ and MAP1B peptide binding to DAPK Core**

| GST Only | GST Kinase |
|----------|------------|
| RLU      | RLU        |

**D -- Binding of MAP1B to DAPK-1**

**E -- Elution of Different Classes of Peptide Motifs from the DAPK-1 Domain**

**F -- Direct Biotinylation**

**DAPK-1 Core**

**MAP1B**

**Elution of Different Classes of Peptide Motifs from the DAPK-1 Domain**

GST-DAPK-1core was adsorbed onto a combinatorial 12-mer peptide-phage library and eluted with either acidic or ATP-containing buffer. Eluted phage were propagated in *E. coli*, amplified, and concentrated. Dose-dependent binding of p53 to the kinase (Fig. 1d, panel i). Furthermore, when GST-DAPK-1core was coated onto a solid phase, we detected dose-dependent binding of p53 to the kinase (Fig. 1d, panel ii). Glutathione captured GST-DAPK-1core also was able to bind to p53 in a dose-dependent manner (Fig. 1d, panel iii). These data indicate that GST-DAPK-1core is active in both peptide docking and in substrate phosphorylation, and this fraction was used as a bait to select for high affinity peptide binding ligands.

**Elution of Different Classes of Peptide Motifs from the DAPK-1 Domain**

GST-DAPK-1core was adsorbed onto a combinatorial 12-mer peptide-phage library and eluted with either acidic or ATP-containing buffer. Eluted phage were propagated in *E. coli*, amplified, and concentrated for the subsequent round of screening. Peptide sequences isolated after three rounds of screening by elution from DAPK-1 with ATP (a) or acid (b) are summarized as indicated. c, binding of MAP1B peptide φ and peptide to DAPK-1. Microtiter wells were coated with anti-GST IgG followed by incubation with GST only (left three panels) or with GST-tagged DAPK-1 (right three panels), as indicated. Synthetic peptide φ with homology to MAP1B (EEHRLRRPG), the corresponding homologous peptide derived from MAP1B (EEHRLRAIG), or control peptides (NT) were added to the wells without or with DAPK-1, and GST-DAPK-1-core complexes were quantified using streptavidin (SA) peroxidase. The data are plotted as peptide binding activity (RLU, relative light units) as a function of target protein in the solid phase. The diagram to the left of c reflects the order of protein complex formation between DAPK-1 and peptide in the solid phase through to the horseradish peroxidase (HRP)-20 antibody detection system. d–f, binding of MAP1B protein fragments to DAPK-1. d, diagram of MAP1B constructs utilized, including the full-length MAP1B gene (MAP1B polyprotein precursor), the splice variant V1 containing the N-terminal DAPK-1-binding peptide, the variant V2 lacking the N-terminal binding peptide, and the sequence of a miniprotein containing the N-terminal 126 amino acids of MAP1B. The boldface region in N126 highlights the location of the DAPK-1-binding peptide. MT, microtubule binding region. e, vectors (1 μg of each) encoding GST-DAPK-1 and V5-tagged MAP1B variants were co-transfected into cells; after 24 h, samples were lysed and processed for two-site ELISA. The binding of MAP1B variants to DAPK-1 was quantified using anti-V5 IgG and secondary antibody linked to peroxidase followed by quantification by ECL. f, co-precipitation of transfected MAP1B with DAPK-1. Vectors encoding V5-tagged MAP1B (V1 [lanes 3–5] or V2 [lanes 6–8]) and GST-tagged DAPK-1 (1 μg of each) were co-transfected into cells; after lysis, the amount of DAPK-1 bound in the anti-V5 immune complex was quantified by immunoblotting with anti-DAPK-1 IgG.
MAP1B and the DAPK-1 family. Using an ELISA-based assay (39), and so we did not evaluate PML as a potential binding partner. Mutation of the calmodulin domain deleted; Cam, kinase-dead).

