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cAMP-stimulated phosphorylation of diaphanous 1 regulates protein stability and interaction with binding partners in adrenocortical cells

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ABSTRACT Diaphanous homologue 1 (DIAPH1) is a Rho effector protein that coordinates cellular dynamics by regulating microfilament and microtubule function. We previously showed that DIAPH1 plays an integral role in regulating the production of cortisol by controlling the rate of mitochondrial movement, by which activation of the adrenocorticotropin (ACTH)/cAMP signaling pathway stimulates mitochondrial trafficking and promotes the interaction between RhoA and DIAPH1. In the present study we use mass spectrometry to identify DIAPH1 binding partners and find that DIAPH1 interacts with several proteins, including RhoA, dynamin-1, kinesin, β-tubulin, β-actin, oxysterol-binding protein (OSBP)–related protein 2 (ORP2), and ORP10. Moreover, DIAPH1 is phosphorylated in response to dibutyryl cAMP (Bt\textsubscript{2}cAMP) at Thr-759 via a pathway that requires extracellular signal-related kinase (ERK). Alanine substitution of Thr-759 renders DIAPH1 more stable and attenuates the interaction between DIAPH1 and kinesin, ORP2, and actin but has no effect on the ability of the protein to interact with RhoA or β-tubulin. Finally, overexpression of a DIAPH1 T759A mutant significantly decreases the rate of Bt\textsubscript{2}cAMP-stimulated mitochondrial movement. Taken together, our findings establish a key role for phosphorylation in regulating the stability and function of DIAPH1.

INTRODUCTION Cortisol biosynthesis occurs in two cellular organelles, where both the first and final reactions occur in mitochondria and the intermediary enzymatic steps take place in the endoplasmic reticulum (ER). We previously reported that adrenocorticotropin (ACTH) regulates cortisol production by stimulating an increase in the rate of mitochondrial trafficking (Li and Sewer, 2010). Inhibition of microtubule polymerization attenuated ACTH/cAMP-stimulated mitochondrial movement and attenuated the secretion of cortisol, whereas agents that perturb the polymerization of actin increased the rate of mitochondrial movement and cortisol secretion both basally and in response to ACTH/cAMP. We also identified key roles for RhoA and the RhoA effector, diaphanous homologue 1 (DIAPH1), in regulating ACTH/cAMP-stimulated mitochondrial movement and cortisol production. Overexpression of a constitutively active DIAPH1 prevented mitochondrial movement and cortisol secretion, suggesting a role for DIAPH1 in coordinating the trafficking of mitochondria in response to ACTH stimulation.

DIAPH1 is a member of the formin family of effector proteins that regulate cytoskeletal dynamics by interacting with actin, microtubules, and other cytoskeletal-associated regulatory proteins (Watanabe et al., 1997; Ishizaki et al., 2001; Palazzo et al., 2001a; Bartolini and Gundersen, 2010; DeWard et al., 2010; Schonichen and Geyer, 2010; Young and Copeland, 2010). The ability of DIAPH1 to promote changes in cell shape and mobility is regulated by signal transduction cascades that are mediated by the small Rho GTPases Rho, Rac, and Cdc42. For example, lysophosphatidic acid (LPA)–stimulated activation of Rho promotes the activation of DIAPH1 and subsequent formation of actin filaments (Watanabe et al., 1997; Palazzo et al., 2001b; Copeland and Treisman, 2002;
DIAPH1 facilitates the stabilization of microtubules in response to LPA (Nagasaki and Gundersen, 1996; Cook et al., 1998) and is required for generating stable microtubules in multiple cell types, including fibroblasts (Eng et al., 2006; Bartolini and Gundersen, 2010), glioma cells (Yamana et al., 2006), and platelets (Higashi et al., 2008). DIAPH1 has also been found to be required for broad lamellipodia formation in response to growth factor stimulation (Zaoui et al., 2008) and for LPA-stimulated phosphorylation of glycogen synthase kinase 3B in migrating fibroblasts (Eng et al., 2006). Finally, roles for DIAPH1 in regulating serum response factor target gene transcription (Copeland and Treisman, 2002; Geneste et al., 2002; Gopinath et al., 2007) and the Pp2c phosphatase family member POPX2 (Xie et al., 2008) have been described. On the basis of our previous studies demonstrating a role for RhoA and DIAPH1 in controlling mitochondrial movement and cortisol production in human adrenocortical cells (Li and Sewer, 2010) and the ability of DIAPH1 to interact with multiple cellular proteins (Bartolini and Gundersen, 2010; Schonichen and Geyer, 2010; Young and Copeland, 2010), we used mass spectrometry to identify proteins that interact with DIAPH1 in H295R adrenocortical cells. In addition to identifying several DIAPH1 binding partners, we show that DIAPH1 is a phosphoprotein and that phosphorylation regulates the stability of the protein, its ability to interact with several binding partners, and the rate of mitochondrial movement.

