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Investigating the Regulation of Brain-specific Kinases 1 and 2 by Phosphorylation*

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Brain-specific kinases 1 and 2 (BRSK1/2) are AMP-activated protein kinase (AMPK)-related kinases that are highly expressed in mammalian forebrain. Studies using transgenic animal models have implicated a role for these kinases in the establishment of neuronal polarity. BRSK1 and BRSK2 are activated by phosphorylation of a threonine residue in the T-loop activation segment of the kinase domain. *In vitro* studies have demonstrated that LKB1, an upstream kinase in the AMPK cascade, can catalyze this phosphorylation. However, to date, a detailed comparative analysis of the molecular regulation of BRSK1/2 has not been undertaken. Here we present evidence that excludes another upstream kinase in the AMPK cascade, Ca^{2+}/calmodulin-dependent protein kinase kinase β, from a role in activating BRSK1/2. We show that equivalent mutations in the ubiquitin-associated domains of the BRSK isoforms produce differential effects on the activation of BRSK1 and BRSK2. Contrary to previous reports, activation of cAMP-dependent protein kinase does not affect BRSK1 or BRSK2 activity in mammalian cells. Furthermore, stimuli that activate AMPK had no effect on BRSK1/2. Finally, we provide evidence suggesting that protein phosphatase 2C is a likely candidate for catalyzing the dephosphorylation and inactivation of BRSK1/2.

BRSK1[4] (also referred to as SAD-B) and BRSK2 (SAD-A) are mammalian serine/threonine kinases that along with 10 other kinases form the AMPK-related family of protein kinases (1). BRSK1 and BRSK2 are evolutionarily conserved, and orthologs exist in mice, *Caenorhabditis elegans* (SAD-1), *Drosophila* (CG6114), and ascidians (HrPOPK-1). Initial studies using *sad-1* mutants in *C. elegans* identified a function for the kinase in regulating synaptic vesicle distribution and development of normal synapses (2). Subsequently, BRSK1 has been shown to localize to and associate with synaptic vesicles in mouse hippocampus and cerebellum as well as in cultured rat primary hippocampal neurons (3). Knock-out mice that lack both BRSK1 and BRSK2 have defects in neuronal polarity and die at ~2 h after birth (4). Embryos from the knock-out mice have visibly thinner cortices when compared with control mice due to disordered subplate layers, and neurons lack distinct axonal and dendritic processes. Information regarding the potential downstream targets for BRSK1/2 is very limited; however, a number of AMPK-related kinases, including BRSK1 and BRSK2, phosphorylate tau, a microtubule-associated protein that regulates stability of the microtubule network (4). The phosphorylation of tau may contribute to the polarity phenotype observed in the BRSK1/2 knock-out mice.

LKB1 is a tumor suppressor kinase linked to the rare hereditary cancer predisposition, Peutz-Jeghers syndrome (5). In complex with regulatory proteins Ste-20 related adaptor (STRAD) and mouse protein 25 (MO25), LKB1 phosphorylates and activates the AMPK-related kinases by phosphorylation of a specific threonine residue within the highly conserved T-loop activation domain (1, 6, 7). A role for LKB1 in cell polarity has been described, and its ortholog in *C. elegans* (*Par4*) is one of six polarity regulators governing embryonic development (reviewed in Ref. 8). Recently, a mouse model was generated in which deletion of the *LKB1* gene was limited to pyramidal neurons of the cortex (9). Although the cortex of these transgenic animals was normal in overall size, the ventricles were larger and the cortical wall thinner when compared with control animals. Immunofluorescence analyses revealed a lack of cortical axons in the LKB1-deficient mice and a phenotype similar to the BRSK1/2 double knock-out mice (4). In another study, down-regulation of LKB1 and STRAD using siRNAs prevented axon differentiation, whereas overexpression of these proteins led to multiple axon formation (10). Previous studies have demonstrated that LKB1 is phosphorylated by cAMP-dependent protein kinase (PKA) at serine 431 in the mouse protein (equivalent to serine 428 in the human sequence), although phosphorylation at this residue had no detectable effect on LKB1 activity (11, 12). Interestingly, however, a role for the PKA-dependent phosphorylation of LKB1 was suggested from the studies examining LKB1 in neuronal polarization (9, 10). Recently, a pathway has been proposed in which extracellular signals are transduced into a phosphorylation cascade requiring PKA, LKB1, and BRSK1/2 and resulting in axon specification through phosphorylation of microtubule-associated proteins (9).

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[4] The abbreviations used are: BRSK, brain-specific protein kinase; AMPK, AMP-activated protein kinase; CaMKKβ, Ca^{2+}/calmodulin-dependent protein kinase kinase β; PKA, cAMP-dependent protein kinase; CREB, cAMP response element-binding protein; HA, hemagglutinin; MO25, mouse protein 25; pfu, plaque-forming units; PP2C, protein phosphatase 2C; STRAD, Ste-20 related adaptor; UBA, ubiquitin-associated.

Although transgenic animal studies indicate an essential role for BRSK1 and BRSK2 in neuronal development, a detailed assessment of the molecular basis for their regulation has not been reported. Here, we present data characterizing the regulation of BRSK1 and BRSK2 by phosphorylation. We find that in contrast to LKB1, CaMKKβ does not activate BRSK1 or BRSK2 in mammalian cells. We show that an equivalent mutation within the ubiquitin-associated (UBA) domains of BRSK1 and BRSK2 has differential effects on their activity. We found that PKA does not directly regulate the activity, or activation by LKB1, of BRSK1 or BRSK2. In addition, a number of stimuli that lead to activation of AMPK in mammalian cells have no effect on the activity of BRSK1/2. Finally, we present data examining the role of protein phosphatases on the regulation of BRSK1/2.

EXPERIMENTAL PROCEDURES

Plasmids—BRSK2 was cloned into the pcDNA3.1a/Myc-His mammalian expression vector (Invitrogen). pCMV-BRSK1-HA and cDNA encoding human LKB1 were kind gifts from Prof. D. Alessi (University of Dundee). cDNA encoding CaMKKβ was a gift from Dr. H Tokumitsu (Kagawa Medical University). Human LKB1 and CaMKKβ were cloned into the pAd-5CMV plasmid for adenovirus production (ViraQuest). cDNA encoding mouse LKB1 was a gift from Prof. A. Ashworth and was cloned into pcDNA3. Point mutations encoding the single amino acid substitutions, G343A for BRSK1, T174A, T174E, and G310A for BRSK2, and D159A, S431A, and S431E for LKB1, were generated using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions.