**In Vitro Studies**

DAPK-1 and MAP1B Interact in vitro—MAP1B has two known transcript variants expressed in rat tissues as follows: transcript variant 1 gives rise to full-length protein, and transcript variant 2 is translated into protein lacking the N-terminal 126 amino acids (33). Transfection of cloned fragments of the rat MAP1B with and without the N-terminal region (1–1367 and 127–1367) showed that overexpression of protein with the N-terminal region caused programmed cell death in cultured neuronal cells (33). Given that the N-terminal region was shown to have such a dramatic effect and that this region has the peptide-binding motif for DAPK-1 (Fig. 2d), we cloned the human equivalent of both fragments for use in in vitro and in vivo studies. In vitro binding assays were performed using GST-DAPK-1 core and V5 epitope-tagged fragments of MAP1B protein synthesized in transfected human cells, including (Fig. 2d): 1) full-length human MAP1B; 2) MAP1B-V1 (1–1367, containing the N-terminal EEHLRRXXG motif); 3) MAP1B-V2 (without the N-terminal EEHLRRXXG motif); and 4) N126 (containing the N-terminal EEHLRRXXG motif). Using DAPK-1 in the solid phase, stable binding could be detected to full-length MAP1B, MAP1BV1, and N126 (Fig. 2e). However, less binding was observed to MAP1B-V2, which lacks the N-terminal EEHLRRXXG motif. The discrimination in binding between MAP1B-V1 and MAP1B-V2 was also observed in a co-immunoprecipitation assay using lysate from human cells. Co-transfection of V5-tagged variants of MAP1B with untagged DAPK-1 was followed by immunoprecipitation of MAP1B with anti-V5 beads and immunoblotting for DAPK-1. Relative to empty vector controls, MAP1B-V1 bound with DAPK-1 (Fig. 2f, lane 4 versus 3) to a higher extent than MAP1B-V2 (Fig. 2f, lane 7 versus 6), again suggesting that the primary interaction site between DAPK-1 and MAP1B is on the N-terminal domain of the MAP1B protein.

**MAP1B Forms a Synthetic Lethal Interaction with DAPK-1 to Inhibit Clonogenic Cell Growth**—Given that MAP1B could interact with DAPK-1 in vitro, we examined whether MAP1B was a positive or negative regulator of DAPK-1 activity. The effect of DAPK-1 on cell growth in a clonogenic assay was measured, because this measures cell survival under long conditions. Alone, MAP1B variants had very little effect on cell growth (Fig. 3). The effect of MAP1B-V2 was dramatic and that this region caused programmed cell death in cultured neuronal cells (33). Given that the N-terminal region was shown to have such a dramatic effect and that this region has the peptide-binding motif for DAPK-1 (Fig. 2d), we cloned the human equivalent of both fragments for use in in vitro and in vivo studies. In vitro binding assays were performed using GST-DAPK-1 core and V5 epitope-tagged fragments of MAP1B protein synthesized in transfected human cells, including (Fig. 2d): 1) full-length human MAP1B; 2) MAP1B-V1 (1–1367, containing the N-terminal EEHLRRXXG motif); 3) MAP1B-V2 (without the N-terminal EEHLRRXXG motif); and 4) N126 (containing the N-terminal EEHLRRXXG motif). Using DAPK-1 in the solid phase, stable binding could be detected to full-length MAP1B, MAP1BV1, and N126 (Fig. 2e). However, less binding was observed to MAP1B-V2, which lacks the N-terminal EEHLRRXXG motif. The discrimination in binding between MAP1B-V1 and MAP1B-V2 was also observed in a co-immunoprecipitation assay using lysate from human cells. Co-transfection of V5-tagged variants of MAP1B with untagged DAPK-1 was followed by immunoprecipitation of MAP1B with anti-V5 beads and immunoblotting for DAPK-1. Relative to empty vector controls, MAP1B-V1 bound with DAPK-1 (Fig. 2f, lane 4 versus 3) to a higher extent than MAP1B-V2 (Fig. 2f, lane 7 versus 6), again suggesting that the primary interaction site between DAPK-1 and MAP1B is on the N-terminal domain of the MAP1B protein.

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**FIGURE 3.** Genetic interaction between DAPK-1 and MAP1B using a clonogenic assay. a, synthetic lethal interaction between MAP1B and DAPK-1, HCT116 cells were co-transfected with the indicated expression vectors conferring antibiotic resistance. 48 h later, 10,000 trypsinized cells were plated in media containing the selective antibiotic. Cell growth was visualized by Giemsa dye staining and quantified using densitometry. Plotted data are relative plate densities as a function of co-transfected expression vectors (Vec). a, indicated MAP1B constructs (full-length MAP1B FL, V1, V2, or N126) and/or DAPK-1 were co-transfected in HCT116 cells before selection of transfected cells using blastocidin. Panel i, photograph shows extent of cell growth after 1 week. Panel ii, graph shows densitometric data from a representative experiment. b, MAP1B attenuation using siRNA reduces DAPK-1 growth inhibition. A375 cells were transfected with MAP1B siRNA for 48 h before a further transfection with the indicated expression vectors for 24 h. Panel i, Western blot showing attenuation of MAP1B using specific siRNA. Panel ii, cells were trypsinized and 10,000 cells plated in media containing geneticin. Graph shows mean cell growth as a function of co-transfected expression plasmids (Vec, empty vector; WT, wild type; ΔCam, calmodulin domain deleted; K42A, kinase-dead). Error bars show standard deviation of data from three experiments (* significance was determined using Student’s t test). c, MAP1B and DAPK-1 reduce cell viability. 24 h after transfection, adherent cells were suspended by trypsin digestion and pooled with floating cells from the growth medium. Suspended cultures were then stained with trypan blue to visualize nonviable cells. Graph shows mean nonviable cells as a function of transfected HA-DAPK-1 expression vectors (error bars show standard deviation of data from three counts).
Regulation of DAPK-1 and MAP1B Interactions