RESULTS

Identification of DIAPH1 binding partners

We previously identified a role for DIAPH1 in regulating glucocorticoid biosynthesis in human adrenocortical cells by controlling the rate of mitochondrial movement (Li and Sewer, 2010). These studies revealed that ACTH activated a signal transduction cascade that led to the acute activation of the small GTPase RhoA and an increased interaction between RhoA and its effector DIAPH1. On the basis of these data, we hypothesized that DIAPH1 coordinates ACTH-stimulated mitochondrial trafficking and cortisol production by forming a larger protein complex that facilitates the exchange of substrate between the ER and mitochondria. To test this hypothesis, we immunoprecipitated DIAPH1 from H295R adrenocortical cells and subjected the immunoprecipitated proteins to SDS-PAGE and mass spectrometry. As shown in Figure 1A, DIAPH1 copurifies with RhoA and DIAPH1 in controlling mitochondrial movement and cortisol production in human adrenocortical cells (Li and Sewer, 2010) and the ability of DIAPH1 to interact with multiple cellular proteins (Bartolini and Gundersen, 2010; Schonichen and Geyer, 2010; Young and Copeland, 2010), we used mass spectrometry to identify proteins that interact with DIAPH1 in H295R adrenocortical cells. In addition to identifying several DIAPH1 binding partners, we show that DIAPH1 is a phosphoprotein and that phosphorylation regulates the stability of the protein, its ability to interact with several binding partners, and the rate of mitochondrial movement.

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Figure 1: (A) Representative gel of H295R cell lysates that were immunoprecipitated using an anti-DIAPH1 antibody and subjected to SDS–PAGE and Coomassie staining. Immunoprecipitation and mass spectrometric analysis was performed on three separate occasions. Asterisks denote bands that were excised for mass spectrometry. (B) GFP-tagged or endogenous DIAPH1 was immunoprecipitated from control or Bt-cAMP-treated (0.4 mM, 30 min) samples and the immobilized proteins were washed, separated by SDS–PAGE, and analyzed by Western blotting using antibodies against kinesin (heavy chain), dynamin-1, β-tubulin, RhoA, or vimentin. Five percent of inputs were subjected to SDS–PAGE and Western blotting using an anti-GFP antibody. Blots shown are representative, and each immunoprecipitation was performed on at least three separate occasions, each time in duplicate. (C) Lysates from control or Bt-cAMP-treated H295R cells were immunoprecipitated using an anti-DIAPH1 antibody and subjected to SDS–PAGE and Coomassie staining. Immuno-precipitated proteins were washed, separated by SDS–PAGE, and analyzed by Western blotting using antibodies against kinesin (heavy chain), dynamin-1, β-tubulin, and SH3 domain–containing proteins (e.g., Src kinase) and that were capable of nucleating actin in the absence of GTPase activation mutants that lack the DAD are constitutively active variants (Alberts, 2001). On GTP- and platelets (Higashi et al., 2008). In addition to regulating the actin polymerization, DIAPH1 also controls microtubule dynamics via binding to the FH2 domain (Ishizaki et al., 2001; Palazzo et al., 2001a; Bartolini et al., 2008).