Cell Culture—Human liver epithelial CCL13 cells and human embryonic kidney HEK293 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 2 mM L-glutamate. For transfection, CCL13 and HEK293 cells were seeded into 10-cm plates at 0.5×10^6 cells/plate and transfected using a calcium phosphate precipitation kit (Clontech). Cells were harvested 48–72 h after transfection. Human neuroblastoma SH-SY5Y cells were maintained in Dulbecco's modified Eagle's medium: F12 1:1 mix containing 10% fetal bovine serum, 2 mM L-glutamate, and 1% non-essential amino acids. SH-SY5Y cells were transfected using the AMAXA nucleofector kit (Amaxa Biosystems) according to manufacturer's instructions for this cell line. Cells were transferred into serum-free medium for treatment with adenovirus, forskolin, ionomycin, carbachol, or sorbitol as indicated in the figure legends (Figs. 2–6).

Cell Lysates—Cells were lysed in Buffer A (50 mM HEPES, pH 7.4, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM EDTA, 10% glycerol, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) containing 1% Triton X-100 for 30 min and then sonicated (10×1 s bursts). Insoluble material was removed by centrifugation at $20{,}000 \times g$ for 10 min. Protein content was determined by a Bradford assay (13).

Tissue Lysates—Mouse tissues were homogenized in 10 volumes (w/v) of homogenization buffer (20 mM Tris-HCl, pH 7.4, 10 mM EGTA, 2 mM EDTA, 50 mM NaF, 5 mM sodium pyrophosphate, 250 mM sucrose, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) and centrifuged for 30 min at $10{,}000 \times g$. The supernatant was used for further analysis.

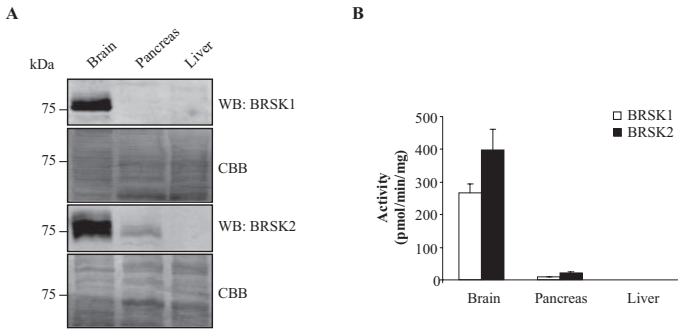

FIGURE 1. **BRSK expression and activity.** Mouse tissue homogenates (30 μg) were analyzed by Western blotting using anti-BRSK1 or anti-BRSK2 antibodies (*A*). Protein loading was visualized by staining the membranes with Coomassie Brilliant Blue (*CBB*) and is shown below each blot. BRSK1/2 were immunoprecipitated from mouse tissue homogenates (100 μg) with anti-BRSK1 or anti-BRSK2 antibodies, and kinase activity of the immune complexes was measured using the peptide substrate LNR (*B*). Results are plotted as pmol/min/mg of lysate and are the mean of at least three experiments ± S.D.

Antibody Production—Glutathione *S*-transferase fusion protein of full-length BRSK2 was generated and used to immunize rabbits.

Western Blotting—Samples (30–50 μg) were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membrane was blocked for 1 h in phosphate-buffered saline containing 5% skimmed milk and incubated with primary antibody overnight at 4 °C. After extensive washing with 20 mM Tris, pH 7.4, 0.5 M NaCl, 0.5% Tween 20, the membrane was incubated in secondary antibody conjugated to Alexa Fluor 680 (Invitrogen) or IRDye800 (LI-COR) and scanned on the LI-COR Odyssey infrared imaging system using Odyssey software 2.0 for band quantification. Hemagglutinin (HA)-tagged BRSK1 was detected using an anti-HA polyclonal antibody (AbCam), Myc-tagged BRSK2 was detected using an anti-Myc antibody (9B11, Cell Signaling), and CaMKKβ was detected using an anti-FLAG antibody (Sigma). Additional antibodies used were anti-BRSK1 (AbCam), anti-LKB1 (Ley37D/G6, Santa Cruz Biotechnology), anti-phospho-CREB (Ser-133; 87G3, Cell Signaling), anti-CREB (Sigma), and anti-glyceraldehyde-3-phosphate dehydrogenase (AbCam).

Dephosphorylation of BRSK2—BRSK2 was immunoprecipitated from 100 μg of cell lysate by incubation with protein G-Sepharose beads coupled to anti-Myc antibody. Immune complexes were incubated with recombinant PP2Cα (14) and 2.5 mM $MgCl_2$ in 50 mM Hepes for 20 min at 37 °C.

Assay of BRSK Activity—BRSK1/2 activity was measured by phosphorylation of the LNR peptide substrate (KKLNRTLSFAEPG (15)). BRSK1/2 was immunoprecipitated from 100 μg of cell lysate by incubation with protein A-Sepharose or protein G-Sepharose beads coupled to anti-HA or anti-Myc antibody, respectively, for 2 h at 4 °C. Following extensive washing with Buffer A, the immune complexes were incubated with 0.5 mM LNR and 0.2 mM [γ^{32}P]ATP, 5 mM $MgCl_2$ in Buffer A for 20 min at 37 °C. Radioactivity incorporated into the LNR peptide was determined by filter assay using phosphocellulose P-81 filter squares. Endogenous AMPK was immunoprecipitated from cell lysates using an anti-pan-β AMPK antibody (16), and