A panel of DAPK-1 mutants, including the kinase-dead version (K42A) and an activated version with the calmodulin domain deleted (ΔCaM), was evaluated to determine whether DAPK-1 kinase activity was required for optimal cooperation of MAP1B. MAP1B expression was efficiently reduced by siRNA transfection 24 h prior to transfection (Fig. 3b, panel i). Overexpression of both wild type DAPK-1 (WT) and ΔCaM inhibited cell growth, but this was attenuated in cells with reduced MAP1B protein expression (Fig. 3b, panel ii). Ablation of DAPK-1 kinase activity by mutation of Lys42 to Ala42 completely eliminated DAPK-1-induced cell growth suppression with or without endogenous MAP1B. These data strongly suggest that MAP1B cooperates with DAPK-1 to reduce cell growth and that this requires functional DAPK-1 kinase activity.

The cell growth assay does not provide any information relating to methods of cell growth inhibition. However, DAPK-1 transfection resulted in an increase in the number of nonviable cells as determined by counting trypan blue-positive cells (Fig. 3c). This effect was reduced by K42A mutation, suggesting that death was dependent on an active kinase domain; however, deletion of the CaM regulatory region had no significant effect.

MAP1B cooperation with DAPK-1 does not induce an apoptotic cell death program. HCT116 cells were transfected with the indicated expression vectors for 24 h before quantitation of apoptotic markers using FACS. Transfected populations were marked by co-transfection of GFP expression marker at a 1:5 ratio. Graphs show representative populations from four experiments. a, sub-G1 DNA content was measured as an indication of apoptosis-specific ordered DNA fragmentation. The transfected cell population is graphed with a gray line and the total cell population area is shaded black (not to scale). b, extent of apoptosis in transfected cell populations was determined by quantitation of PS externalization. allophycocyanin-conjugated annexin V signal is shown on the abscissa and propidium iodide (PI) staining on the ordinate. Percentage population in the LR quadrant is shown. LL, lower left; UR, upper right; UL, upper left.

DAPK-1-induced loss of cell viability was enhanced by co-transfection of full-length MAP1B (Fig. 3c, shaded bars), demonstrating that MAP1B can synergize with DAPK-1 to reduce cell viability. These data suggest that cell death is a mechanism that could account for MAP1B and DAPK-1 co-growth suppression.

MAP1B Interaction with DAPK-1 Does Not Induce Apoptosis—Multiple studies have show that DAPK-1 can induce cell death via an apoptotic program. Therefore, the effect of MAP1B and DAPK-1 on levels of apoptosis was analyzed by quantification of ordered DNA fragmentation and phosphatidylserine (PS) externalization using FACS. Cultures transfected with MAP1B, DAPK-1, or with both genes did not contain a detectable proportion of cells with sub-G1 DNA content (Fig. 4a). Therefore, MAP1B and DAPK-1 transfection does not induce apoptotic fragmentation. Using the annexin V assay, the extent of apoptosis after expression of DAPK-1 alone was determined as 8.72% of cells scored in the lower right (LR) quadrant (Fig. 4b). Expression of MAP1B was 8.60% in the LR quadrant. Co-expression from both vectors resulted in 9.99% of cells in the LR quadrant. These data could indicate that MAP1B and DAPK-1 ectopic expression alone can induce PS externalization. However, this is only a small induction when compared with TNFR1-transfected cells that scored 12.6% in the LR quadrant. Nevertheless, co-expression of both exogenous proteins did not enhance PS externalization. Together, these data suggest that it is highly unlikely that the synergistic growth suppression and reduction in cell viability (Fig. 3) precede though an apoptotic program. Therefore, further cellular assays were used to elucidate the mechanism of DAPK-1 and MAP1B co-growth suppression.

DAPK-1-induced Plasma Membrane Blebbing and Autophagosome Accumulation Is Enhanced by Interaction with MAP1B—In addition to the documented role of DAPK-1 during type I apoptotic cell death, DAPK-1 is also implicated in...

