The Formin mDia Regulates GSK3 β through Novel PKCs to Promote Microtubule Stabilization but Not MTOC Reorientation in Migrating Fibroblasts

Christina H. Eng,^{*,†} Thomas M. Huckaba,^{*} and Gregg G. Gundersen^{*}

^{*}Department of Anatomy and Cell Biology and [†]Integrated Program in Cellular, Molecular, and Biophysical Studies, Columbia University, New York, NY 10032

Submitted October 3, 2005; Revised August 14, 2006; Accepted September 6, 2006
Monitoring Editor: Erika Holzbaur

In migrating cells, external signals polarize the microtubule (MT) cytoskeleton by stimulating the formation of oriented, stabilized MTs and inducing the reorientation of the MT organizing center (MTOC). Glycogen synthase kinase 3 β (GSK3 β) has been implicated in each of these processes, although whether it regulates both processes in a single system and how its activity is regulated are unclear. We examined these issues in wound-edge, serum-starved NIH 3T3 fibroblasts where MT stabilization and MTOC reorientation are triggered by lysophosphatidic acid (LPA), but are regulated independently by distinct Rho GTPase-signaling pathways. In the absence of other treatments, the GSK3 β inhibitors, LiCl or SB216763, induced the formation of stable MTs, but not MTOC reorientation, in starved fibroblasts. Overexpression of GSK3 β in starved fibroblasts inhibited LPA-induced stable MTs without inhibiting MTOC reorientation. Analysis of factors involved in stable MT formation (Rho, mDia, and EB1) showed that GSK3 β functioned upstream of EB1, but downstream of Rho-mDia. mDia was both necessary and sufficient for inducing stable MTs and for up-regulating GSK3 β phosphorylation on Ser9, an inhibitory site. mDia appears to regulate GSK3 β through novel class PKCs because PKC inhibitors and dominant negative constructs of novel PKC isoforms prevented phosphorylation of GSK3 β Ser9 and stable MT formation. Novel PKCs also interacted with mDia in vivo and in vitro. These results identify a new activity for the formin mDia in regulating GSK3 β through novel PKCs and implicate novel PKCs as new factors in the MT stabilization pathway.

INTRODUCTION

Microtubule (MT) arrays become polarized to perform their functions in development, differentiation, and cell migration. One of the major ways MT arrays become polarized is through the generation of a subset of stabilized MTs (Gundersen and Bulinski, 1988; Bulinski and Gundersen, 1991; Gundersen *et al.*, 2004). In contrast to dynamic MTs, selectively stabilized MTs display longer half-lives and increased resistance to MT-depolymerizing drugs (Webster *et al.*, 1987; Khawaja *et al.*, 1988). Selectively stabilized MTs are abundant in differentiated cells, but are not restricted to differentiated cells: in wounded monolayers of fibroblasts, stable MTs form selectively so that they orient toward the leading edge (Gundersen and Bulinski, 1986; Gundersen and Bulinski, 1988; Palazzo *et al.*, 2004). Selective MT stabilization is thought to occur by the formation of a plus-end cap that prevents the MT from growing or shrinking (Infante *et al.*, 2000), and factors implicated in selective MT stabilization are localized at the ends of these MTs (Wen *et al.*, 2004).

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E05-10-0914>) on September 20, 2006.

Address correspondence to: Gregg G. Gundersen (ggg1@columbia.edu).

Abbreviations used: GSK3 β , glycogen synthase kinase 3 β ; PKC, protein kinase C; MTs, microtubules; MTOC, microtubule organizing center; LPA, lysophosphatidic acid; pSer9, phosphorylated serine 9.

Stabilized MTs accumulate posttranslationally modified tubulin, including detyrosinated and acetylated tubulin, and this may contribute to the recognition of stabilized MTs by factors that selectively interact with modified tubulin, such as kinesin motors (Liao and Gundersen, 1998). The functions of these stabilized, detyrosinated MTs in polarized cells are beginning to be understood, and studies have highlighted their importance in vimentin intermediate filament extension (Gurland and Gundersen, 1995; Kreitzer *et al.*, 1999), endosomal recycling (Lin *et al.*, 2002), migration (Wen *et al.*, 2004), and virus infection (Naranatt *et al.*, 2005). In addition, mice lacking the detyrosination/tyrosination cycle die shortly after birth and have neuronal defects (Erck *et al.*, 2005).

In mammalian fibroblasts, the formation of long-lived stable MTs is regulated by the serum factor lysophosphatidic acid (LPA), which stimulates a signaling pathway that involves the heterotrimeric G protein, G $\alpha_{12/13}$, the small GTPase Rho, its effector, the formin mDia, and the MT plus-end binding proteins EB1 and APC (Cook *et al.*, 1998; Palazzo *et al.*, 2001a; Wen *et al.*, 2004; Goulmari *et al.*, 2005). Rho and mDia also appear to be involved in MT stabilization in embryonic endodermal cells, where they function along with the plakin, ACF-7 (Kodama *et al.*, 2003). Binding of activated Rho to mDia is thought to relieve mDia's autoinhibited state and lead to its activation (Alberts, 2001). Activated forms of mDia bind both EB1 and APC, and all three proteins can be found on the ends of stable MTs, consistent with a capping mechanism (Wen *et al.*, 2004). MTs stabilized by this Rho-mDia signaling pathway do not grow or shrink (Cook *et al.*, 1998; Palazzo *et al.*, 2001a; Wen *et al.*, 2004),

making this form of stabilization different from that of MAPs, which stabilize MTs by binding along their length, but do not block tubulin exchange at the ends.

The MT cytoskeleton is also polarized by positioning the MT organizing center (MTOC) at a specific place in the cell. In migrating fibroblasts, astrocytes, macrophages, and endothelial cells, the MTOC is reoriented so that it lies between the nucleus and the leading edge (Gotlieb *et al.*, 1981; Kupfer *et al.*, 1982, 1983; Gundersen and Bulinski, 1988; Etienne-Manneville and Hall, 2001). Cdc42, Par6, atypical PKC (aPKC), and dynein comprise a common "MTOC reorientation" pathway in fibroblasts, astrocytes, and endothelial cells (Etienne-Manneville and Hall, 2001, 2003; Palazzo *et al.*, 2001b; Tzima *et al.*, 2003; Gomes *et al.*, 2005). In fibroblasts, this pathway, like that regulating MT stabilization, is triggered by LPA (Palazzo *et al.*, 2001b). In fibroblasts, it is known that LPA triggers a second Cdc42-regulated pathway that functions to move the nucleus rearward, whereas the Par6-aPKC-dynein pathway maintains the MTOC at the cell centroid. The combined effects of the two pathways are necessary for MTOC reorientation (Gomes *et al.*, 2005). The second Cdc42-regulated pathway involves the Cdc42 effector, MRCK (myotonic dystrophy kinase-related Cdc42-binding kinase), myosin and actin retrograde flow, which is thought to drive the nucleus rearward (Gomes *et al.*, 2005).

GSK3 β was named for its role in regulating glycogen synthase, although it is now known to participate in many signaling pathways and cellular activities. GSK3 β is usually constitutively active and most signaling pathways result in the inhibition of GSK3 β . For example, insulin stimulates protein kinase B (PKB) to phosphorylate Ser9 (pSer9) of GSK3 β and this inhibits its activity (Cross *et al.*, 1995). Insulin treatment of starved 3T3 fibroblasts does promote a small increase in the levels of stable MTs (Gundersen *et al.*, 1994), suggesting that regulation of GSK3 β activity may influence stable MT formation. In the Cdc42 pathway involved in MTOC reorientation in astrocytes, activation of atypical PKC also increases GSK3 β pSer9 levels (Etienne-Manneville and Hall, 2003), but whether GSK3 β participates in MTOC reorientation in fibroblasts or other systems that orient their MTOC has not been tested.