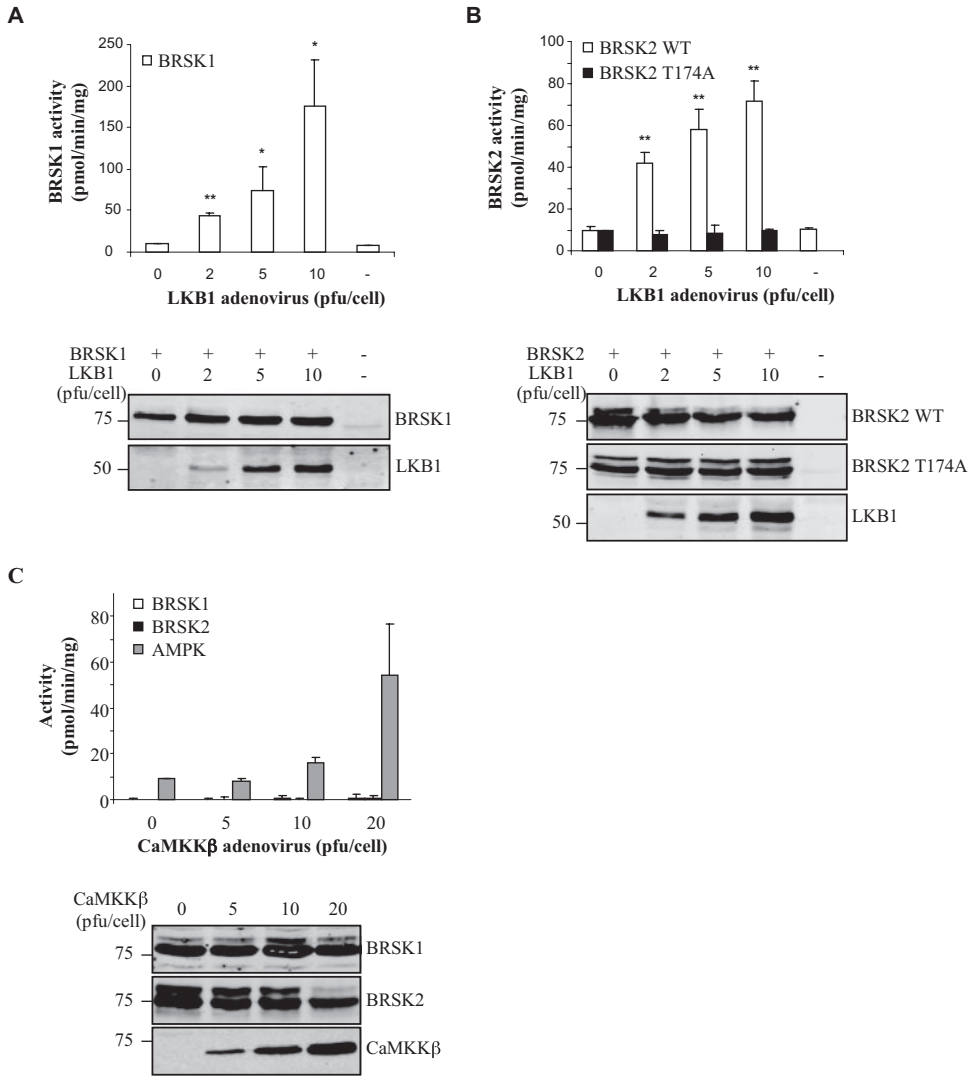

FIGURE 2. **Activation of BRSK1 and BRSK2 by LKB1 but not CaMKKβ.** CCL13 cells were transfected with BRSK1 (HA-tagged), wild-type (*WT*) BRSK2, or BRSK2 harboring the T174A mutation (both Myc-tagged) and infected with varying amounts of adenovirus, measured as pfu/cell, containing either LKB1 (*A* and *B*) or CaMKKβ (FLAG-tagged (*C*)). BRSK1 and BRSK2 were immunoprecipitated from cell lysates (100 μg) with either anti-HA or anti-Myc antibodies, and kinase activity was assayed using the synthetic peptide substrate LNR (*A* and *B*). Endogenous AMPK was immunoprecipitated from cell lysates, and activity was determined by the SAMS peptide assay (*C*). Results are plotted as pmol/min/mg of lysate and are the mean of at least three experiments ± S.D.; *, $p < 0.05$ or **, $p < 0.01$, significantly different from activity in the absence of LKB1. Protein expression was analyzed by Western blotting of cell lysates (30 μg) with anti-HA (BRSK1), anti-Myc (BRSK2), anti-LKB1, or anti-FLAG (CaMKKβ) antibodies, and in each case, a representative blot is shown.

AMPK activity was measured by phosphorylation of the SAMS peptide (17).

Statistical Analyses—Data are expressed as mean ± standard deviation from three or more independent experiments. Statistical significance was established by Student's *t* test. Data were considered significant at $p < 0.05$.

RESULTS

Expression of BRSK1 and BRSK2 in Mouse Tissues—Previous studies have demonstrated that BRSK1 and BRSK2 are expressed predominantly in the brain (3, 4). Recently, however, expression of both BRSK1 and BRSK2 was reported in pancreas (18). Since none of the previous studies compared the relative expression of BRSK isoforms, we determined expression and activity of BRSK1 and BRSK2 in the brain and pancreas (Fig. 1). As judged by Western blotting, both BRSK1 and BRSK2 were highly expressed in brain, with low level expression of BRSK2 in pancreas. However, we were unable to detect expression of BRSK1 in pancreas. Neither BRSK1 nor BRSK2 expression was detected in liver nor in any other tissues we examined (data not shown). Consistent with the protein expression, the activity of both BRSK1 and BRSK2 was readily detected in immune complexes isolated from brain. Low, but significant, activity of both isoforms was also detectable in pancreas. No activity was detected in other tissues, including liver.

LKB1 but Not CaMKKβ Activates BRSK Activity—To begin characterizing the regulation by upstream kinases, we transfected CCL13 cells with BRSK1 and BRSK2. As CCL13 do not express LKB1 (7), we manipulated the levels of LKB1 expression using an adenoviral delivery system (Fig. 2*A* and *B*, *bottom panels*). In the absence of LKB1 expression, virtually no activity was detected for either BRSK1 or BRSK2. Increasing levels of LKB1 expression (as judged by Western blotting) resulted in increasing levels of BRSK1 and BRSK2 activity, in the absence of any additional stimuli (Fig. 2, *A* and *B*). Increasing the dosage of LKB1 beyond 10 plaque-forming units (pfu)/cell did not increase BRSK1 or BRSK2 activity further (data not shown). Expression of LKB1 had no obvious effect on the level of expression of either BRSK1 or BRSK2, as judged by Western blotting. To confirm that BRSK2 activation required phosphorylation of Thr-174 within the T-loop activation domain (1), we expressed a mutant construct of BRSK2 in which threonine 174 was mutated to alanine (T174A). No activation of this mutant form of BRSK2 was observed, even at the highest concentration of LKB1, confirming that this residue is critical for kinase activity of BRSK2. Mutation of Thr-174 to alanine had no detectable effect on expression of BRSK2 (Fig. 2*B*). However, Western blot analysis of BRSK2 revealed the presence of a distinct doublet. This doublet corresponds to BRSK2 since the bands are only detected following expression of BRSK2. Although we have been unable to determine the reason for BRSK2 migrating as a doublet on SDS-PAGE, it appears to be related