Wen et al., 2004). DIAPH1 binds directly to actin via its formin homology (FH)-2, thus facilitating actin nucleation (Shimada et al., 2004; Schonichen and Geyer, 2010). The ability of DIAPH1 to interact with actin (and other binding partners) is controlled by a diaphanous autoregulatory domain (DAD) in the carboxy terminus of the protein. In the absence of activated signaling, the DAD binds to the amino terminus–localized Rho-binding domain, thereby rendering the FH1 and FH2 domains inaccessible for proteins such as actin, tubulin, and SH3 domain–containing proteins (e.g., Src kinase) to bind to DIAPH1 (Watanabe et al., 1999; Alberts, 2001). On GTP-bound Rho binding, the autoinhibitory conformation is relieved and DIAPH1 is able to bind to downstream effector proteins. Thus truncation mutants that lack the DAD are constitutively active variants that are capable of nucleating actin in the absence of GTPase activation (Alberts et al., 1998; Watanabe et al., 1999).

In addition to regulating the actin polymerization, DIAPH1 also controls microtubule dynamics via binding to the FH2 domain (Ishizaki et al., 2001; Palazzo et al., 2001a; Bartolini et al., 2008).
cAMP stimulates DIAPH1 phosphorylation at T759

To define the functional significance of phosphorylation of DIAPH1 in controlling complex assembly and steroid hormone biosynthesis, we generated a phosphospecific antibody to Thr-759. To test the specificity of the antibody, we transfected cells with green fluorescent protein (GFP)–tagged wild-type or T759A mutant DIAPH1, and subjected them to immunoprecipitation and Western blotting. As shown in the representative Western blots and in the graph of the densitometric analysis (Figure 3A), Bt2cAMP increased the phosphorylation of wild-type DIAPH1 by 3.8-fold at this site, whereas cells expressing the T759A mutant produced a greatly diminished phosphospecific signal and did not exhibit an increase in phosphorylation in response to Bt2cAMP treatment. Bt2cAMP-stimulated phosphorylation of the endogenous protein was inhibited when the cells were pretreated with H89 or U0126, suggesting that both protein kinase A (PKA) and mitogen-activated protein kinase (MAPK) signaling are required for increased DIAPH1 phosphorylation at this site (Figure 3B). The inhibitory effect of U0126 on cAMP-mediated DIAPH1 phosphorylation prompted us to determine the effect of PKA and MAPK inhibition on extracellular signal-regulated kinase (ERK) activity. Consistent with the work of other laboratories that demonstrated a role for ERK in cAMP signaling in adrenocortical cells (Lotfi et al., 2000;
Phosphorylation regulates DIAPH1

We next determined the effect of phosphorylation at T759 on the stability of DIAPH1 by treating cells transfected with wild-type or T759A mutant DIAPH1 with cycloheximide (CHX). As shown in Figure 4A, CHX reduced the expression of wild-type (WT), GFP-tagged DIAPH1, whereas the T759A mutant was resistant to the effect of the translation inhibitor. Six hours of incubation with CHX decreased the expression of the WT protein by 80%, whereas the expression of the T759A mutant was reduced by only ∼35% (Figure 4B). Proteasome inhibition for 6 h increased WT DIAPH1 to levels similar to that observed for T759A (Figure 4C), consistent with a possible resistance of T759A to proteasomal targeting.

Of interest, a recent global proteomics study discovered that DIAPH1 is ubiquitinated at five sites (Kim et al., 2001), which prompted us to speculate that phosphorylation at T759 might alter (in this case, increase) the propensity for ubiquitin attachment to the former site. Of note, ubiquitination regulates the degradation of DIAPH2 at the end of mitosis (DeWard and Alberts, 2009), supporting a role for this posttranslational modification in controlling the stability of the DIAPH family of proteins. To determine whether DIAPH1 is ubiquitinated, H295R cells were treated with Bt2cAMP and DIAPH1 immunoprecipitated for analysis by SDS–PAGE and Western blotting. Although we were unable to detect an increase in the ubiquitination of the endogenous protein in response to Bt2cAMP (unpublished data), cAMP stimulation did increase the cellular amount of sumoylated DIAPH1 (Figure 4E).

Phosphorylation of DIAPH1 regulates protein stability

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TABLE 2: Effect of Bt2cAMP on the interaction of DIAPH1 with binding partners.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control SC1</th>
<th>Control SC2</th>
<th>Control Total</th>
<th>Bt2cAMP SC1</th>
<th>Bt2cAMP SC2</th>
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<tr>
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Proteins are sorted based on ratio of Bt2cAMP/control spectral counts (from high to low).