LPA is another cytokine that causes the inhibition of GSK3 β by phosphorylation on Ser9, and although PKCs seem to be involved, the specific isoform(s) has not been identified (Fang *et al.*, 2002). Because LPA stimulates both MT stabilization and MTOC reorientation in starved NIH 3T3 fibroblasts, we were interested in whether the inhibition of GSK3 β was important for the effects of LPA on either or both of these MT-polarizing events. Given the evidence that GSK3 β is involved in MTOC reorientation in astrocytes, we predicted that it would also be involved in both processes in fibroblasts. However, we find that GSK3 β is not involved in MTOC reorientation in fibroblasts, but instead is involved in MT stabilization. Because LPA-induced MT stabilization in fibroblasts is regulated by a Rho-mDia pathway, this suggested that there might be a novel pathway involved in regulating GSK3 β in response to LPA stimulation. Indeed, we find that GSK3 β pSer9 is stimulated by both LPA and active forms of mDia1 or mDia2 and that mDia1 was both necessary and sufficient for enhancing GSK3 β pSer9. mDia1 appears to regulate GSK3 β phosphorylation through a pathway involving PKC ϵ , a novel PKC isoform, because mDia1 directly binds PKC ϵ , and PKC ϵ was necessary for both the LPA- and mDia-stimulated phosphorylation of GSK3 β and for formation of stable MTs. These results describe a novel activity for a formin in regulating kinase activity and show

that in fibroblasts, GSK3 β contributes to the Rho-mDia-EB1-APC pathway that regulates MT stabilization.

MATERIALS AND METHODS

Materials, Cell Culture, and Microinjection

All materials were purchased from Sigma (St. Louis, MO) unless otherwise indicated. NIH 3T3 cells were maintained as previously described (Cook *et al.*, 1998; Kreitzer *et al.*, 1999; Palazzo *et al.*, 2001a). For microinjection, confluent NIH 3T3 cells were serum starved for 2 d, wounded, and microinjected as previously described (Cook *et al.*, 1998; Kreitzer *et al.*, 1999; Palazzo *et al.*, 2001a). Plasmid DNA was microinjected in H-KCl (10 mM HEPES, pH 7.4, 140 mM KCl) at 100–200 μ g/ml and allowed to express 2–4 h after microinjection. To induce stable MTs and MTOC reorientation, 5 μ M LPA was added for 90–120 min. GSK3 β inhibitors LiCl (20 mM) or SB216763 (Tocris, Ballwin, MO) were added to starved cells and incubated for 2 h. SB216763 was used at 2–10 μ M with similar results.

DNA Plasmids

All HA-tagged PKC constructs were a gift from J. W. Soh (Soh *et al.*, 1999), and flag-tagged dominant negative PKC α was from C. Carpenter (Coghlan *et al.*, 2000). Full-length HA-Rok α was from L. Lim (Leung *et al.*, 1996). mDia1 and mDia2 constructs were from A. Alberts (Alberts, 2001), except for pEGFP-C1-521-1040-mDia2, which was made by subcloning 521-1040-mDia2 from pEFm-EGFP (Wen *et al.*, 2004) into pEGFP-C1 (Clontech, Palo Alto, CA). HA-tagged GSK3 β was constructed by PCR amplification of pcDNA3-GSK3 β (a gift from J. Kitajewski) with primers 5'-GGCGGCTCGACAGAAGAGC-CATCATG-3' and 3'-GCCTCCAACCTCTACCTCTAGAGCCCCC-5' and cloned into pKHA $_3$ (a gift from D. Brautigen). To prepare GFP-GSK3 β , GSK3 β was subcloned from pKHA $_3$ into pEGFP-C1 (Clontech). The GFP-GSK3 β fusion was then subcloned into pCAGGS/ES (a gift from P. Scheiffele) downstream of the chicken β -actin promoter. All constructs were verified by sequencing, and DNA was purified with Qiagen midiprep kits (Chatsworth, CA).

mDia1 siRNA

COS-7 cells were transfected with mDia1 siRNA in a six-well dish with 4 μ l of 20 μ M siRNA and 10 μ l of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in 1 ml of growth media (DMEM with 10% FBS). The mDia1 siRNA sequence was 5'-GCUGGUCAGAGCCAUGGAU-3' (Arakawa *et al.*, 2003) and the control GFP sequence was 5'-GGCUACGUCCAGGAGCGCACC-3'. Both siRNAs were from Dharmacon (Boulder, CO). After 24 h, the medium was changed to serum-free DMEM for another 24 h. Cells were lysed and analyzed for GSK3 β phosphorylation. Similar knockdown results in COS-7 cells were obtained with a second mDia1 siRNA constructed using the Ambion Silencer siRNA construction kit (Austin, TX). This mDia1 sequence was 5'-AAGGUGAAG-GAGGACCGCUUUU-3'. The GAPDH siRNA used was the control provided with the kit. For microinjection of siRNA into NIH 3T3 fibroblasts, confluent monolayers were serum-starved and wounded. A few hours later, Ambion kit-synthesized siRNAs were diluted in H-KCl and microinjected at 10 μ M with 1 mg/ml 500 kDa FITC-dextran (Molecular Probes, Eugene, OR) as an injection marker. After microinjection (48 h), cells were fixed for 5–10 min in methanol at -20° C and processed for immunofluorescence. To visualize individual siRNA-treated cells, the Ambion kit-synthesized siRNA was labeled with CX-rhodamine using the Mirvus Label IT siRNA Tracker kit (Boston, MA) according to the manufacturer's instructions. NIH 3T3 fibroblasts passaged at least three times in FBS were transfected with 2 μ l Mirvus TKO Transfection reagent and 200 nM siRNA in 300 μ l of growth media in a 24-well plate. After transfection (24 h), cells were replated onto coverslips and allowed to adhere another 24 h. Cells were then starved for 24 h, treated with LPA, and fixed and processed for immunofluorescence as above. To examine GSK3 β pSer9 levels (see below) in siRNA treated cells, NIH 3T3 cells were transfected with siRNA to mDia1 or GAPDH (Invitrogen) with Lipofectamine Plus. Cells were split 24 h after transfection and transfected a second time 48 h after transfection. After the second transfection, cells were washed, serum-starved overnight, treated with LPA or insulin, and processed for immunofluorescence (see below).

Immunofluorescence Microscopy

Staining of fixed cells was performed as previously described (Gundersen *et al.*, 1984). Glu MTs were detected with a polyclonal rabbit antibody (Ab) SG that specifically recognizes dephosphorylated (Glu) tubulin (Gundersen *et al.*, 1984; 1:400), and tyrosinated (Tyr) MTs were detected with the rat mAb YL1/2 (1:10, European Collection of Animal Cell Cultures, Salisbury, United Kingdom). The following Abs were used for detection of overexpressed or endogenous proteins: mouse mAb to HA (12CA5, 1:100, Roche, Indianapolis, IN), mouse mAb to GSK3 β (0011-A, 1:100, Santa Cruz Biotechnology, Santa Cruz, CA), mouse mAb to mDia1 (clone 51, 1:100, BD Transduction, Lexington, KY), and mouse mAb to GAPDH (6C5, 1:1000, Ambion). For certain experiments, cells were also stained with mouse mAb to actin (C4D6, 1:200, a

gift from J. Lessard, University of Cincinnati), β -tubulin (3F3, 1:200), or γ -tubulin (GTU-88, 1:200, Sigma). For localizing F-actin, cells were fixed in 4% paraformaldehyde for 10 min, permeabilized with 0.5% Triton X-100 for 5 min, and stained with rhodamine-phalloidin (1:500, Molecular Probes). Digital images were acquired as previously described (Palazzo *et al.*, 2001a).

For staining with GSK3 β pSer9 Ab (1:100, Cell Signaling Technology, Beverly, MA), cells were fixed with 2% paraformaldehyde + 0.2% glutaraldehyde in PBS for 20 min and then permeabilized with 0.2% Triton X-100 in PBS for 10 min. Cells were then blocked in 2% BSA in TBS and then stained as previously described, except primary antibody incubations were for 1 h. To quantify GSK3 β pSer9 in individual cells, digital images were acquired with equivalent exposure times as previously described (Palazzo *et al.*, 2001a). The average fluorescence intensity of the entire cell was measured using MetaMorph software (Universal Imaging, West Chester, PA) on a per cell basis after subtracting the background intensity (measured in an adjacent cell-free area of the coverslip). We attempted to correct the fluorescence measurements for nonspecific staining of the secondary antibody; however, these readings were too variable to give consistent results between the experiments and so were not corrected for.