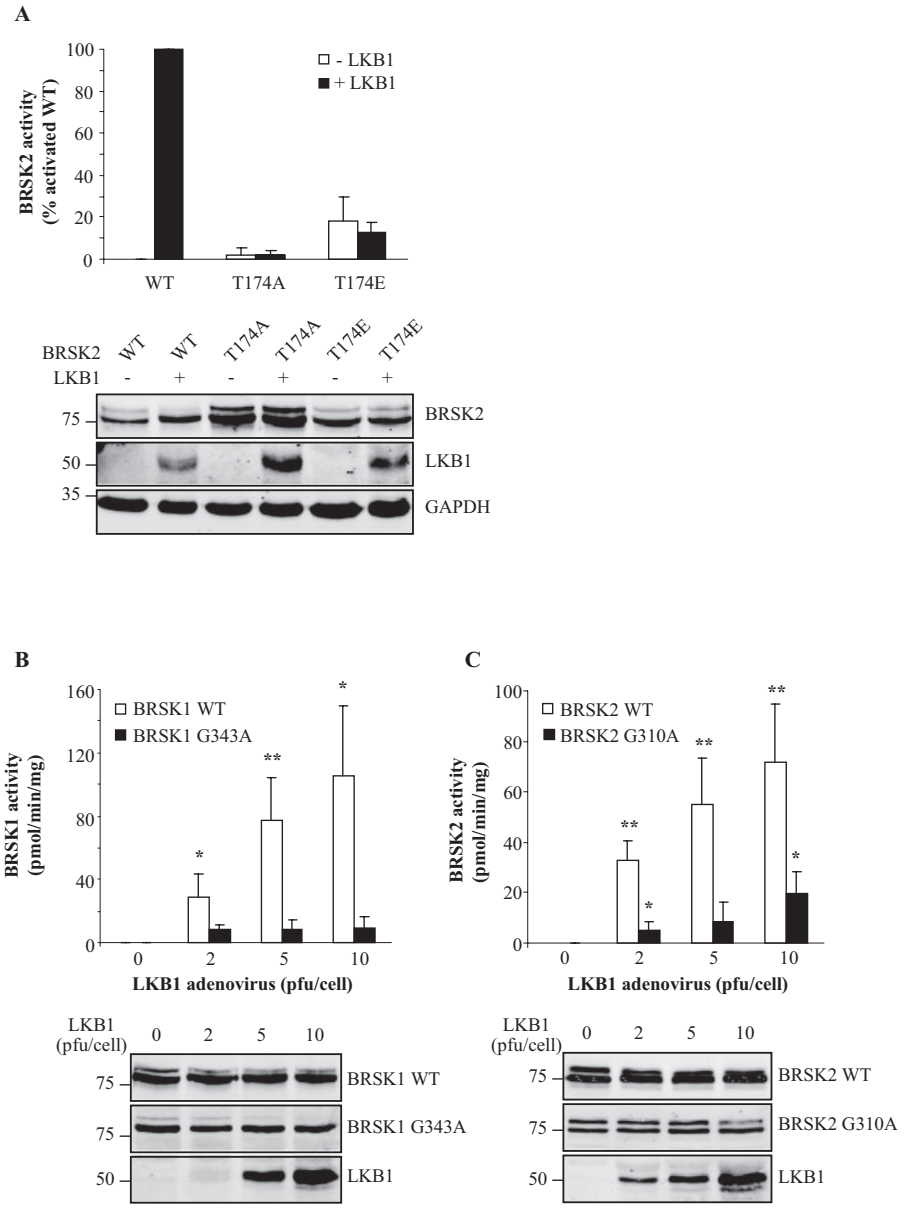

FIGURE 3. **Activation of BRSK1 and BRSK2 mutants by LKB1.** CCL13 cells were transfected with wild-type BRSK2 (*WT*) or BRSK2 harboring either the T174A or the T174E mutation (*A*), wild-type BRSK1 or BRSK1 harboring the G353A mutation (*B*), or wild-type BRSK2 or BRSK2 harboring the G310A mutation (*C*). Cells were infected with the indicated amount of adenovirus containing LKB1 (10 pfu/cell in *A*), and BRSK1 and BRSK2 activity was measured in immune complexes using the LNR peptide assay. Results are plotted either as the percentage of activity of wild-type BRSK2 in the presence of LKB1 (*A*) or pmol/min/mg of lysate (*B* and *C*) and are the mean of at least three experiments ± S.D.; *, $p < 0.05$ or **, $p < 0.01$, significantly different from activity in the absence of LKB1. In each case, a representative Western blot is included to show protein expression levels. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

to the activation state of BRSK2 since the intensity of the slower migrating form diminishes in parallel with activation. A doublet is also detected for the T174A mutant, although in this case, the relative intensities do not change following expression of LKB1 (Fig. 2*B*).

In addition to LKB1, AMPK has been shown to be phosphorylated and activated by Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ (19–21)). Increasing intracellular Ca^{2+} levels lead to activation of AMPK through a CaMKKβ-mediated pathway (19, 21–23). In an attempt to determine whether CaMKKβ activates BRSK1/2, we co-expressed either BRSK1 or BRSK2 with CaMKKβ in CCL13 cells and determined BRSK activity. We were unable to detect any activity for either BRSK1 or BRSK2 following expression of CaMKKβ, despite significant activation of endogenous AMPK (Fig. 2*C*). These results suggest that although there is a high degree of conservation surrounding the T-loop residue within AMPK and BRSK1/2, CaMKKβ is not capable of phosphorylating and activating BRSK1 or BRSK2.

Role of T-loop Phosphorylation and UBA Domain on BRSK1 and BRSK2 Activity—As demonstrated by us and others (1), phosphorylation of threonine 174 is essential for activation of BRSK2. The sequence surrounding the equivalent threonine residue in BRSK1 (Thr-189) is identical to that in BRSK2, and a previous study reported that phosphorylation of Thr-189 is essential for BRSK1 activity (1). In that same study, it was reported that mutation of the T-loop residue to glutamic acid led to a constitutively active form of the kinase expressed in *Escherichia coli* (1). We generated the T174E mutant for BRSK2 and expressed it in CCL13 cells. Irrespective of LKB1 expression, BRSK2 activity of this mutant was ~15% of the activated, wild-type kinase (Fig. 3*A*). Our results suggest that the glutamic acid substitution is not sufficient for maximal activity of BRSK2 in mammalian cells.