FIGURE 2: (A) H295R cells were transfected with WT or phosphomutant pEGFP-DIAPH1 and lysates immunoprecipitated using an anti-GFP antibody and protein A/G agarose. Immobilized proteins were subjected to SDS–PAGE, followed by Western blotting using an anti–phospho-Ser/Thr antibody. Ten percent of inputs were subjected to SDS–PAGE and Western blotting using an anti-GFP antibody. Densitometric analysis of phosphor-Ser/Thr and GFP expression is expressed as fold change compared with wild type, and data represent mean ± SD of four experiments, each performed in duplicate. (B) Location of the putative phosphorylation sites on the primary structure of DIAPH1. (C) T-Coffee sequence alignment of DIAPH1, highlighting the position of a conserved threonine residue (T759 in humans) across different species.
Mutation of T759 significantly reduces the ability of SUMO to be conjugated to the protein (Figure 4F), suggesting a role for cAMP-stimulated phosphorylation in triggering sumoylation.

**Phosphorylation at T759 regulates complex formation**

We next sought to define the role of phosphorylation in regulating the ability of DIAPH1 to interact with members of the complex that were identified by mass spectrometry (Figure 1). Although there was no difference between the interaction of wild type or T759A mutant with RhoA (Figure 5A), alanine substitution at T759 decreased the interaction of kinesin with DIAPH1 by 65% (Figure 5B). Given that the FH2 domain of DIAPH1 is required for its interaction of DIAPH1 with actin and microtubules (Eng et al., 2006; Bartolini and Gundersen, 2010) and T759 lies in the FH2 domain (Figure 2C), we also determined the effect of phosphorylation on the interaction of DIAPH1 with actin and tubulin. As shown in Figure 5C, there was a 86% decrease in the interaction of the T759A DIAPH1 mutant with β-actin but no significant decrease in the interaction with β-tubulin (Figure 5D). Of interest, whereas mutation of T759 led to a 78% decrease in the interaction between DIAPH1 and ORP-2 (Figure 5E), the interaction between the T759A DIAPH1 mutant and ORP-10 was reduced by only 33% (Figure 5F).

**Phosphorylation at T759 modulates mitochondrial movement**

We previously showed that cells expressing a constitutively active DIAPH1 mutant exhibit an elongated cell shape and impaired mitochondrial trafficking (Li and Sewer, 2010). However, we also found that silencing DIAPH1 renders the cells unable to secrete cortisol. Collectively these data indicate that the actin-nucleating and/or microtubule-stabilizing activity of the protein must be precisely controlled to ensure optimal cortisol output. To define the functional significance of phosphorylation of the protein in modulating mitochondrial movement, we transfected H295R cells with GFP-tagged wild-type or alanine-substituted mutant DIAPH1 and quantified the rate of mitochondrial trafficking by real-time video imaging, as previously described (Li and Sewer, 2010). As shown in Figure 6, the rate of mitochondrial movement in cells expressing the wild-type protein was increased 1.8-fold by Bt2cAMP. In contrast, the rate of
mitochondrial movement in unstimulated cells expressing the DIAPH1 T759A mutant was reduced by 70%. Of interest, although the rate of mitochondrial trafficking was significantly decreased in control cells expressing mutant DIAPH1, Bt$_2$CAMP stimulation increased the rate of mitochondrial movement by 1.9-fold (Figure 6), suggesting that cAMP signaling might modulate DIAPH1 function via mechanisms that are independent of phosphorylation at T759.