Analysis of GSK3 β Phosphorylation Levels by Western Blotting

COS-7 cells were transfected in growth medium (DMEM with 10% CS) with Lipofectamine 2000 according to the manufacturer's specifications. After transfection (6–24 h), cells were washed twice with serum-free medium (SFM; DMEM + 10 mM HEPES) and incubated in SFM for an additional 18–24 h. Cells were treated with 10 μ M LPA or 10–100 μ g/ml insulin for 5 min. To inhibit PKC, 10 μ M bisindolylmaleimide I (Calbiochem, La Jolla, CA) was added 1 h before treatment with LPA or insulin. Cells were washed once with cold PBS and lysed in 1% Triton X-100, 150 mM NaCl, 20 mM Tris, pH 7.4 or 8.0, phosphatase inhibitors (5 mM β -glycerophosphate, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate), and Protease Inhibitor Cocktail (Sigma). After 10 min extraction on ice, lysates were clarified by centrifugation (13,000 $\times$ g, 10 min, 4°C). Protein concentration was determined with a BCA protein assay and equal amounts of protein were run on SDS-PAGE, transferred to nitrocellulose and analyzed by Western blotting with rabbit Ab to GSK3 β pSer9 (1:5000, Cell Signaling Technology), mouse mAb GSK3 β (0011-A, 1:5000, Santa Cruz), mouse mAb to mDia1 (clone 51, 1:1000, BD Transduction), rat mAb to HA (3F10, 1:5000, Roche), and mouse mAb to vinculin (VIN-11-5, 1:5000, Sigma).

Binding Experiments

COS-7 cells were transfected with myc-GFP- Δ GBD-mDia1 or myc-GFP as a control along with pHACE-PKCe, pHACE-PKC η , pHACE-PKC ζ , or HA-ROK α plasmids using Lipofectamine 2000 according to the manufacturer's protocol. After 24 h transfection, cells were washed once with cold PBS and lysed in RIPA buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.4, 150 mM NaCl, and phosphatase and protease inhibitors). After 10-min extraction on ice, lysates were clarified by centrifugation (13,000 $\times$ g, 10 min, 4°C). To each extract, rabbit Ab to full-length GFP (1:500, BD Transduction) and protein A-Sepharose beads were added, and lysates were mixed overnight at 4°C. Beads were washed in RIPA buffer, eluted with SDS sample buffer and loaded on gels. Expressed proteins were detected by Western blotting with mouse mAb to myc (9E10, 1:5000, Santa Cruz) and rat mAb to HA (3F10, 1:5000, Roche). For endogenous binding, 5 mg of pre-cleared mouse brain extract was immunoprecipitated with 20 μ g rabbit anti-PKCe (Santa Cruz) or control rabbit IgG overnight at 4°C in lysis buffer containing 1% TX-100, 150 mM NaCl, 20 mM Tris, pH 7.4, and phosphatase and protease inhibitors. Protein G-Sepharose beads were then added and mixed for an additional 3 h. Beads were washed with lysis buffer, eluted with SDS sample buffer, and analyzed by SDS-PAGE followed by Western blotting. Antibodies used for blotting were mouse mAb to mDia1 (1:1000, BD Transduction) and rabbit Ab to PKCe (1:1000, Santa Cruz). For direct *in vitro* binding, 20 μ g GST-521-1040-mDia2 or an equal molar amount of GST was bound to glutathione-agarose beads for 1–2 h at 4°C. Then, 6 pmol recombinant purified human PKCe or PKC ζ (Calbiochem) was added in lysis buffer with or without lipids (100 μ g/ml phosphatidylserine and 10 μ g/ml diacylglycerol; Avanti, Alabaster, AL) and mixed overnight at 4°C. Beads were washed with lysis buffer, and bound proteins were eluted with SDS sample buffer and run on gels. Bound proteins were detected by Western blotting with rabbit Ab to PKCe (1:2000, Santa Cruz), rabbit Ab to PKC ζ (1:2000, Upstate Biotechnology, Lake Placid, NY), or mouse mAb to GST (1:20,000, Cell Signaling Technology). For blot overlay analysis, equimolar amounts of PKCe or PKC ζ were subject to SDS-PAGE and transferred to nitrocellulose. The membrane was incubated overnight at 4°C with 10 μ g/ml GST-521-1040-mDia2 or an equimolar amount of GST in TBST containing 5% BSA, 100 μ g/ml phosphatidylserine and 10 μ g/ml diacylglycerol. The bound GST-mDia2 or GST was detected by mouse Ab to GST, and quantified using the LI-COR Odyssey Infrared Imaging System (Cincinnati, OH).

RESULTS

GSK3 β Is a Negative Regulator of LPA-stimulated MT Stabilization

We first tested whether GSK3 β was involved in the LPA-stimulation of MT stabilization and/or MTOC reorientation in wound-edge serum-starved NIH 3T3 fibroblasts. For MT stabilization, we assayed the formation of stabilized MTs by immunofluorescently staining cells for post-translationally detyrosinated tubulin (or Glu tubulin after its C-terminus), which accumulates in MTs after they are stabilized (Webster *et al.*, 1987; Khawaja *et al.*, 1988). We then scored cells for their content of Glu MTs. Cells containing five or more brightly stained, extended Glu MTs were counted as positive for Glu MTs. For MTOC reorientation, we assessed the position of the MTOC (detected with antibodies to γ -tubulin) relative to the leading edge and nucleus, scoring MTOCs as reoriented if they were in a pie-shaped segment facing the wound; random orientation of the MTOC gives a value 33% "reoriented" by this method (see Palazzo *et al.*, 2001b).

Because LPA stimulates inhibition of GSK3 β (Fang *et al.*, 2002; and see below), we first tested whether known inhibitors of GSK3 β , LiCl, or SB216763 mimicked the effect of LPA treatment of starved fibroblasts. When starved fibroblasts were treated with either GSK3 β inhibitor, the level of Glu MTs increased without significantly affecting the levels of the bulk, dynamic MTs detected with Tyr tubulin antibody (Figure 1, A and B). As with LPA treatment, Glu MTs induced by the GSK3 β inhibitors constituted a subset of the total MTs. However, unlike those triggered by LPA, they were not preferentially oriented toward the wound edge (Figure 1A). Glu MTs induced by GSK3 β inhibitors were not noticeably bundled, indicating that MAP-like proteins were not involved. SB216763 treatment of starved NIH 3T3 fibroblasts also promoted an increase in nocodazole-resistant MTs (Figure 1C), indicating that inhibition of GSK3 β affects MT stability and not the enzymes responsible for tubulin post-translational modifications. These results show that inhibition of GSK3 β is sufficient to trigger the formation of stabilized MTs in fibroblasts.

To test whether inhibition of GSK3 β was necessary for stable MT formation, serum-starved fibroblasts were microinjected with DNA encoding full-length GSK3 β and after expression (1–2 h), treated with LPA to induce stable MTs. Overexpression of wild-type GSK3 β inhibited the formation of Glu MTs induced by LPA (Figure 1, A and B). These results demonstrate that GSK3 β is a negative regulator of LPA-stimulated MT stability.