A number of the AMPK-related kinases, including BRSK1 and BRSK2, contain a UBA domain, immediately C-terminal to the kinase domain (24). The precise function of these domains in the AMPK-related kinases is unclear, although some studies have suggested that they may play a role in the stability of the kinase domain (24, 25). In addition, there is evidence that the presence of the UBA domains is required for phosphorylation and activation by LKB1 (24). To determine the effect of the UBA domain on BRSK activity, we generated constructs for expression of BRSK1 and BRSK2 harboring a mutation of a glycine to alanine residue (Gly-343 in BRSK1, Gly-310 in BRSK2) within the UBA domain. These glycine residues are conserved throughout the AMPK-related kinase family and have been identified previously as critical for UBA function (26). The G343A mutation almost completely abolished activa-

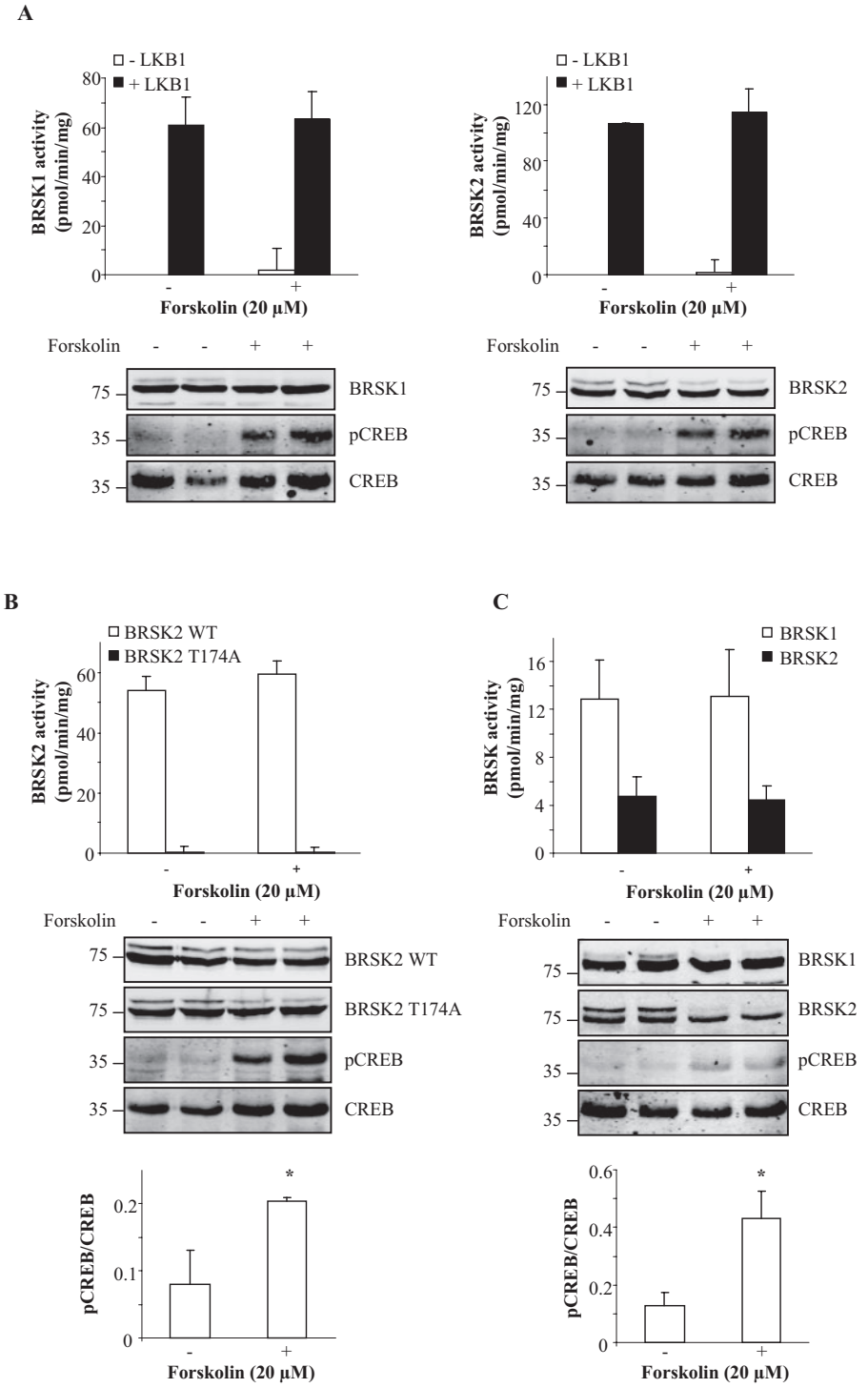

FIGURE 4. **Activation of PKA by forskolin has no effect on BRSK1 or BRSK2 activity.** CCL13 (*A*), HEK293 (*B*), or SH-SY5Y (*C*) cells were transfected with BRSK1, wild-type (*WT*) BRSK2, or BRSK2 harboring the T174A mutation. Additionally, CCL13 cells were infected with adenovirus containing LKB1 (10 pfu/cell) as indicated on the figure. 48 h after transfection, cells were incubated with 20 μM forskolin or a vehicle control for 15 min prior to harvesting. BRSK1 and BRSK2 activity was determined in immune complexes by the LNR peptide assay. Results are plotted as pmol/min/mg of lysate and are the mean of at least three experiments ± S.D. Activation of PKA was monitored by determining the level of phosphorylation of CREB relative to total CREB by Western blotting. Bar charts showing the ratio of phospho-CREB (*pCREB*):total CREB are shown (±S.D.) as well as representative blots. Significant differences in the ratio of phospho-CREB in the presence of forskolin relative to vehicle are shown, *, $p < 0.05$.

tion of BRSK1 by LKB1 (Fig. 3*B*) without significantly affecting the level of expression of BRSK1. In contrast, although the G343A mutation reduced BRSK2 activity, there was still significant activation of the mutant form of the kinase, which increased following increasing expression of LKB1 (Fig. 3*C*). At the maximum level of LKB1 expression (10 pfu/cell), BRSK1 G343A activity was less than 10% of the wild-type activity, whereas BRSK2 G310A was 30% of wild type. These results suggest that a functional UBA domain is not as essential for BRSK2 activity, or phosphorylation by LKB1, as it is for BRSK1 activation.

Activation of the PKA Pathway Does Not Affect BRSK Activity—A previous study reported that *in vitro* PKA phosphorylates threonine 260 within BRSK2, increasing its activity (27). Taken together with the finding that LKB1 is phosphorylated by PKA (11, 12), these results imply that PKA may act upstream of a LKB1-BRSK pathway. To test whether this might be the case in a mammalian cell system, we co-expressed either BRSK1 or BRSK2 together with LKB1 in CCL13 cells and measured BRSK activity following activation of the PKA pathway with the adenylate cyclase activator, forskolin. Consistent with previously published data (12), forskolin treatment (15 min, 20 μM) resulted in a significant increase in phosphorylation of cAMP-response element-binding protein (CREB), a well established substrate of PKA (Fig. 4*A*). However, there was no effect of forskolin treatment on either BRSK1 or BRSK2 activity, either in their inactive, unphosphorylated forms or in their active, phosphorylated forms (Fig. 4*A*). We carried out a similar experiment in HEK293 cells, in which LKB1 is expressed endogenously (7). As with the CCL13 cells, forskolin treatment increased phosphorylation of CREB but had no effect on the activity of wild type BRSK2 (Fig. 4*B*). Similarly, forskolin treatment did not activate the T174A mutant form of BRSK2. Finally, we expressed BRSK1 and BRSK2 in the human neuroblastoma cell line, SH-SY5Y. Since BRSK1/2 are predominantly expressed in the brain, these cells are more likely to provide a closer physiologically relevant background for BRSK activity. In addition, these