**FIGURE 4:** (A) H295R cells were transfected with WT or T759A-mutant pEGFP-DIAPH1 and treated with 50 μg/ml CHX for 3 or 6 h. Cell lysates were harvested and separated by SDS–PAGE, followed by Western blotting using anti-GFP and anti-GAPDH antibodies. (B) Graphical analysis of data obtained from Western blotting studies of WT and phospho(T579) DIAPH1 protein expression in cells treated with 50 μg/ml CHX for 0–6 h. Data represent mean ± SEM of four separate experiments, each carried out in duplicate. Asterisks denote statistical significance (p < 0.05) from WT 0-h and T759A 0-h groups, respectively. (C) H295R cells were transfected with WT or T759A-mutant pEGFP-DIAPH1 and treated with 20 μM MG132 for 6 h. Cell lysates were harvested and separated by SDS–PAGE, followed by Western blotting using anti-GFP and anti-GAPDH antibodies. Left, representative blots; right, densitometric analysis. Graph data represent mean ± SD of four separate experiments, each in duplicate. (D) H295R cells were treated for 30 min with 0.4 nM Bt$_2$CAMP in the presence or absence of 20 μM MG132. Cell lysates were harvested and separated by SDS–PAGE, followed by Western blotting using anti–phospho(T759)-DIAPH1, anti-DIAPH1, and anti-GAPDH antibodies. Left, representative blots; right, densitometric analysis. Data represent the mean ± SD of four experiments, each in duplicate. Asterisks denote statistical significance (p < 0.05) compared with untreated control. (E) Lysates from control or Bt$_2$CAMP-treated cells transfected with GFP-tagged DIAPH1 were incubated with anti-GFP antibody and protein A/G agarose and the immunoprecipitated protein analyzed by SDS–PAGE and Western blotting for SUMO-1. Representative blots of study performed on several occasions, each time in duplicate. (F) Lysates from cells that were transfected with WT or T759A GFP-tagged DIAPH1 were immunoprecipitated with anti-GFP and the immobilized proteins assessed by SDS–PAGE and Western blotting for SUMO-1. Representative blots of study performed on two separate occasions, each time in duplicate.

**DISCUSSION**

In the human adrenal cortex ACTH coordinates multiple regulatory mechanisms to tightly control the production of cortisol. We previously identified a novel role for ACTH signaling in regulating the rate of mitochondrial movement in H295R human adrenocortical cells (Li and Sewer, 2010). ACTH-stimulated activation of the cAMP/PKA signaling pathway rapidly activates RhoA, promotes the interaction of the GTPase with DIAPH1, and stimulates the subsequent phosphorylation of RhoA. Overexpression of either a dominant-negative RhoA or a constitutively active DIAPH1 mutant attenuated the ability of ACTH or Bt$_2$CAMP to increase the rate of mitochondrial movement and decreased the secretion of cortisol, indicating key roles for these proteins in glucocorticoid production. However, silencing DIAPH1 also reduced cortisol biosynthesis, suggesting that the expression and activity of the protein needs to be precisely fine tuned to achieve optimal steroid hormone capacity. These findings also led us to hypothesize that DIAPH1 may act as a transducer in ACTH/cAMP-stimulated cortisol production in the adrenal cortex and that interaction of the protein with other downstream effectors in adrenocortical cells is required for cortisol biosynthesis. We show here that DIAPH1 copurifies with multiple proteins (Table 1) involved in regulating cytoskeletal dynamics, cell signaling, and lipid homeostasis.