FIGURE 4. MAP1B cooperation with DAPK-1 does not induce an apoptotic cell death program. HCT116 cells were transfected with the indicated expression vectors for 24 h before quantitation of apoptotic markers using FACS. Transfected populations were marked by co-transfection of GFP expression marker at a 1:5 ratio. Graphs show representative populations from four experiments. a, sub-G1 DNA content was measured as an indication of apoptosis-specific ordered DNA fragmentation. The transfected cell population is graphed with a gray line and the total cell population area is shaded black (not to scale). b, extent of apoptosis in transfected cell populations was determined by quantitation of PS externalization. allophycocyanin-conjugated annexin V signal is shown on the abscissa and propidium iodide (PI) staining on the ordinate. Percentage population in the LR quadrant is shown. LL, lower left; UR, upper right; UL, upper left.
type II autophagic cell death where overexpression of DAPK-1 leads to caspase-independent cell death in conjunction with accumulation of mature autophagic vesicles, plasma membrane blebs, and nuclear condensation without DNA degradation. This is accompanied by no measurable loss of mitochondrial membrane potential or release of cytochrome c (17). Reduction of DAPK-1 protein levels by antisense RNA also reduces autophagic cell death induced by serum withdrawal or amino acid starvation in MCF7 cells, providing strong evidence that DAPK-1 is necessary for autophagic cell death in this system (17). Cell membrane blebbing is often thought of as a hallmark of apoptosis rather than autophagic cell death. Nevertheless, plasma membrane blebbing has been shown to be a robust marker of DAPK-1 action in cells, is independent of caspase activity, and is accompanied by accumulation of autophagic vesicles leading to type II cell death (40). Therefore, a membrane blebbing assay was set up to quantify DAPK-1 activity independent of apoptosis. Transfection of DAPK-1 protein efficiently induced membrane blebbing relative to that induced by control vector transfection (Fig. 5a, panel i, micrograph I versus III). Overexpression of MAP1B alone leads to only a slight stimulation of membrane blebbing (Fig. 5a, panel i, micrograph II). Co-transfection of MAP1B synergized with transfected DAPK-1 to stimulate membrane blebbing in over 80% of co-transfected cells (Fig. 5a, panel i, micrograph IV and quantified in Fig. 5a, panel ii). These data provide molecular evidence that a functional cooperation exists between MAP1B and DAPK-1 to induce the morphologic changes that are characteristic of DAPK-1 action in cells.

We next determined the effect of DAPK-1 kinase activity in this assay. Kinase-activated ΔCAM had enhanced activity, whereas the K42A mutant had a reduced ability to cause membrane blebbing activity (Fig. 5b, panel i). This is a reproduction of previous work showing that the membrane blebbing function of DAPK-1 is kinase activity-dependent (17). When endogenous levels of MAP1B were depleted by siRNA, the ability of WT and the activated (ΔCaM) DAPK-1 to induce membrane blebbing was reduced (Fig. 5b, panel i). This was in contrast to the situation after K42A transfection, where MAP1B depletion had no significant effect on the proportion of cells with membrane blebbing morphology. The MAP1B mutants V1 and V2 were co-transfected with DAPK-1 to determine whether the N terminus of MAP1B, which forms a DAPK-1 docking site (Fig. 2) and which forms a synthetic lethal interaction with DAPK-1 (Fig. 3), plays a role in membrane blebbing. Co-transfection of DAPK-1 with the MAP1B mutant lacking the N-terminal DAPK-1 binding site (MAP1B-V2) resulted in reduced membrane blebbing activity, relative to full-length MAP1B and MAP1B-V1 which have the N-terminal binding site (Fig. 5b, panel ii). Thus, the N-terminal domain of MAP1B is also required to stimulate the membrane blebbing activity of DAPK-1.

In addition to membrane blebbing as an established DAPK-1 assay, the protein is also able to induce the production of autophagosomes. Autophagic vesicles can be distinguished from regular lysosomes at the molecular level because they are decorated with light chain 3 (LC3) (36). The effect of MAP1B and DAPK-1 co-expression on autophagy in HEK293 cells that stably express the autophagy marker GFP-LC3 was assessed. During autophagy, this marker decorates autophagosomes so that they can be identified and quantified as GFP-LC3 foci,
Regulation of DAPK-1 and MAP1B Interactions

which fluoresce much brighter than the diffuse GFP-LC3 background (Fig. 6a, white arrows). Cells were treated with MAP1B siRNA or siRNA scramble control for 32 h before transfection with HA-DAPK-1. HA-DAPK-1 efficiently induced GFP-LC3 foci formation (Fig. 6, a and b, panel i). MAP1B siRNA reduced the number of GFP-LC3 foci formed in HA-DAPK-1 transfected cells by approximately half (Fig. 6, a and b, panel i). During autophagy, soluble LC3 (LC3-I) is post-translationally cleaved and lipidated to form LC3-II, and this cleavage event can be monitored by protein analysis using Western blotting. As expected, accumulation of GFP-LC3 foci was accompanied by GFP-LC3 modification after HA-DAPK-1 transfection (Fig. 6b, panel ii). The extent of modification was also reduced by MAP1B siRNA. These observations showing that MAP1B depletion attenuates DAPK-1-induced autophagosome formation provide evidence to suggest that MAP1B is involved in the DAPK-1-induced autophagic program as well as membrane blebbing (Fig. 5).