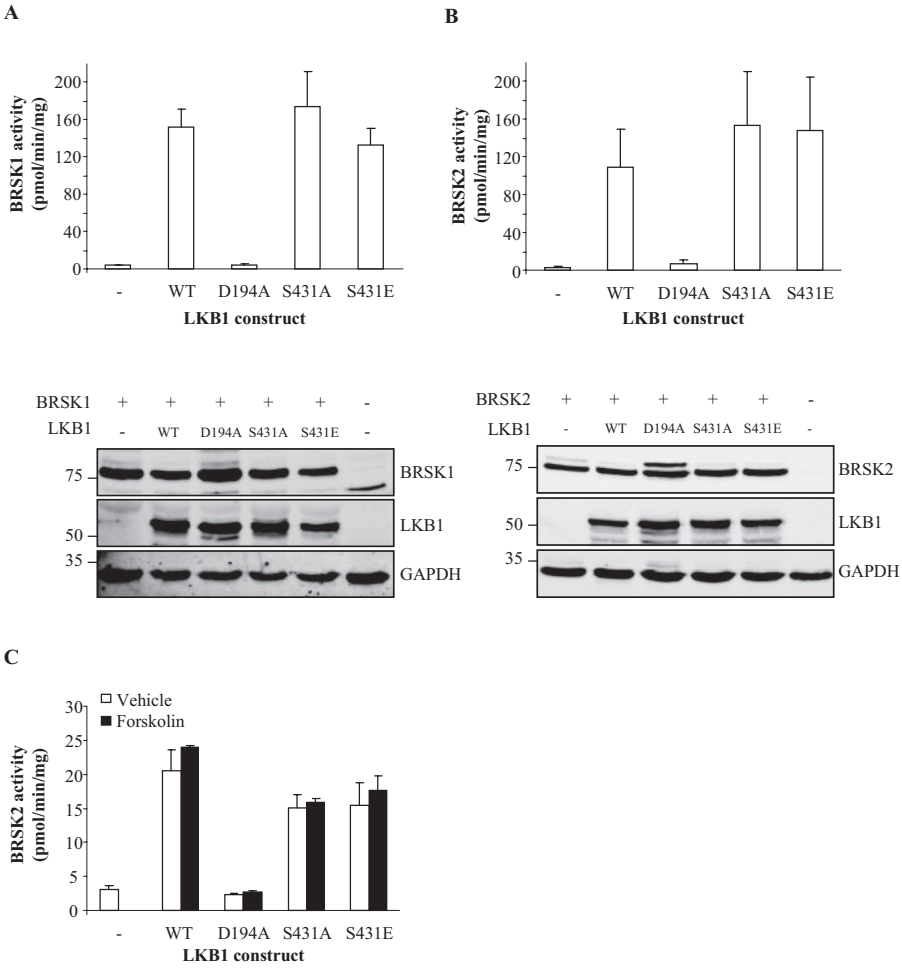

FIGURE 5. **Activation of BRSK1 and BRSK2 by LKB1 mutants.** CCL13 cells were co-transfected with BRSK1 (*A*) or BRSK2 (*B*) and either wild-type (*WT*) LKB1 or the various LKB1 mutants, as indicated. BRSK activity was determined using the LNR peptide assay. Results are plotted as pmol/min/mg of lysate and are the mean of at least three experiments ± S.D. In each case, representative Western blots show protein expression levels. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. *C*, 48 h after transfection with BRSK2 and the various LKB1 mutants indicated, CCL13 cells were treated with either 20 μM forskolin or vehicle control for 15 min prior to harvesting and determination of BRSK2 activity, as above. Results are plotted as pmol/min/mg of lysate and are the mean of three experiments ± S.D.

cells also express endogenous LKB1.[5] Although the activity of both BRSK1 and BRSK2 was lower in the SH-SY5Y cells when compared with either CCL13 cells or HEK293 cells, forskolin treatment had no effect on their activity (Fig. 4*C*), although it significantly increased phosphorylation of CREB.

Mutating the PKA Phosphorylation Site within LKB1 Has No Effect on BRSK Activity—PKA has been shown to phosphorylate LKB1 at serine 431 (in mouse LKB1, equivalent to serine 428 in human LKB1) (11, 12). Although our results described above do not support a role for the PKA pathway in the activation of BRSK1 or BRSK2 by LKB1, it remained possible that phosphorylation of LKB1 at serine 431 was important in the activation process. Therefore, to test this possibility, we generated two mutant forms of LKB1, harboring a substitution of serine 431 to either alanine (S431A) or glutamic acid (S431E) and used these to co-express with either BRSK1 or BRSK2 in CCL13 cells. As a control, we also constructed a catalytically inactive form of LKB1, harboring a mutation of aspartic acid 194 (in the ATP-binding site of the kinase domain) to alanine (D194A). Consistent with our previous studies, co-expression of wild-type LKB1 was essential for activation of BRSK1 and BRSK2, whereas no activity was detected following expression of kinase-dead LKB1 (Fig. 5). Co-expression of LKB1 harboring either the S431A or the S431E mutations resulted in BRSK1 and BRSK2 activities similar to those obtained following expression of wild-type LKB1. The mutations within LKB1 had no obvious effect on the level of expression of either LKB1 or the BRSK isoforms, as judged by Western blotting. As we noted in our earlier experiments, under conditions where BRSK2 was inactive, Western blotting revealed a distinct doublet. A similar double, albeit with a fainter slower migrating band, was also detected for BRSK1. As with BRSK2, the intensity of the slower migrating band was strongest in conditions where BRSK1 was inactive. Forskolin treatment of cells co-expressing the LKB1 mutants and BRSK2 had no effect on BRSK2 activity (Fig. 5*C*). These results strongly suggest that activation of PKA does not directly activate BRSK2 or increase its activation by LKB1.

Implicating PP2C in the Regulation of BRSK Activation—Despite growing interest, the signaling pathways leading to activation of the AMPK-related kinase, including BRSK1/2, remain poorly understood. In an attempt to identify stimuli that lead to activation of BRSK1/2, we treated cells with ionomycin (a calcium ionophore), carbachol (an acetylcholine mimetic), and sorbitol (hyperosmotic stress). However, none of these treatments had any effect on the activity of endogenous BRSK2 in SH-SY5Y neuronal cells (Fig. 6*A*). Under these conditions, the activity of BRSK1 was barely detectable, and there was no evidence of activation. In contrast, all the treatments led to significant activation of AMPK. Next, we treated cells with okadaic acid at a concentration that would be expected to inhibit both protein phosphatase 1 and protein phosphatase 2A activity (28). Okadaic acid markedly increased phosphorylation of CREB (Fig. 6*B*), proving its efficacy in these cells. However, there was no significant effect of okadaic acid treatment on the activity of either BRSK1 or BRSK2 (Fig. 6*B*), indicating that dephosphorylation and inactivation of BRSK1/2 is not catalyzed by okadaic acid-sensitive phosphatases. As an initial attempt to identify the physiologically relevant phosphatase involved in regulating BRSK1/2, we determined whether

[5] N. J. Bright, D. Carling, and C. Thornton, unpublished results.