Our present finding that DIAPH1 interacts with RhoA is consistent with both our previous studies (Li and Sewer, 2010) and findings of other laboratories (Watanabe et al., 1997, 1999; Ishizaki et al., 2001; Lammers et al., 2005; Otomo et al., 2006; Higashi et al., 2008; Zaoui et al., 2008; Narumiya et al., 2009; Tanizaki et al., 2010). Moreover, the identification of tubulin and actin as DIAPH1 binding partners is consistent with a large body of literature (Palazzo et al., 2001; Otomo et al., 2006; Bartolini et al., 2008). In addition to these proteins, we now show that DIAPH1 copurifies with several other proteins, including vimentin, microtubule-associated protein 4 (MAP4), son of sevenless (SOS), kinesin, dynine, and A kinase anchor protein 13 (also known as AKAP-Lbc). Coimmunoprecipitation assays confirmed interactions with kinesin, vimentin, and dynamin-1 (Figure 1B). Roles for kinesin and dynamin-1 in regulating organelle positioning and cytoskeletal dynamics are well established. Given that DIAPH1 interacts with microtubules and kinesin regulates the transport of cargo and organelles along microtubules (Allan and Schroer, 1999; Gross, 2004; Frederick and Shaw, 2007; Verhey and Hammond, 2009), the identification of kinesin as a DIAPH1 binding partner is consistent with the
role of this motor protein in cellular transport. Of note, targeted disruption of kinesin heavy chain, kif5B, results in abnormal perinuclear clustering of mitochondria (Tanaka et al., 1998), a phenotype that we observed in cells overexpressing constitutively active DIAPH1 (Li and Sewer, 2010). Our finding that DIAPH1 copurifies with other proteins involved in vesicle and organelle transport, including dynein and dynamin-1 (Table 1), suggests that these proteins act to coordinate communication between mitochondria and the ER for the transport of 11-deoxycortisol during cortisol biosynthesis. Of note, vimentin was recently found to play a pivotal role in ovarian and adrenocortical steriodogenesis, where targeted disruption of the gene resulted in reduced corticosterone production in both male and female mice (Shen et al., 2010). By virtue of the ability of several members of this family to bind to cholesterol and oxysterols, ORPs are implicated in cholesterol metabolism by serving as transporters between organelles, signaling mediators, and sensors of cellular lipid concentrations (Lehto et al., 2001; Olkkonen and Levine, 2004; Fairn and McMaster, 2008; Ridgway, 2010). By virtue of the ability of several members of this protein family to bind to cholesterol and oxysterols, ORPs are implicated in cholesterol metabolism (Wang et al., 2002; Du et al., 2011). However, ORPs have also been shown to bind to phospholipids, notably phosphatidic acid and phosphatidylinositol phosphates (Xu et al., 2001; Stefan et al., 2011).

Our finding that DIAPH1 is a phosphoprotein is consistent with published proteomic studies using a Cdk1–cyclin B substrate trap, which demonstrated that DIAPH1 is phosphorylated at Thr-759 in HeLa cells (Blethrow et al., 2008). In H295R cells, Bt2cAMP-stimulated phosphorylation of this site is dependent on ERK phosphorylation via an ERK-dependent mechanism, as demonstrated by the lack of a significant difference in Thr-759 phosphorylation between unstimulated and stimulated cells (Figure 5F).
kinase activity (Figure 3B). Thr-759 lies in a putative MAP kinase phosphorylation motif, which suggests that other members of this family may target the protein in response to specific cellular cues. Our findings that the T759A mutant is more stable than the wild-type protein and that the protein is sumoylated (Figure 4) support a role for cAMP signaling in activating the phosphorylation and subsequent sumoylation of the protein, potentially phosphorylation triggering the activation of a degradative clock. The ubiquitin-mediated degradation of mDia2 (DIAPH2) is required for completion of mitosis in HeLa and HEK293T cells (DeWard and Alberts, 2009). Moreover, we have preliminary evidence to suggest that silencing DIAPH1 leads to aberrant cortisol secretion (Li and Sewer, 2010), supporting our previous finding that overexpression of a constitutively active DIAPH1 impairs cortisol secretion (Li and Sewer, 2010) and suggesting that tight control of DIAPH1 expression is required for coordinating optimal steroid hormone production in response to ACTH.

In summary, we show that DIAPH1 interacts with multiple proteins in H295R human adrenocortical cells and that cAMP signaling modulates the interaction of a subset of proteins with DIAPH1. We also show that Bt2cAMP promotes the PKA- and ERK-dependent phosphorylation of DIAPH1 at T759 and that mutation of this site alters the stability of the protein and the rate of cAMP-stimulated mitochondrial movement. These findings support a model in which DIAPH1 cAMP signaling triggers posttranslational modification of the protein, thus allowing for tight control of both the expression and activity of the protein, and ultimately cortisol biosynthesis.

MATERIALS AND METHODS

Chemicals

Bt2cAMP was obtained from Sigma-Aldrich (St. Louis, MO). H89, CHX, MG132, and U0126 were purchased from EMD Biosciences (La Jolla, CA).

Cell culture and transient transfection

H295R adrenocortical cells were donated by William E. Rainey (Health Sciences University of Georgia, Augusta, GA) and cultured in Dulbecco's modified Eagle's/F12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% Nu-Sera (BD Biosciences, Palo Alto, CA), 1% ITS Plus (BD Biosciences, San Diego, CA), and antibiotics.