DAPK-1-induced Membrane Blebbing Is Downstream of Autophagy—The data generated by the blebbing and autophagy assays (Figs. 5 and 6) correlate with the data generated by the clonogenic growth suppression assay (Fig. 3), where in each case MAP1B cooperates with DAPK-1. Therefore, it is likely that MAP1B and DAPK-1 co-growth suppression proceeds though a type II cell death program. However, it was necessary to establish if the observed correlation between the biochemical interaction, induction of autophagy, the stimulation of membrane blebbing, and inhibition of cell growth was in fact because of a causal relationship stimulated by cooperation between the two proteins. The methylated nucleotide 3-methyladenine (3-MA) (41) is a well characterized pharmacological inhibitor of autophagy. Therefore, it was predicted that if there was a causal relationship between autophagy and membrane blebbing, then DAPK-1 induced blebbing would be decreased by 3-MA.

3-MA was added to HA-DAPK-1-transfected cells to determine the extent of DAPK-1-induced membrane blebbing that was because of autophagy. 3-MA was able to reduce membrane blebbing at low concentrations (2–5 mM) and completely blocked blebbing at higher concentrations (10 mM) (Fig. 7, a and c). This dose-dependent reduction in blebbing demonstrates that there is a causal relationship between DAPK-1-induced autophagy and DAPK-1-induced membrane blebbing. Furthermore, this places the induction of autophagy upstream of blebbing and suggests that under these conditions DAPK-1-induced blebbing is a result of autophagy. To determine whether blebbing induced by cooperation between DAPK-1 and MAP1B was because of autophagy, cells were co-transfected for 18 h and treated with 10 mM 3-MA for 6 h before fixing and staining for co-transfected cells. 3-MA treatment completely blocked membrane blebbing in co-transfected cells (Fig. 7b, dark gray bars), although it was not as efficacious as 3-MA under these conditions (Fig. 7b, black bars). The pan-caspase inhibitor, benzoyloxycarbonyl-VAD-fluoromethyl ketone, did not have any effect on blebbing, providing evidence that DAPK-1- and MAP1B-induced blebbing results from caspase-independent autophagy and not apoptosis. These data demonstrate that MAP1B cooperates with DAPK-1 to induce autophagy and that there is a relationship between DAPK-1 and MAP1B co-induced blebbing and autophagy with autophagic signaling being upstream of membrane blebbing. As a control, either starvation by amino acid removal or blocking the mammalian target of rapamycin pathway using rapamycin partially stimulated membrane blebbing in HA-DAPK-1-transfected cells (Fig. 7d) validating the integrity of these assays.
Interestingly, MAP1B siRNA blocked stimulation of membrane blebbing by starvation or rapamycin suggesting that the DAPK-1-MAP1B interaction is downstream of the starvation signaling and the mammalian target of rapamycin pathway.

DAPK-1 and MAP1B Co-localize with Microtubules before Accumulation at the Cell Cortex—Having established a site of physical contact between DAPK-1 and MAP1B in vitro, and having determined that the two proteins cooperate to induce autophagy-dependent membrane blebbing, a series of cell imaging and biochemical studies were conducted to determine whether these two known cytoskeleton-associating proteins interact with each other and the cytoskeleton (43, 44). HA-DAPK-1 was co-transfected with full-length native MAP1B in A375 cells for 10, 18, and 32 h before fixing and staining for MAP1B, HA, A-tubulin, and F-actin using phalloidin counterstain. During fixation of cells, the cytoplasm was partially extracted using 0.01% Triton X-100 in microtubule stabilizing buffer, a technique often used to aid visualization of the cytoskeleton in cells. After 10 h of treatment HA-DAPK-1 and MAP1B clearly co-localized with microtubules, especially at or near the microtubule organizing center (Fig. 8, open arrows). After 18 h, transfected cells had gross morphological changes with blebs, and co-localization with tubulin was less pronounced. By contrast, the transfected proteins strikingly decorated cortical F-actin fibers (Fig. 8, closed-headed arrows). This was accompanied by an overall reduction in tubulin staining intensity in transfected cells, and the microtubule organizing center was less well defined. After 32 h all co-transfected cells had shrunk and had extensive membrane blebs. In these cells, the cytoplasmic extraction fixation method revealed that both MAP1B and DAPK-1 decorate cortical F-actin filaments around the surface of the blebs. These data indicate that DAPK-1 and MAP1B transiently interact with microtubules and with cortical F-actin. In addition to this, overall microtubule bundling was reduced suggesting that co-transfection of the two proteins might induce general collapse of the microtubule cytoskeleton after 18 h.