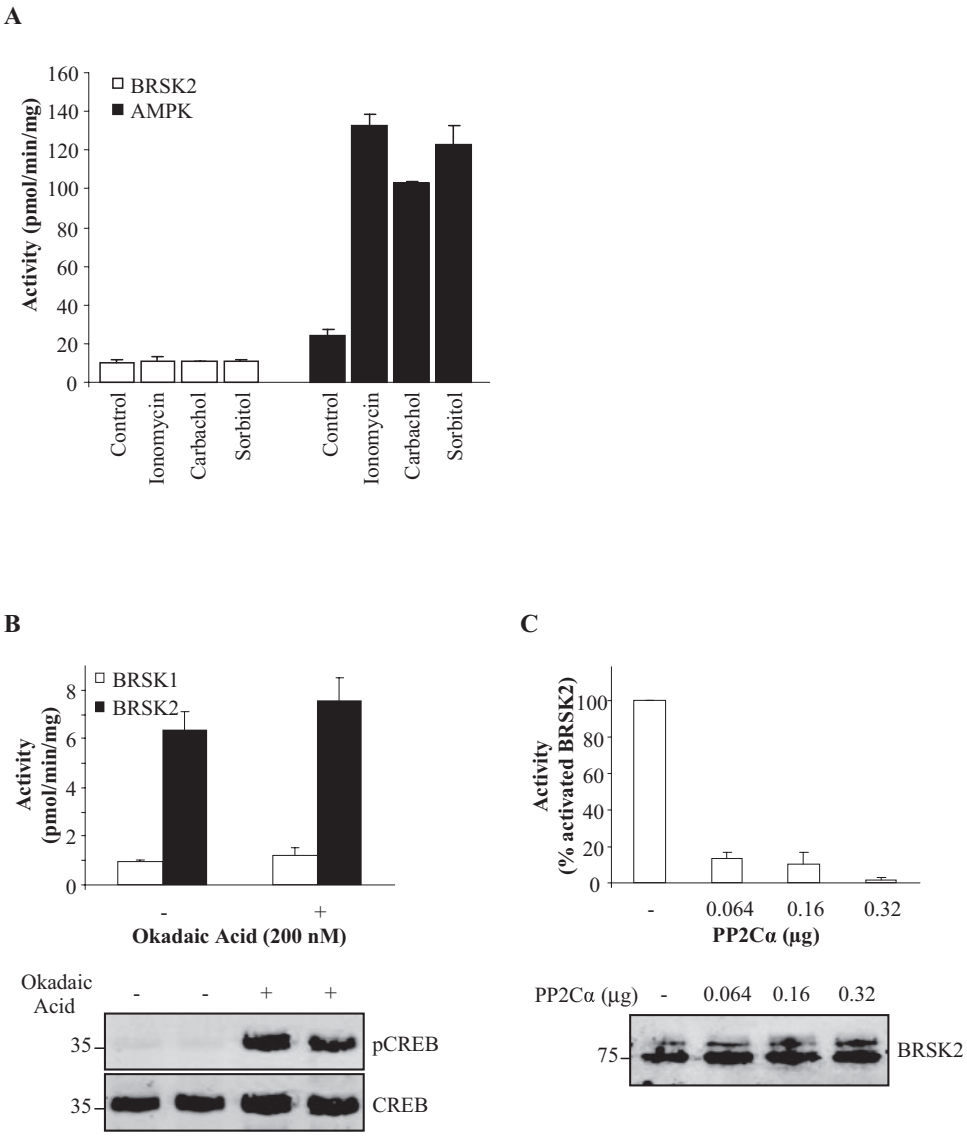

FIGURE 6. **Effect of various treatments on BRSK2 activity.** SH-SY5Y cells were incubated with ionomycin (1 μM, 5 min), carbachol (10 μM, 5 min), or sorbitol (0.5 M, 30 min). BRSK2 was immunoprecipitated from SH-SY5Y lysates (100 μg) and activity measured by LNR substrate assay (*A*). SH-SY5Y cells were treated with okadaic acid (200 nM, 45 min), and BRSK2 activity was determined as above (*B*). In both cases, results are plotted as pmol/min/mg and are the average of at least three experiments ± S.D. Phosphatase inhibition by okadaic acid was monitored by determining the levels of phospho-CREB (*pCREB*) relative to total CREB (*B*, *bottom panel*). *C*, active BRSK2 immunoprecipitated from CCL13 co-expressing LKB1 was incubated with increasing amounts of recombinant PP2Cα for 20 min. Following washing of the immune complexes to remove PP2C, BRSK2 activity was determined. Results are plotted as the percentage of activity determined in the absence of PP2C and are the mean value from three experiments ± S.D. Western blot analysis was used to verify the presence of equal amounts of BRSK2 (*C*, *bottom panel*).

PP2C, an okadaic acid-insensitive phosphatase, could dephosphorylate BRSK2 *in vitro*. As can be seen from Fig. 6*C*, recombinant PP2C efficiently inactivated BRSK2.

DISCUSSION

Emerging data on the roles of LKB1, BRSK1 (SAD-B), and BRSK2 (SAD-A) suggest that this kinase cascade is essential for regulating polarity in embryonic cortical development. Recently, the function of LKB1 has been studied in a variety of systems, and our understanding of the role of this kinase in regulating cell growth and metabolism is becoming clearer. However, for the most part, the function of the AMPK-related kinases, including BRSK1 and BRSK2, remains enigmatic. In this study, we have begun to address the regulation of BRSK1 and BRSK2 by phosphorylation.

Although most studies have focused on the role of BRSK1/2 in the brain, a recent report showed that they are also expressed in pancreas and that they may be involved in the induction of acinar polarity (18). In this study, we detected expression of BRSK1 and BRSK2 in pancreas, although at a greatly reduced level when compared with brain. In both tissues, BRSK2 activity is substantially greater than BRSK1.