GSK3 β Does Not Affect LPA-stimulated MTOC Reorientation

To test whether GSK3 β was involved in MTOC reorientation in fibroblasts, we treated wounded, starved NIH 3T3 fibroblast monolayers with SB216763 in the absence or presence of LPA to promote MTOC reorientation. SB216763 did not induce MTOC reorientation in starved cells and did not inhibit MTOC reorientation induced by LPA (Figure 2, A and B). To extend this finding, starved NIH 3T3 fibroblasts were microinjected with wild-type GSK3 β in the presence or absence of LPA to examine whether overexpression of GSK3 β inhibited MTOC reorientation. Expression of wild-type GSK3 β , which inhibited MT stabilization induced by LPA (Figures 1, A and B, and 2A), did not inhibit MTOC reorientation induced by LPA or promote MTOC reorientation on its own (Figure 2, A and B). Thus, in starved NIH 3T3 fibroblasts, GSK3 β is involved in regulating MT stabilization but not MTOC reorientation. This is consistent with our

Figure 1. GSK3 β negatively regulates LPA-induced stable MT formation. (A) Distribution of Glu and Tyr MTs in wounded, starved NIH 3T3 fibroblasts left untreated or treated with 5 μ M LPA, 20 mM LiCl, 2 μ M SB216763, or microinjected with DNA encoding GSK3 β (arrow) and then treated with LPA (inset shows GSK3 β expression in the injected cell). After 2 h, cells were fixed and immunostained for Glu and Tyr tubulin to monitor changes in stable and bulk MTs, respectively. Note that Glu MTs induced by LiCl or SB216763 were not oriented toward the wound-edge as were those induced by LPA. (B) Quantification of percentage of wounded, starved NIH 3T3 fibroblasts with Glu MTs after the indicated treatments (untr, untreated). Data are mean $\pm$ SEM from at least three experiments ($n > 70$ cells). (C) Nocodazole-resistant microtubules in wounded, starved NIH 3T3 fibroblasts treated with or without 2 μ M SB216763 for 2 h. Nocodazole resistance was assessed by treating cells with 2 μ M nocodazole for 30 min, detergent extracting, and fixing before immunostaining for β -tubulin. Scale bars, (A and C) 20 μ m.

previous studies showing that factors working in one of the MT regulatory pathways in fibroblasts do not function in the other pathway (Palazzo *et al.*, 2001b; Wen *et al.*, 2004).

GSK3 β Functions in the Rho-mDia-EB1-APC MT Stabilization Pathway

To examine whether GSK3 β functions with the known components of the MT stabilization pathway or alternatively acts through a parallel pathway, we tested whether inhibition of GSK3 β was able to promote MT stabilization in the presence of known inhibitors of the MT stabilization pathway. Botulinum C3 toxin, which specifically inhibits Rho (Aktories *et al.*, 1989), blocks LPA-stimulated formation of stable MTs (Cook *et al.*, 1998). Microinjection of C3 toxin did not prevent the induction of stable MTs by LiCl (Figure 3, A and B), but did reduce the levels of actin fibers, demonstrating that the injected protein was functional. The MT tip protein EB1 acts downstream of both Rho and mDia in the stabilization pathway, and the C-terminal fragment of EB1 (EB1-C) inhibits stable MTs induced by LPA or mDia (Wen *et al.*, 2004). Expression of EB1-C inhibited the induction of stable MTs by LiCl (Figure 3, A and B).

These results suggest that GSK3 β functions in the MT stabilization pathway and acts downstream of Rho but upstream of EB1. As a further test of this, we explored whether GSK3 β inhibition in starved cells stimulated the formation

of actin filaments. If GSK3 β acts downstream of Rho and mDia, it should not affect the actin cytoskeleton. Indeed, addition of LiCl to serum-starved cells did not promote formation of actin stress fibers, compared with LPA (Figure 3C), which activates Rho and mDia and induces stress fiber formation (Ridley and Hall, 1992; Watanabe *et al.*, 1997). This result confirms that GSK3 β functions downstream of Rho but upstream of EB1 in the MT stabilization pathway and also suggests that stable MTs formed by the inhibition of GSK3 β do not require actin stress fibers, consistent with earlier studies (Palazzo *et al.*, 2001a; Wen *et al.*, 2004).

mDia Is Required for the LPA-stimulated Phosphorylation of GSK3 β

LPA treatment of starved Swiss 3T3 fibroblasts was shown earlier to increase GSK3 β Ser9 phosphorylation, although the mechanism for this was not fully delineated (Fang *et al.*, 2002). We confirmed that LPA treatment of starved COS-7 cells or NIH 3T3 fibroblasts increased GSK3 β pSer9 levels (Figure 4, A and B). In both cases, the levels of GSK3 β pSer9 triggered by LPA was similar to that induced by insulin, which stimulates GSK3 β pSer9 through a pathway involving PKB/Akt (Cross *et al.*, 1995).

The cellular factors involved in LPA-induced GSK3 β pSer9 are unknown. Given that GSK3 β appears to function downstream of Rho-mDia (but upstream of EB1) in the MT stabiliza-

Figure 2. GSK3 β does not affect MTOC re-orientation. (A) Merged images depicting Glu tubulin (red), γ -tubulin (green), and DAPI staining (blue) of wounded, starved NIH 3T3 fibroblasts treated 10 μ M SB216763 for 1–2 h or microinjected with plasmid encoding GSK3 β (arrows) and either left untreated (–LPA) or treated with 5 μ M LPA (+LPA) for 2 h (insets show the cells expressing GSK3 β). Wound edge is toward the top in each panel and is depicted with a dashed white line. (B) Quantification of percentage of wounded, starved NIH 3T3 fibroblasts with oriented MTOCs after the indicated treatments. Data are mean $\pm$ SEM from at least three experiments ($n > 40$ cells). Random distribution of the MTOC would lead to 33% of the cells with oriented MTOCs. Scale bar, (A) 20 μ m.

zation pathway, upstream factors such as mDia are potential candidates for mediating the effect of LPA on GSK3 β . The GSK3 β pSer9 levels were assessed in COS-7 cells transiently transfected with HA-tagged GSK3 β with or without constitutively active versions of mDia (Δ GBD-mDia1 or Δ GBD-mDia2). By examining the pSer9 levels of HA-tagged GSK3 β , which was distinguishable from the endogenous GSK3 β by a difference in migration on SDS gels (see Figure 4A), we could specifically determine the effect of mDia in the cotransfected cells. Expression of either active mDia1 or mDia2 increased HA-GSK3 β pSer9 levels (Figure 4A). The levels of HA-GSK3 β pSer9 stimulated by active mDia1 or mDia2 were about half of that stimulated by LPA. Because $\sim 50\%$ of the cells were cotransfected (unpublished data), these results suggest that activation of mDia alone may be sufficient to account for the LPA-stimulated increase in GSK3 β pSer9 levels.

Next, we determined whether activation of endogenous mDia1 was sufficient to stimulate endogenous GSK3 β phosphorylation on Ser9. NIH 3T3 cells were transfected with GFP-tagged Dia autoregulatory domain (GFP-DAD), a fragment of mDia1 that is able to activate the endogenous protein by relieving autoinhibition (Alberts, 2001). Transfection with DAD was sufficient to promote phosphorylation of endogenous GSK3 β in NIH 3T3 cells, whereas the control transfection with GFP alone had no effect (Figure 4B). In addition, expression of GFP-521-1040-mDia2, which contains the FH1 and FH2 domains and is the minimal domain

of mDia that is sufficient to stimulate stable MT formation (Wen *et al.*, 2004), was also sufficient to promote GSK3 β pSer9 in NIH3T3 cells (Figure 4B).

To test whether endogenous mDia1 was required for the LPA-stimulated increase in GSK3 β pSer9 levels, we knocked down mDia1 with siRNA. COS-7 cells were transfected with HA-GSK3 β as before, in combination with siRNA specific for mDia1 or control siRNA specific for GFP. mDia1 siRNA decreased endogenous mDia1 by $\sim 75\%$, whereas the control siRNA against GFP did not affect mDia1 levels (Figure 4C). mDia1 siRNA, but not GFP siRNA, reduced LPA-stimulated HA-GSK3 β pSer9 by $\sim 50\%$, but did not affect basal HA-GSK3 β pSer9 levels in unstimulated cells or the induction of HA-GSK3 β pSer9 in cells stimulated with insulin (Figure 4C). These data demonstrate that mDia1 is responsible for at least part of the enhanced GSK3 β pSer9 levels induced by LPA and that mDia1 does not participate in insulin-stimulated regulation of GSK3 β , which occurs through the PKB/Akt pathway (Cross *et al.*, 1995).