To further demonstrate that DAPK-1 co-localizes with MAP1B at cortical actin fibers, HA-DAPK-1 was transfected
32 Hours 18 Hours 10 Hours

FIGURE 8. Co-transfected MAP1B and DAPK-1 co-localize with microtubules and microfilaments. A375 cells were co-transfected with HA-DAPK-1 and MAP1B for 10, 18, or 32 h before fixing by partial cytosol extraction with 3% formaldehyde in PHEM buffer supplemented with 0.01% Triton for 15 min. Cytoskeletons were then stained with rat anti-HA (2A10), mouse anti-MAP1B (AA6), and rabbit anti-tubulin antibodies followed by the appropriate highly cross-adsorbed Alexa dye-conjugated secondary antibodies. F-actin fibers were visualized using phalloidin counter-stain. Open arrows highlight areas of microtubule co-localization and closed arrows areas of microfilament co-localization.

into cells before analysis of the distribution of HA-DAPK-1 and endogenous MAP1B staining in relation to cortical F-actin. After 24 h, transfected DAPK-1 induced accumulation of endogenous MAP1B with exogenous DAPK-1 co-staining with cortical F-actin (Fig. 9a, cell i). This in contrast to nontransfected cells where endogenous MAP1B was evenly diffuse throughout the cytoplasm (Fig. 9a, cells ii and iii). In HA-DAPK-1-transfected cells, MAP1B staining at the cortex is double that in the cytoplasm after 24 h (Fig. 9b). Deletion of the CaM regulatory region from DAPK-1 (ΔCaM) did not significantly increase cortical accumulation of MAP1B, whereas inactivation of the kinase blocked cortical MAP1B accumulation.

To substantiate the evidence that DAPK-1 associates with microtubules (Fig. 8, open arrows), the ability of DAPK-1 to bind to microtubules in vitro was assessed after three rounds of microtubule depolymerization and depolymerization cycles (45). After three cycles DAPK-1 strongly associates with microtubules (Fig. 10A, lane 10), as does MAP1B. We were confident that only specifically bound proteins were present in the preparation because actin was not present in the pellet after three rounds (Fig. 10A, lanes 9 and 10), where most of actin was removed after two rounds (lanes 3 and 4). This demonstrates that nonbinding contaminants were removed. However, it is interesting that actin did not purify with DAPK-1, given that DAPK-1 is a known actin-binding protein. This could indicate that DAPK-1 associates with microtubules prior to any interaction with actin.

Amino Acid Withdrawal Stimulates the Formation of Protein Complexes Containing Endogenous MAP1B and DAPK-1—Given that DAPK-1 and MAP1B bind in vitro (Fig. 2), synergize to reduce clonogenic cell growth and reduce cell viability (Fig. 3), and induce membrane blebbing (Fig. 5), dependent on an active autophagic program (Figs. 6 and 7), it was predicted that...
Regulation of DAPK-1 and MAP1B Interactions

The two endogenous proteins would interact in cells after stimulation by amino acid starvation. Therefore, HEK293 cells were starved for 4 h before lysis and determination of DAPK-1: MAP1B binding by co-immunoprecipitation. Also, given that both proteins interact strongly with the cytoskeleton (Figs. 8–10), cells were treated for a further 1 h with the cytoskeletal depolymerizing drugs latrunculin B and nocodazole. As expected, the extent of MAP1B co-precipitating with DAPK-1 was increased after starvation (Fig. 10B, lanes 3 and 5 versus lanes 7 and 9). Treatment with nocodazole and latrunculin B resulted in liberation of MAP1B and DAPK-1 complexes from the insoluble cytoskeleton increasing the extent of co-immunoprecipitation (Fig. 10B, lanes 7 and 9 versus 5 and 3). The combination of starvation and drug treatment resulted in a clear co-immunoprecipitation demonstrating that endogenous DAPK-1 and MAP1B are present in complexes in cells under stressed conditions.

DISCUSSION

One of the difficulties in human cancer research is that dissection of a pathway that is not evolutionarily conserved in eukaryotic systems like yeast cannot necessarily rely on classic genetic screens to reveal signaling pathways. The absence of the DAPK family in yeast precludes such rapid genetic dissection of its function and regulation. Therefore, methods in the cancer field to identify such novel protein-signaling interactions have generally included antibody-based immunoprecipitation methods, tandem affinity protein-tagging immune precipitation techniques, yeast two-hybrid, and peptide aptamers. These techniques have advantages and disadvantages ranging from in vivo relevance through incomplete representation of a library.