LKB1 acts as a master upstream kinase activating AMPK and the AMPK-related kinases (1). More recent studies identified CaMKKβ as an alternative upstream kinase in the AMPK pathway (19–21). Surprisingly, although BRSK1 and BRSK2 are the closest neighbors to the catalytic subunits of AMPK in the human kinome, the results of our present study indicate that CaMKKβ is unable to activate BRSK1 or BRSK2 in mammalian cells. In contrast, LKB1 was able to activate both BRSK1 and BRSK2. LKB1 forms a heterotrimeric complex with two other proteins, STRAD and MO25 (29, 30), and association with these proteins is essential for optimal kinase activity of LKB1. One possibility that may distinguish phosphorylation of downstream targets by LKB1 *versus* CaMKKβ may be related to the heterotrimeric nature of the LKB1 complex. It is possible that either STRAD or MO25 is involved in binding to substrate proteins, facilitating phosphorylation by LKB1. Indeed, the crystal structure of MO25 (1UPK) reveals that it may act as a scaffold protein, allowing binding of proteins to the LKB1-STRAD-MO25 complex (31). However, we have not been able to detect a direct interaction between BRSK1 or BRSK2 and LKB1 in co-immunoprecipitation assays from cell lysates[5], suggesting that if there is an interaction, it does not survive the conditions we have employed.

An important technical point to note from our study is that in contrast to a previous study in which mutation of Thr-174 within BRSK2 to an acidic glutamic acid residue resulted in full kinase activity (1), we observed very low activity of this mutant.

Similar findings to ours have been reported recently for other AMPK-related kinases, SIK (32), Par1 in *Drosophila* (33), and QIK.[5] These data suggest that glutamic acid mutations of the T-loop residue in the AMPK-related kinases do not lead to full, constitutive activity, at least not in mammalian cells. The initial report that these mutations led to active kinases was carried out using expression in *E. coli*, and it is possible that in the bacterial system, differences in protein folding may allow the mutant proteins to retain full activity. Nonetheless, care should be taken before considering that the mutant forms of the AMPK-related kinases are constitutively active in mammalian cells.

Ten out of the twelve AMPK-related kinases possess a UBA domain (24), an ~45-amino-acid domain that is involved in binding ubiquitin (34). However, the function of these UBA domains within the AMPK-related kinases is unclear. Recent biochemical analysis suggests that none of the UBA domains in the AMPK-related kinases bind polyubiquitin or other ubiquitin-related molecules (24). Structural studies of the AMPK-related kinases, MARK2 and MARK3, have indicated that the function of the UBA domain may be to regulate phosphorylation, and therefore activity, of the kinase, through conformational changes (24, 25). Based on structural analysis of the catalytic and UBA domains of MARK3 (25), it has been proposed that the ubiquitin-binding function of the UBA domain may have evolved to interact with the kinase domain, at the expense of binding ubiquitin. This interaction can stabilize the open conformation of the kinase domain, allowing phosphorylation by LKB1. We found that mutation of a key residue (Gly-343) within the UBA domain of BRSK1 almost completely abolished its activation by LKB1. However, mutation of the equivalent residue in BRSK2 (Gly-310) reduced, but did not prevent, activation by LKB1. It is possible that the kinase domain of BRSK2 can exist in an open conformation, even in the absence of a functional UBA domain, whereas this is not the case with BRSK2. In a previous study, removal of the UBA domain or mutation of the conserved glycine residue in a number of the AMPK-related kinases reduced, but did not abolish, kinase activity (24). However, in that study, the effects on BRSK1 were not reported.

BRSK2 has been reported previously to be phosphorylated by PKA on Thr-260, leading to increased BRSK2 activity *in vitro* (27). LKB1 has also been identified as substrate for PKA, although there is no evidence to date that this directly regulates LKB1 activity (11, 12). In our current study, we were unable to find any evidence for a direct role of PKA in the regulation of BRSK1 or BRSK2 or in their activation by LKB1. In previous studies, phosphorylation of LKB1 by PKA had no effect on its subcellular localization (11, 12). Additionally, we have been unable to detect any alteration in the subcellular localization of either BRSK isoforms or LKB1 under any of the conditions we have examined.[5] Despite this lack of evidence for a direct role of PKA on the LKB1/BRSK pathway, it was recently reported that phosphorylation of LKB1 by PKA is required for the regulation of polarity in cortical neurons (9). Furthermore, it was speculated that this signaling pathway required activation of BRSK1/2. Although we cannot rule out the possibility that direct regulation of either LKB1 or BRSK1/2 by PKA may occur in specific types of cells, *e.g.* subpopulations of neuronal cells, our data using a neuroblastoma cell line are not consistent with this hypothesis. An alternative possibility is that PKA could play an indirect role, *e.g.* altering the long term expression or localization of LKB1 and/or BRSK1 and BRSK2. In our system, we have looked at the acute (15-min) effects of stimulating the PKA pathway. It is conceivable that longer term changes in expression or localization could be important for alterations in cell polarity and growth.

In this study, we have examined the regulation of two of the AMPK-related kinases by phosphorylation. Interestingly, in contrast to AMPK, activation in mammalian cells was dependent on LKB1 expression and could not be achieved via CaMKKβ. At the moment, the basis for this specificity in upstream signaling is unknown. We have shown unambiguously that PKA has no direct effect on activation of BRSK1/2. How activation of BRSK1 and BRSK2 is achieved *in vivo* remains a key issue. In an attempt to address this, we treated SH-SY5Y neuroblastoma cells with a number of stimuli but were unable to detect activation of BRSK2. We are left, therefore, with the conclusion that there must be as yet unidentified signals that regulate BRSK1 and BRSK2 activation. The evidence to date strongly suggests that LKB1 is constitutively active. In our study, we find that co-expression of BRSK1 or BRSK2 with LKB1 results in significant activity in the absence of any additional stimuli. It is difficult to understand the relevance of a system in which the downstream kinase in a kinase cascade is permanently active. However, a consequence of our findings is that we predict that these signals will work via the dephosphorylation of BRSK1/2, rather than through stimulation of LKB1. This would be similar to the mechanism underlying the regulation of the LKB1-AMPK cascade. In this case, an increase in AMP inhibits dephosphorylation of Thr-172 within AMPK (35, 36), whereas for BRSK1 and BRSK2, the signaling molecule has not yet been identified. As a first step to determine whether this is the case, we have shown in this study that BRSK1/2 do not appear to be substrates for protein phosphatase 1 or 2A in neuronal cells. However, *in vitro* studies support the hypothesis that PP2C is a relevant phosphatase for BRSK1/2 *in vivo*. Interestingly, this would be similar to the case for AMPK, where PP2C appears to be the physiologically important phosphatase. Further studies are required to determine whether modulating dephosphorylation of BRSK1/2 by PP2C is the mechanism for control of BRSK1/2 activity.

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