Mass spectrometry

Five hundred milligrams of total cellular protein isolated from H295R cells was precleared with immunoglobulin G (IgG) and incubated with anti-DIAPH1 (Millipore, Billerica, MA) and protein A/G (Santa Cruz Biotechnology, Santa Cruz, CA) overnight on a tube rotator at 4°C. Immunoprecipitants were washed twice with RIPA buffer (50 mM Tris–Cl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 0.4 mM EDTA, 150 mM aprotinin, 1 mM leupeptin, 1 mM E-64, 500 mM 4-(2-aminoethyl)benzenesulfonylfluoride) and three times with phosphate-buffered saline (PBS) and then subjected to SDS–PAGE and Coomassie staining. Bands were excised, destained, reduced, alkylated, and trypsinized using an iRGD Kit (Sigma-Aldrich). Peptides were concentrated using ZipTips (C18, Millipore), mixed with matrix, and spotted onto a matrix-assisted laser desorption ionization plate for tandem time-of-flight mass spectrometric analysis (4700 Proteomics Analyzer; Applied Biosystems, Foster City, CA).

Coimmunoprecipitation

H295R cells were plated onto 100-mm dishes and transfected with 5 μg of murine pEGFP-mDia (generously donated by Shuh Narumiya, Kyoto University Faculty of Medicine, Kyoto, Japan) using GeneJuice (3 μl/μg DNA; EMD Biosciences). At 72 h after transfection, cells were treated for 30 min with 0.4 mM Bt2cAMP and harvested into 100 μl of RIPA buffer. In some experiments, cells were transfected with GFP-tagged wild-type or T751A DIAPH1 (corresponds to human T759A). For sumoylation studies, cells were transfected with wild-type or T559A pEGFP-mDia and pSG5-His-SUMO1 (generated in the laboratory of Stephen Goff, Columbia University, New York, NY; Yueh et al., 2006; purchased from Addgene [Cambridge, MA; plasmid 17271]) and harvested in RIPA containing 20 mM N-ethylmaleimide. Five percent of the lysate was retained (input), and the remaining volume was diluted 10-fold with RIPA. Lysates were precleared with 1 μg of rabbit IgG, 10 mg/ml bovine serum albumin, and 25 μl of protein A/agarose (Santa Cruz Biotechnology) for 30 min at 4°C with rotation. Samples were centrifuged for 2 min at 4000 × g and the supernatants incubated with 5 μg of rabbit anti-GFP (A11122; Invitrogen) and 50 μl of protein A/agarose for 16 h at 4°C with rotation. The immobilized proteins were washed three times in RIPA and twice in PBS (5 min each wash), and the eluted proteins were separated by SDS–PAGE (10% acrylamide). Output proteins were transferred to polyvinylidene fluoride (PVDF) membranes, incubated with anti-dynamin 1 (1:1000; sc-12724; Santa Cruz Biotechnology), anti-kinase, heavy chain (1:1000; MAB1613; Millipore), anti-β-tubulin (1:1000; sc-23949; Santa Cruz Biotechnology), anti-β-actin (1:1000; sc-81178; Santa Cruz Biotechnology), anti-vimentin (1:7500; 574P; Cell Signaling), anti-ORP2 (1:1000; sc-665567; Santa Cruz Biotechnology), anti-ORP4 (1:1000; sc-376601; Santa Cruz Biotechnology), anti-ORP10 (1:1000; GenexTex, Irvine, CA), anti-RhoA (1:1000; sc-418; Santa Cruz Biotechnology), anti-SUMO1 (1:500; sc-9060; Santa Cruz Biotechnology), and alkaline phosphatase–conjugated secondary antibodies (1:5000; ECF Western Blotting Kit; GE Biosciences, Piscataway, NJ), and imaged using a VersaDoc 4000 Imager (Bio-Rad, Hercules, CA). Input samples
were subjected to SDS–PAGE and Western blotting using an anti-GFP antibody (Invitrogen).