DAPK-1 is a relatively large protein with multiple domains and docking motifs that will likely drive its regulation and function. There is a growing realization that small linear peptide interaction sites or docking motifs play an important role in protein function (46). Throughout evolution, proteins have diverged and have been extensively duplicated into families that share a similar function. This divergent evolution has given rise to protein modular structure, each module being a discrete region assigned a discrete function. There are over 7000 known protein globular domain modules that form folded independent compact structures, and these perform a vastly diverse set of functions ranging from death signaling to catalysis during metabolism. However, globular protein domains cover only a fraction of the total amino acid sequence of an organism. The remaining peptide sequence is either of low complexity or is intrinsically disordered. Disordered regions have a critical functional role in signaling pathways. For example, phosphorylation motifs and binding sites are often located in linear disordered regions of proteins, and like globular domains they conform to evolutionarily conserved sequence patterns (47). Although globular domains bind partners relatively strongly, down to picomolar affinities, linear motifs often have weaker binding kinetics (48). Thus, linear motifs are often involved in transient interactions such as those in signaling networks.

FIGURE 10. DAPK-1 interactions with MAP1B and tubulin. A, DAPK-1 co-precipitates with microtubules during temperature-dependent microtubule polymerization/depolymerization cycling. A375 cells were transfected for 24 h with vector control or HA-DAPK-1. Cell cultures were then harvested and lysed in microtubule polymerization buffer supplemented with 0.1% Triton X-100 and then centrifuged three times for 15 min. Lysates were then incubated at 37 °C for 40 min with 1 mM GTP to allow formation of microtubules. After each cycle an aliquot of precipitated microtubules was increased after starvation (Fig. 10B, lanes 3 and 5 versus lanes 7 and 9). Treatment with nocodazole and latrunculin B resulted in liberation of MAP1B and DAPK-1 complexes from the insoluble cytoskeleton increasing the extent of co-immunoprecipitation (Fig. 10B, lanes 7 and 9 versus 5 and 3). The combination of starvation and drug treatment resulted in a clear co-immunoprecipitation demonstrating that endogenous DAPK-1 and MAP1B are present in complexes in cells under stressed conditions.

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Regulation of DAPK-1 and MAP1B Interactions

(e.g. at phosphorylation sites or through the Src homology 3 domain or the 14-3-3 domain etc.).

Sensitive regulation of cellular processes requires transient signals from many weakly interacting components interacting in synergy. Useful information could not be provided by strong and long term interactions between only pairs of proteins. Therefore, signal transduction among many components interacting via linear motifs with weaker binding kinetics can provide specific and sensitive regulation of cellular signal transduction (49). As such, bioinformatic approaches identifying bioreactive linear peptide-binding motifs will assist in expanding the “interactome” of a target signaling protein.

Use of phage peptide display technology has classically been geared toward characterizing contact sites for known binding partners. For example, a second lower affinity MDM2 interaction site in the DNA binding domain of p53, a second p300-docking site in the p53 PXXP repeat domain, and a novel XYL docking domain have both been identified in this manner (50–52). Also, because the purified bait can be manipulated relatively easily in vitro, phage display has often been used to determine binding affinity changes upon alteration of the target proteins conformation. However, broader use of phage display to identify truly novel interaction partners has only recently been developed. Expansion of putative protein interaction maps has been achieved using MDM2 as prototypical bait target (53). These studies demonstrate that phage display technology can be used to rapidly discover novel binding partners and expand on protein interaction maps involving signal transduction pathways.

There are relatively few binding proteins identified for DAPK-1, one of which was identified for the death domain using yeast two-hybrid as ERK (10). In this study we have used DAPK-1 core kinase domain as bait using peptide aptamer libraries in a biochemical approach to identify DAPK-1-binding proteins, which led to the identification of a novel interaction for DAPK-1 with MAP1B. MAP1B functions in neuronal differentiation and neurite outgrowth, where it is present in the growth cone of extending axons (54) (55). In growing axons for example, MAP1B is phosphorylated at multiple sites within the heavy chain by GSK3β regulating axon growth (55, 56). Both MAP1B and the related MAP1A are post-translationally cleaved to yield two regulatory heavy chains (MAP1B heavy chain and MAP1A heavy chain) and two light chains (LC1 and LC2) that can form a dimer involving any one heavy chain and light chain. The combination of chains in the resulting dimer is governed by post-translational modification, including phosphorylation on the microtubule-associated proteins. Interestingly, LC3 is a member of the MAP1 family of microtubule proteins and is homologous to the light chains. A report has recently been published showing that LC3 interacts with high affinity to MAP1B (32), where phosphorylated MAP1B associates with autophagosomes. In rats, two transcript variants of MAP1B have been discovered as follows: the overexpression of transcript variant 1 (equivalent to V1 in this study) in cultured cortical neurons resulted in the acceleration of neuronal death, but expression of the alternative MAP1B isoform (V2) had no significant effect on cell death (33). The DAPK-1-MAP1B interaction, uncovered by the peptide screen in this study, occurs in part within the N-terminal region of the regulatory heavy chain (V1 isoform). We cannot determine as yet whether there are other DAPK-1 contact sites on MAP1B nor whether DAPK-1 directly phosphorylates MAP1B. However, the requirement for the kinase activity of DAPK-1 for MAP1B-stimulated growth suppression (Fig. 3), membrane blebbing (Fig. 5), and MAP1B translocation (Fig. 9) identifies a potential DAPK-1 catalytic kinase function in these processes.