To quantify the effect of mDia1 knockdown on endogenous Ser9 phosphorylation of GSK3 β in NIH 3T3 cells, a quantitative immunofluorescence approach was taken because the transfection efficiency was not sufficient to permit a Western blot assay. NIH 3T3 were transfected with siRNA to mDia1, or siRNA to GAPDH as a control, serum-starved, and then treated with LPA or insulin. Cells were fixed and

Figure 3. GSK3 β functions between Rho and EB1 in the MT stabilization pathway. (A) Microinjected C3 toxin does not inhibit LiCl-induced Glu MTs, whereas expression of microinjected EB1C-GFP DNA does inhibit LiCl-induced Glu MTs. C3 was coinjected with human IgG as an injection marker. Cells were fixed and stained for actin, and Tyr and Glu MTs. Arrows indicate injected cells. (B) Quantification of percentage of wounded, starved NIH 3T3 fibroblasts with Glu MTs after the indicated treatments (untr., untreated). Data are mean $\pm$ SEM from at least three experiments ($n > 50$ cells). (C) LiCl does not induce changes in actin organization. Starved, wounded NIH 3T3 cells were left untreated or treated with either 20 mM LiCl or 5 μ M LPA for 90 min, fixed, and stained for F-actin with rhodamine-phalloidin. Scale bars, (A and C) 20 μ m.

stained for GSK3 β pSer9, and mDia1 or GAPDH. The average fluorescence intensity per cell was measured in cells stained for GSK3 β pSer9 that had visible mDia1 (or GAPDH) knockdown (for examples, see Figure 4E). This assay showed a consistent 30–50% increase in GSK3 β pSer9 fluorescence in LPA- and insulin-treated cells, demonstrating the validity of this assay (Figure 4D). Knockdown of

mDia1, but not GAPDH, resulted in a significant decrease in LPA-stimulated GSK3 β pSer9 (Figure 4D). There was also a decrease in insulin-stimulated GSK3 β pSer9 in mDia1 siRNA-treated cells, but this decrease was less significant compared with LPA (Figure 4D). These data demonstrate that mDia1 is required for at least some of the enhanced GSK3 β -pSer9 levels induced by LPA.

Figure 4. mDia is required for GSK3 β phosphorylation and MT stabilization induced by LPA. (A) Western blot of starved COS-7 cells transfected with HA-tagged GSK3 β and either left untreated, treated with 10 μ g/ml insulin, 10 μ M LPA (both for 5 min) or cotransfected with GFP-tagged Δ GBD-mDia constructs. Blots were probed with antibody to GSK3 β pSer9 (top) or total GSK3 β (bottom). Positions of HA-GSK3 β pSer9, expressed HA-GSK3 β and endogenous GSK3 α / β are shown on the left. (B) Western blot of endogenous GSK3 β pSer9 levels in starved NIH 3T3 cells either left untreated, treated with 10 μ g/ml insulin, 10 μ M LPA (both for 5 min), or transfected with GFP alone, GFP-521-1040-mDia2, or GFP-DAD. Blots were probed with antibody to GSK3 β pSer9 (top) or total GSK3 β (bottom). Positions of endogenous GSK3 α / β are shown on the left. (C) Western blots of COS-7 cells treated as in A, except that siRNA specific for mDia1 or GFP was cotransfected along with HA-GSK3 β . Western blots were probed with antibodies to mDia1, GSK3 β pSer9, total GSK3 β and vinculin (as a loading control). Mock-treated cells received transfection reagents alone. (D) Quantification of average fluorescent intensity of GSK3 β pSer9 in immunofluorescently stained cells that had been mock-transfected, or transfected with mDia1 or GAPDH siRNA. Cells were left untreated (untr) or stimulated with 10 μ g/ml insulin or 10 μ M LPA (both for 5 min) as indicated. Average fluorescent intensity is expressed as the percentage of mock-transfected, serum-starved cells. * $p < 0.01$ compared with mock LPA; ** $p < 0.05$ compared with mock insulin by Student's t test. Data are mean $\pm$ SEM from three experiments in which >70 cells were measured. (E) Immunofluorescence images of starved NIH 3T3 fibroblasts microinjected with 10 μ M mDia1 or GAPDH siRNAs and FITC-dextran as an injection marker. Cells were serum-starved

mDia1 Is Required for Stable MT Formation

Previous studies identified mDia as the likely Rho effector for LPA-mediated MT stabilization (Palazzo *et al.*, 2001a). Given that mDia1 is required for LPA-induced increase in GSK3 β pSer9 and that GSK3 β appeared to be involved in the MT stabilization pathway, it was important to confirm that mDia was necessary for MT stabilization induced by LPA as previously reported (Goulimari *et al.*, 2005). Wound-edge starved NIH 3T3 fibroblasts, which express mDia1 but not mDia2 (Tominaga *et al.*, 2000; and unpublished data), were microinjected with siRNA against mDia1 that we had validated in COS-7 cells (Figure 4C). Two days after mDia1 siRNA treatment or GAPDH siRNA as a control, the cells were treated with LPA to stimulate formation of stable MTs. The majority of cells injected with siRNA had reduced levels of mDia1 or GAPDH as detected by immunofluorescence, confirming the efficacy of the knockdown in NIH 3T3 fibroblasts (Figure 4E). In cells with visible knockdown of mDia1, there was a decrease in the level of Glu MTs in response to LPA compared with uninjected cells or GAPDH siRNA injected cells (Figure 4E). Quantification of this effect in cells transfected with labeled siRNA (to identify the transfected cells) showed that mDia1 knockdown significantly reduced the induction of Glu MTs by LPA compared with untransfected or siRNA GAPDH controls (Figure 4F).

Novel PKCs Are Required for GSK3 β Phosphorylation and MT Stabilization by LPA

LPA-stimulated GSK3 β pSer9 is blocked by PKC inhibitors (Fang *et al.*, 2002; Yang *et al.*, 2005), and we confirmed this result in NIH 3T3 and COS-7 cells, where treatment with the general PKC inhibitor, bisindolylmaleimide I, strongly inhibited LPA-stimulated, but not insulin-stimulated, GSK3 β pSer9 (Figure 5, A and B). Next, we tested whether mDia-stimulated GSK3 β pSer9 also required PKC. COS-7 cells transfected with HA-GSK3 β were pretreated with bisindolylmaleimide I and then treated with LPA or insulin or cotransfected with constitutively active Δ GBD-mDia2, and their levels of GSK3 β pSer9 were assayed. GSK3 β pSer9 induced by LPA or active mDia2 was inhibited by bisindolylmaleimide I, indicating that LPA/mDia-induced GSK3 β pSer9 required PKC(s) (Figure 5B). Insulin-stimulated GSK3 β pSer9 was less affected, as expected (Figure 5B).

There are three classes of PKC isoforms: conventional, novel, and atypical. We tested whether a specific class of PKC was involved in the increase in GSK3 β pSer9 and stable MTs triggered by mDia1. Initially, we screened a set of dominant negative PKC isoforms for effects on LPA-induced stable MT formation. Expression of either dominant negative PKC ϵ or PKC η , both novel isoforms of PKC, prevented LPA-induced stable MTs in starved NIH 3T3 fibroblasts (Figure 5, C and D). In contrast, expression of dominant negative conventional (α , β 1, γ) or atypical (ζ , λ) PKC isoforms had no effect on LPA-induced stable MTs (Figure 5, C and D). Cells expressing dominant negative PKC ϵ or PKC η still retained their ability to reorient their MTOC in response

to LPA (Figure 5E), again confirming that proteins involved in MT stabilization do not affect MTOC reorientation. Expression of atypical PKC ζ or λ isoforms interfered with LPA-induced MTOC reorientation (Figure 5C and unpublished data), as previously reported (Etienne-Manneville and Hall, 2001; Gomes *et al.*, 2005), confirming the activity of these proteins.