**Site-directed mutagenesis and DIAPH1 phosphomutant screening**

PCR was used to generate alanine-substituted variants of the pEGFP-mDia expression plasmid using the Stratagene QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). All mutants were confirmed by sequencing (Retrogen, San Diego, CA). Putative phosphomutants were tested by transfecting cells with wild-type or mutant pEGFP-mDia and then immunoprecipitating the isolated lysates with anti-GFP (Invitrogen). The purified proteins were separated by SDS–PAGE, transferred to PVDF membranes, and incubated with anti–phospho-Ser/Thr-Pro antibody (1:1000; MAB1613; Millipore). Ten percent of the lysates were (input samples) subjected to SDS–PAGE and Western blotting using an anti-GFP antibody (Invitrogen).

**Generation of phosphospecific DIAPH1 antibody**

An affinity-purified phosphospecific antibody was produced to recognize DIAPH1 phosphorylated at T759 by Pacific Immunology (Ramona, CA). Two splice variants of DIAPH1 have been identified in humans, isoforms 1 and 2. Isoform 1 is the longer form (1272 amino acids), and isoform 2 (1263 amino acids) lacks an in-frame exon in the 5′-coding region. However, the amino acid sequence of the antigenic region is identical. The phosphopeptide antigen, LPGLT[PPO3] PKLYKPKCQLLR-C, corresponds to the region between amino acid region 754–772 of isoform 2 and regions 763 and 781 of isoform 1. A cysteine residue was added to the C-terminus of the peptide to facilitate chemical cross-linking. Sera from immunized rabbits were tested for antibody titer, and specificity for the phosphorylated peptide was tested by enzyme-linked immunosorbent assay and subjected to affinity purification. A nonphosphorylated antibody raised against the same peptide sequence was also produced.

**Western blotting**

H295R cells were treated with 10 μM H89 or 10 μM U0126 for 15 min and treated with Bt2cAMP (0.4 mM) for 30 min, and whole-cell extracts were prepared in RIPA. Lysates were separated by SDS–PAGE and transferred to PVDF membranes. In some experiments, cells were transfected with wild-type or T751A pEGFP-mDia and then treated with 0.4 mM Bt2cAMP for 30 min or with 20 μM MG132 for 6 h. In other experiments, cells were treated with 1 μM S1P for 15–60 min or with 50 μg/ml CHX for 3 or 6 h. Some lysates were incubated with λ-phosphatase (EMD Biosciences) before SDS–PAGE. Whole-cell lysates were immunoprecipitated with anti-GFP antibodies and protein A/G plus-agarose and washed, and the purified protein was separated by SDS–PAGE. Blots were incubated with anti-DIAPH1 (1:2000; AB3888; Millipore) or anti–phospho Thr-759 DIAPH1 antibody (1:250; Pacific Immunology) and alkaline phosphate-conjugated secondary antibody (1:5000; ECF Western Blotting Kit; GE Healthcare, Piscataway, NJ) and imaged on a VersaDoc 4000 (Bio-Rad). Densitometric analysis was carried out using Quantity One software (Bio-Rad).

**Confocal microscopy and time-lapse video imaging**

Cells were plated onto coverslips and transfected with 50 ng of wild-type or T759A pEGFP-DIAPH1 (transfection efficiency ranged from 70 to 85%) using Gene Juice (EMD Biosciences) and Opti-MEM (Invitrogen) for 48 h. Confocal images were collected in control and treated cells using an LSM510 confocal microscope (Carl Zeiss, Thornwood, NY) equipped with a helium–neon coherent laser, as previously described (Kulik et al., 2002; Li and Sewer, 2010). Emissions were collected with a C-Apochromat 40/1.3 numerical aperture oil immersion objective (Carl Zeiss) using a 560-nm long-pass filter. The Zeiss Imaging Physiology platform was used to determine the velocity of movement of individual mitochondria from taped recordings. To quantify the rate of movement, we set the time of exposure of each frame to 1 s, with a 3-s interval between successive frames. The track of mitochondria was obtained by subtracting the change in position after each frame interval. Only mitochondria that changed position during a given time interval were calculated. For these mobile mitochondria, the translocation between neighbor frames was measured, and the mean rate of movement was calculated. Each experiment was performed at least three times, and the movement of at least 20 mitochondria was analyzed in each experimental condition.

**Statistical analysis**

One-way analysis of variance, Tukey–Kramer multiple comparison, and unpaired Student’s t tests were performed using InStat software (GraphPad Software, San Diego, CA). Significant differences from a compared value were defined as p < 0.05.

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