A cell growth assay was developed to measure the effect of MAP1B on DAPK-1-induced growth suppression. Using this assay it was established that MAP1B is a positive regulator of DAPK-1-induced growth suppression, synergizing with DAPK-1 in this assay (Fig. 3). However, this did not provide mechanistic data, and so it led us to undertake a series of assays to obtain information pertaining to the mode of growth inhibition. No evidence was obtained suggesting that DAPK-1 and MAP1B cooperate to induce apoptosis (Fig. 4) and yet cell morphological assessment of transfected cells clearly demonstrated that MAP1B cooperates to induce plasma membrane blebbing (Fig. 5) commensurate with accumulation of autophagic vesicles (Fig. 6). This reproduces independent studies showing that DAPK-1 induces caspase-independent membrane blebbing autonomous of apoptosis. Also, DAPK-1-induced membrane blebbing is completely blocked by inhibition of autophagy (Fig. 7). This corroborating evidence clearly demonstrates that membrane blebbing functions independently of apoptosis and has a role in autophagic cell death.

The existence of autophagic cell death remains a topic for debate because autophagy is also a mechanism to maintain homeostasis. Controlled breakdown of intracellular components provides a nutrient supply during starvation and other stresses where removal of damaged organelles, such as mitochondria with reduced membrane potential, can protect cells from damage. The mechanism of how autophagy contributes to cell death is largely unknown. Both apoptotic and autophagic processes may be regulated by the same pathways, although one form of cell death can be enhanced when the other is blocked. As such, activation of autophagy during death may be a cause of lethality or may actually be a futile attempt at rescue. The DAPK-1 family proteins are one of the first molecules described to directly regulate autophagy as a mode of cell death. It has been proposed that autophagic cell death is a more ancient-based type of cell death program, because many of the autophagy-associated genes are evolutionarily conserved between yeast and mammalian organisms. However, the DAPK-1 family does not have related paralogues in yeast, and so it probably evolved afterward to link the basic evolutionarily conserved autophagic machinery and the cell signaling machinery.

Cell membrane blebbing involves complex regulation of the local proteome of the contractile cortex underneath the cell membrane. This involves changes in the abundance and makeup of cytoskeletal elements around the contractile ring of forming blebs, providing motor forces. Given that both DAPK-1 and MAP1B locate to the cell cortex and to the
surface of blebs, it is likely that they positively regulate membrane blebbing by interaction with the contractile ring and cortex of the blebs. However, previously published data suggest that DAPK-1-induced membrane blebbing involves interaction with actin stress fibers (17, 18) leading to perturbation of contractile forces and cell detachment. During the course of our studies, no evidence of DAPK-1 interaction with stress fibers was found; therefore, further study is required to reconcile this discrepancy. Until recently, it has been thought that DAPK-1 exerts its pro-death effects through association with actin where it affects the microfilament network within cells. However, DAPK-1 strongly interacts with the microtubule cytoskeleton as well as microfilaments. It could be that the microtubule-associated fraction of DAPK-1 has not been previously observed because the cytoplasmic fraction shrouds the decorated microtubules so that they can only be observed after partial detergent extraction of the cytoplasm during fixation (as in Fig. 8).

DAPK-1 also disrupts the cytoskeletal integrity of cells by down-regulating integrin-mediated cell adhesion leading to suppression of focal adhesion kinase-associated integrin survival signals commensurate with activation of the p53 pathway (18). Furthermore, the cytoskeleton is disrupted by perturbation of the balance between cell contractile forces, via stress fibers, and cell attachment via integrins, contributing to DAPK-1-mediated death-promoting activity via an anoikis-like mechanism. DAPK-1 is therefore activated during cell detachment and can also help to actively detach a cell from the extracellular matrix leading to cell death. This presumably creates a feedback circuit to suppress cell movement events or to prevent tumor metastasis. It is interesting that DAPK-1-mediated blebbing is stimulated by amino acid starvation and can be blocked by autophagy inhibition. These novel observations may provide a link between regulation of cell migration and nutrient availability. This could have implications for the regulation of tumor metastasis where abhorrent cell migration could be stimulated by changes in the local concentration of metabolites. DAPK-1 would therefore be a prime candidate for regulation of this process.

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Regulation of DAPK-1 and MAP1B Interactions

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