To test whether novel PKCs were sufficient to promote stable MTs, plasmids encoding the isolated catalytic (cat) domain were expressed in starved cells. Expression of PKC ϵ -cat or PKC η -cat did not induce stable MTs in the absence of LPA treatment (unpublished data). Thus, although novel PKCs are required for the formation of stable MTs induced by LPA, their catalytic activity alone is not sufficient.

To check whether the novel PKCs affected GSK3 β pSer9, we analyzed the ability of the dominant negative constructs to inhibit LPA- and mDia-induced GSK3 β pSer9. Coexpression of dominant negative PKC ϵ in COS-7 cells with GFP-GSK3 β as a reporter construct inhibited the increase in GSK3 β pSer9 levels in response to LPA and Δ GBD-mDia1, but not insulin (Figure 5F). Thus, dominant negative PKC ϵ that inhibits MT stabilization also inhibits GSK3 β phosphorylation.

To quantify the effect of dominant negative PKC ϵ on endogenous GSK3 β pSer9, we again performed quantitative immunofluorescence to measure GSK3 β pSer9. COS-7 cells were transfected with dominant negative PKC ϵ , GFP- Δ GBD-mDia1, or both, serum-starved, and then treated with LPA or insulin as indicated. Cells were fixed and stained, and individual cells expressing one or both proteins were measured for their levels of GSK3 β pSer9. Again, this assay showed a consistent increase in GSK3 β pSer9 fluorescence in LPA- and insulin-treated cells, similar to what was observed in NIH 3T3 cells (Figure 5G). In addition, transfection with active mDia1 significantly increased endogenous GSK3 β pSer9 over levels in untreated cells, as had been observed for transfected GSK3 β . Expression of PKC ϵ -KR significantly reduced the Δ GBD-mDia1 and LPA stimulation of GSK3 β pSer9 to background levels (Figure 5G). PKC ϵ -KR also decreased GSK3 β pSer9 stimulation due to insulin, but this decrease was not significant and did not reduce the levels to those of untreated cells (Figure 5G).

mDia Interacts Directly with Novel PKCs

The above results raise the possibility that mDia acts in conjunction with novel PKC isoforms to phosphorylate and inhibit GSK3 β . To explore whether this might be a direct effect of mDia on novel PKCs, we tested whether the two proteins interacted *in vivo*. Mouse brain extract was immunoprecipitated with antibody to PKC ϵ . A small amount of mDia1 was visualized in the immunoprecipitate, demonstrating that PKC ϵ and mDia1 interact at physiological levels (Figure 6A). Because we found that only novel PKC isoforms specifically affected stable microtubules, we examined whether other isoforms of PKC were capable of binding to mDia. Tagged versions of active mDia1 and full-length PKC isoforms were transfected into COS-7 cells and immunoprecipitated. Immunoprecipitation of myc-GFP- Δ GBD-mDia1, but not myc-GFP alone, coimmunoprecipitated the novel PKC isoforms, PKC η and PKC ϵ , and also the atypical PKC isoform, PKC ζ (Figure 6B). Another HA-tagged kinase, ROK α , did not coimmunoprecipitate with mDia1. Thus, active mDia1 is capable of binding multiple PKC isoforms *in vivo* when overexpressed.

To examine whether the interaction between mDia and PKCs is direct, the binding of purified recombinant PKC ϵ or

Figure 4 (cont). for 40–48 h after injection and then treated with 5 μ M LPA (90 min) before fixation and staining for mDia1 (or GAPDH), Glu, and Tyr MTs. Arrows indicate siRNA-injected cells with reduced levels of mDia1 or GAPDH. (F) Quantification of percentage of wounded, starved NIH 3T3 fibroblasts with Glu MTs after transfection with labeled mDia1 or GAPDH siRNA. Cells were stimulated with LPA (5 μ M, 90 min) as indicated. * p < 0.001 compared with mock LPA. Data are mean $\pm$ SEM from three experiments. n > 180 cells. Scale bar, (E) 20 μ m.

Figure 5. Novel PKCs are involved in GSK3 β phosphorylation and MT stabilization. (A) Western blot of starved NIH 3T3 cells left untreated (untr) or treated with 100 μ g/ml insulin or 10 μ M LPA for 5 min. Some cells were pretreated with 10 μ M bisindolylmaleimide I (BIM-1) for 1 h before stimulation. (B) Western blot of COS-7 cells transfected with HA-tagged GSK3 β and either cotransfected with GFP-tagged Δ GBD-mDia2 or treated with 100 μ g/ml insulin or 10 μ M LPA for 5 min after 24-h serum starvation. Some cells were treated with 10 μ M bisindolylmaleimide I (BIM-1) for 1 h before stimulation. Blots were probed with antibodies to GSK3 β pSer9 or total GSK3 β . The position of HA-GSK3 β pSer9, HA-GSK3 β , and endogenous GSK3 α / β are indicated on the left. (C) Immunofluorescence images of Glu and Tyr MTs in wounded, starved NIH 3T3 fibroblasts expressing microinjected dominant negative HA-tagged PKC constructs (K to R mutations in the

Figure 6. mDia interacts with novel PKC isoforms in vivo and in vitro. (A) Mouse brain extract was immunoprecipitated with antibody to PKC ϵ or an equal amount of rabbit IgG as a control. Bound proteins were eluted and analyzed by Western blotting with antibodies against PKC ϵ and mDia1. The input (2%) of each reaction is depicted in the left panels. (B) Western blots of COS-7 cells cotransfected with myc-GFP-ΔGBD-mDia1 (or myc-GFP as a control) and HA-tagged full-length PKCs or ROK α . After 24-h expression, cell lysates were prepared and immunoprecipitated with GFP antibody. Western blots of immunoprecipitates were probed with antibody against HA or myc. (C) Equimolar amounts of PKC were electrophoresed and transferred to nitrocellulose. The membrane was Ponceau-stained to confirm equal loading. The membrane then was incubated with 10 μ g/ml GST-521-1040-mDia2, and the mDia2 bound to PKCs was detected with an antibody to GST. The amount of bound mDia2 was quantified, and the ratio of PKC ϵ to PKC ζ binding was calculated from three independent experiments. (D) Purified recombinant PKC ϵ or PKC ζ was added to equimolar amounts of GST or GST-521-1040-mDia2 prebound to glutathione-agarose beads in buffer with and without phosphatidylserine and diacylglycerol ($\pm$ lipids). Bound proteins were eluted and analyzed by Western blotting with antibody against PKC ϵ or PKC ζ . Five percent of the input was analyzed by Western blotting with antibody to PKC ϵ , PKC ζ , or GST to determine equal loading.

Figure 5 (cont). ATP binding domain) and treated with 2 μ M LPA for 90 min. Arrows show expressing cells; insets show HA staining. (D) Quantification of percentage of wounded, starved NIH 3T3 fibroblasts with Glu MTs after expression of microinjected DNA encoding dominant negative HA-tagged PKC isoforms and treatment with 2 μ M LPA for 90 min. Results are mean $\pm$ SEM from at least three experiments. $n \geq 30$ cells. (E) Quantification of percentage of wounded, starved NIH 3T3 fibroblasts with oriented MTOCs after the indicated treatments. Data are mean $\pm$ SEM from at least three experiments. $n \geq 20$ cells. Random distribution of the MTOC would lead to 33% of the cells with oriented MTOCs. (F) Western blots of COS-7 cells transfected with GFP-tagged GSK3 β and either cotransfected with pcDNA3 (as a control) or HA-tagged dominant negative PKC ϵ (HA-PKC ϵ -KR), and myc-GFP-tagged ΔGBD-mDia1. After 24-h transfection and 24-h serum-starvation, cells were left untreated or treated with 10 μ g/ml insulin or 10 μ M LPA for 10 min. Blots were probed with antibodies to GSK3 β pSer9, total GSK3 β , and HA tags to show expression of the dominant negative HA-PKC ϵ . (G) Quantification of average fluorescence intensity of GSK3 β pSer9 in immunofluorescently stained cells that had been transfected with GFP-ΔGBD-mDia1, HA-PKC ϵ -KR, or both. Cells were stimulated with 10 μ g/ml insulin or 10 μ M LPA (both for 5 min) as indicated. Average fluorescence intensity is expressed as the percentage of untransfected, serum-starved cells. * $p < 0.05$ compared with untreated; ** $p < 0.05$ compared with ΔGBD-mDia1; *** $p < 0.01$ compared with LPA; + $p > 0.05$ compared with insulin by Student's t test. Data are mean $\pm$ SEM from at least three experiments. $n > 70$ cells. Bar, (C) 20 μ m.

PKC ζ with a GST-tagged active fragment of mDia2 was examined by a blot overlay assay. Increasing equimolar amounts of PKC ϵ or PKC ζ immobilized on nitrocellulose were incubated with 10 μ g/ml GST-521-1040-mDia2 (Figure 6C). Before the overlay, the membrane was Ponceau-stained to demonstrate equal loading. Both PKC ϵ and PKC ζ bound to GST-521-1040-mDia2, although PKC ϵ consistently bound more GST-mDia2 than did PKC ζ (Figure 6C). At the highest concentration, PKC ϵ bound 1.8-fold more than PKC ζ (± 0.14) to GST-mDia2, with similar differences in binding at lower concentrations. Binding of both PKC isoforms to GST was minimal compared with GST-521-1040-mDia2, and correcting for the GST binding did not alter the higher level of binding observed with PKC ϵ compared with PKC ζ . Novel PKCs are characterized by the ability to be activated by lipids, but not calcium; thus we wanted to analyze the lipid dependence of mDia binding to PKC ϵ . The interaction of PKC ϵ with GST-521-1040-mDia2 was increased in the presence of the PKC activating lipids phosphatidylserine and diacylglycerol (Figure 6D). Binding of PKC ζ , which does not require diacylglycerol for activation, was not affected (Figure 6D). Thus, both PKC ϵ and PKC ζ bind mDia2 directly, but PKC ϵ has a higher affinity for mDia2 than PKC ζ , and the binding of PKC ϵ is also enhanced by the presence of lipids.

DISCUSSION

We demonstrate that GSK3 β functions and is phosphorylated in the Rho-mDia mediated MT stabilization pathway, acting downstream of Rho and upstream of EB1. GSK3 β is negatively regulated by signals that are sufficient to promote MT stabilization, because knockdown of both mDia1 and dominant negative novel PKCs block MT stabilization and GSK3 β phosphorylation. These data provide a novel role for a formin protein in regulating the action of a kinase, although earlier work showed mDia could interact with Src kinase (Tominaga *et al.*, 2000). Recent studies of formins in mammalian cells have focused on their effects on the actin and MT cytoskeletons (reviewed in Wallar and Alberts, 2003; Zigmond, 2004). Here, we demonstrate that LPA, through mDia1, can regulate the activity of GSK3 β by controlling its phosphorylation.

Our results point to novel PKCs as the likely candidates for mediating the effects of mDia on GSK3 β . We found that dominant negative novel PKCs blocked active mDia induced GSK3 β pSer9 and that mDia interacted directly with PKC ϵ . Although mDia also bound to another PKC isoform, PKC ζ , the affinity of PKC ϵ was higher for mDia and could account for the specific activity of novel PKCs toward stable MTs. Furthermore, although the binding of PKC ϵ to mDia was enhanced in the presence of lipids, the binding of PKC ζ was not. These results suggest that binding of active mDia to novel PKCs may regulate their activity toward substrates such as GSK3 β . Previous work showed that novel PKCs can phosphorylate GSK3 β on Ser9 (Fang *et al.*, 2002). We suggest that mDia may regulate the activity or localization of novel PKCs to enhance their activity toward GSK3 β . This LPA-stimulated pathway involving mDia and novel PKC regulation of GSK3 β functions independently of insulin-PKB regulation of GSK3 β .

This work identifies two new components, novel PKCs and GSK3 β that function in the Rho-mDia-EB1-APC pathway, which regulates plus-end mediated stabilization of MTs. Our work points to a new role for novel PKCs in governing MT stabilization. Novel PKCs are characterized by their dependence on lipids for activation, but they do not require calcium. Novel PKCs (δ , θ , ϵ , and η) are widely and differentially expressed in tissues and each isoform has unique functions. We do not know how mDia binding to PKC may regulate its activity. Perhaps the binding of PKC ϵ to mDia brings it into the vicinity of MTs, where it is sometimes localized *in vivo* (Kato *et al.*, 2001; Palazzo *et al.*, 2001a; Wen *et al.*, 2004). PKC ϵ is also localized to the actin cytoskeleton in some cells (Prekeris *et al.*, 1996; Hernandez *et al.*, 2001), and it is also possible that mDia regulates its activity toward the actin cytoskeleton.

GSK3 β is the first negative regulator to be identified in the Rho-mDia-EB1-APC pathway for MT stabilization. A number of earlier studies have used inhibitors to show that GSK3 β regulates MT stability (Goold *et al.*, 1999; Krylova *et al.*, 2000; Akhmanova *et al.*, 2001). Our work extends these studies by showing how a known MT regulatory pathway controls GSK3 β phosphorylation in response to a physiological stimulus. Most previous studies have implicated GSK3 β in regulating MT stability through its effects on MAPs or CLASPs binding laterally to MTs (Lovestone *et al.*, 1996; Wagner *et al.*, 1996; Goold *et al.*, 1999; Krylova *et al.*, 2000; Akhmanova *et al.*, 2001). Our results indicate that GSK3 β also regulates MT stability through its action on factors involved in MT capture at the cell cortex. Glu MTs are a stabilized subset of MTs that are capped at their distal ends, preventing subunit addition or loss and their ends are found

in close proximity to the cell cortex (Infante *et al.*, 2000; Wen *et al.*, 2004). Direct interaction of mDia with the MT tip proteins EB1 and APC contributes to this end-mediated MT stabilization (Wen *et al.*, 2004). The interaction of these proteins with each other or with MTs are potential targets for regulation by GSK3 β . The only known substrate of GSK3 β among these proteins is APC, and GSK3 β phosphorylation of APC decreases its interaction with MTs *in vitro* (Zumbrunn *et al.*, 2001). APC interaction with EB1 is also regulated by phosphorylation, although this is by other kinases (Askham *et al.*, 2000). It will be interesting to test whether other interactions among these proteins and with MTs are regulated by GSK3 β phosphorylation. GSK3 β also appears to negatively regulate the interaction of CLASPs with microtubules (Akhmanova *et al.*, 2001; Wittmann and Waterman-Storer, 2005), so this is another candidate target for GSK3 β .

The observation that inhibition of GSK3 β induces stable MTs that, in contrast to LPA-stimulated stable MTs, are not polarized toward the leading edge, is likely due to the global inactivation caused by GSK3 β inhibitors and the fact that GSK3 β functions downstream of Rho. Selective stabilization of MTs oriented toward the wound edge occurs because of integrin engagement in the front of the cell, leading to activation of FAK (Palazzo *et al.*, 2004). This adhesion signal is thought to restrict the ability of active Rho to stimulate mDia to the region near the leading edge. Consistent with this idea, overexpression of active Rho promotes the formation of a polarized array of stable MTs, but overexpression of constitutively active mDia does not (Cook *et al.*, 1998; Palazzo *et al.*, 2001a). GSK3 β bypasses the adhesion signal that polarizes the newly formed stable MTs by acting downstream of mDia. Indeed, we have found that GSK3 β inhibition by LiCl is able to promote MT stabilization in nonadherent fibroblasts that lack integrin signals (Palazzo and Gundersen, unpublished observations).

The MT array is also polarized in migrating cells by reorientation of the MTOC to a position between the nucleus and the leading edge of the cell (Gotlieb *et al.*, 1981; Kupfer *et al.*, 1982; Gundersen and Bulinski, 1988). We previously found that inhibition of Cdc42, dynein, or dynactin, proteins involved in MTOC reorientation, does not block MT stabilization and that inhibition of Rho or EB1, proteins involved in MT stabilization, does not block MTOC reorientation (Palazzo *et al.*, 2001b; Wen *et al.*, 2004). We find that GSK3 β and novel PKCs function in the MT stabilization pathway, which is consistent with these results, but not in the MTOC reorientation pathway in NIH 3T3 fibroblasts and that atypical PKCs function in MTOC reorientation, as reported previously (Etienne-Manneville and Hall, 2003; Gomes *et al.*, 2005), but not in MT stabilization. That inhibition of GSK3 β is both necessary and sufficient for MT stabilization, but not for MTOC reorientation, is in contrast to results in wounded astrocytes, where GSK3 β overexpression or inhibition has been found to inhibit MTOC reorientation (Etienne-Manneville and Hall, 2003). A role for GSK3 β in MT stabilization in astrocytes has not yet been tested. We do not know the reason for the different effects of GSK3 β in the two systems, but they may be related to differences in cell types, conditions used to stimulate MTOC reorientation, or to the much longer time to reorient MTOCs in astrocytes (6 h) compared with fibroblasts (1 h).

Despite the differences between the responses of different cell types to GSK3 β , it is clear that GSK3 β is an important regulator of microtubule polarity, and thus a key determinant of polarity during development and differentiation. GSK3 β has a crucial role in Wnt signaling involving APC,

Dvl, Axin, and β -catenin, which mediates embryonic polarity. Recent studies in neurons have also pointed to the significance of GSK3 β in the establishment of axons and maintenance of neuronal polarity via APC, PI3K, and CRMP-2 (Shi *et al.*, 2004; Jiang *et al.*, 2005; Yoshimura *et al.*, 2005). Whether the establishment of polarity by GSK3 β in these situations requires the formation of stable microtubules will be an interesting question to pursue.

ACKNOWLEDGMENTS

We thank J. W. Soh, A. Alberts, D. Brautigan, J. Kitwajewski, P. Scheiffele, C. Carpenter, and F. Bartolini for DNA constructs; Y. Wen for preparation of GST protein; and members of the Gundersen lab for comments on the manuscript. C.H.E. was supported by a Howard Hughes Medical Institute Predoctoral Fellowship. This work was supported by National Institutes of Health Grant GM 62939 (G.G.G.).

REFERENCES

- Akhmanova, A., *et al.* (2001). CLASPs are CLIP-115 and -170 associating proteins involved in the regional regulation of microtubule dynamics in motile fibroblasts. *Cell* 104, 923–935.
- Aktories, K., Braun, U., Rosener, S., Just, I., and Hall, A. (1989). The rho gene product expressed in *E. coli* is a substrate of botulinum ADP-ribosyltransferase C3. *Biochem. Biophys. Res. Commun.* 158, 209–213.
- Alberts, A. S. (2001). Identification of a carboxyl-terminal diaphanous-related formin homology protein autoregulatory domain. *J. Biol. Chem.* 276, 2824–2830.
- Arakawa, Y., Bito, H., Furuyashiki, T., Tsuji, T., Takemoto-Kimura, S., Kimura, K., Nozaki, K., Hashimoto, N., and Narumiya, S. (2003). Control of axon elongation via an SDF-1 α /Rho/mDia pathway in cultured cerebellar granule neurons. *J. Cell Biol.* 161, 381–391.
- Askham, J. M., Moncur, P., Markham, A. F., and Morrison, E. E. (2000). Regulation and function of the interaction between the APC tumour suppressor protein and EB1. *Oncogene* 19, 1950–1958.
- Bulinski, J. C., and Gundersen, G. G. (1991). Stabilization of post-translational modification of microtubules during cellular morphogenesis. *Bioessays* 13, 285–293.
- Coghlan, M. P., Chou, M. M., and Carpenter, C. L. (2000). Atypical protein kinases Clambda and -zeta associate with the GTP-binding protein Cdc42 and mediate stress fiber loss. *Mol. Cell. Biol.* 20, 2880–2889.
- Cook, T. A., Nagasaki, T., and Gundersen, G. G. (1998). Rho guanine triphosphatase mediates the selective stabilization of microtubules induced by lysophosphatidic acid. *J. Cell Biol.* 141, 175–185.
- Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378, 785–789.
- Erck, C., *et al.* (2005). A vital role of tubulin-tyrosine-ligase for neuronal organization. *Proc. Natl. Acad. Sci. USA* 102, 7853–7858.
- Etienne-Manneville, S., and Hall, A. (2001). Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKC ζ . *Cell* 106, 489–498.
- Etienne-Manneville, S., and Hall, A. (2003). Cdc42 regulates GSK-3 β and adenomatous polyposis coli to control cell polarity. *Nature* 421, 753–756.
- Fang, X., Yu, S., Tanyi, J. L., Lu, Y., Woodgett, J. R., and Mills, G. B. (2002). Convergence of multiple signaling cascades at glycogen synthase kinase 3, Edg receptor-mediated phosphorylation and inactivation by lysophosphatidic acid through a protein kinase C-dependent intracellular pathway. *Mol. Cell. Biol.* 22, 2099–2110.
- Gomes, E. R., Jani, S., and Gundersen, G. G. (2005). Nuclear movement regulated by Cdc42, MRCK, myosin, and actin flow establishes MTOC polarization in migrating cells. *Cell* 121, 451–463.
- Goold, R. G., Owen, R., and Gordon-Weeks, P. R. (1999). Glycogen synthase kinase 3 β phosphorylation of microtubule-associated protein 1B regulates the stability of microtubules in growth cones. *J. Cell Sci.* 112(Pt 19), 3373–3384.
- Gotlieb, A. I., May, L. M., Subrahmanyam, L., and Kalnins, V. I. (1981). Distribution of microtubule organizing centers in migrating sheets of endothelial cells. *J. Cell Biol.* 91, 589–594.
- Goulmari, P., Kitzing, T. M., Knieling, H., Brandt, D. T., Offermanns, S., and Grosse, R. (2005). Galpha12/13 is essential for directed cell migration and localized Rho-Dia1 function. *J. Biol. Chem.* 280, 42242–42251.
- Gundersen, G. G., and Bulinski, J. C. (1986). Microtubule arrays in differentiated cells contain elevated levels of a post-translationally modified form of tubulin. *Eur. J. Cell Biol.* 42, 288–294.
- Gundersen, G. G., and Bulinski, J. C. (1988). Selective stabilization of microtubules oriented toward the direction of cell migration. *Proc. Natl. Acad. Sci. USA* 85, 5946–5950.
- Gundersen, G. G., Gomes, E. R., and Wen, Y. (2004). Cortical control of microtubule stability and polarization. *Curr. Opin. Cell Biol.* 16, 106–112.
- Gundersen, G. G., Kalnoski, M. H., and Bulinski, J. C. (1984). Distinct populations of microtubules: tyrosinated and nontyrosinated alpha tubulin are distributed differently in vivo. *Cell* 38, 779–789.
- Gundersen, G. G., Kim, I., and Chapin, C. J. (1994). Induction of stable microtubules in 3T3 fibroblasts by TGF-beta and serum. *J. Cell Sci.* 107(Pt 3), 645–659.
- Gurland, G., and Gundersen, G. G. (1995). Stable, detyrosinated microtubules function to localize vimentin intermediate filaments in fibroblasts. *J. Cell Biol.* 131, 1275–1290.
- Hernandez, R. M., Wescott, G. G., Mayhew, M. W., McJilton, M. A., and Terrian, D. M. (2001). Biochemical and morphogenic effects of the interaction between protein kinase C-epsilon and actin in vitro and in cultured NIH3T3 cells. *J. Cell. Biochem.* 83, 532–546.
- Infante, A. S., Stein, M. S., Zhai, Y., Borisy, G. G., and Gundersen, G. G. (2000). Detyrosinated (Glu) microtubules are stabilized by an ATP-sensitive plus-end cap. *J. Cell Sci.* 113, 3907–3919.
- Jiang, H., Guo, W., Liang, X., and Rao, Y. (2005). Both the establishment and the maintenance of neuronal polarity require active mechanisms: critical roles of GSK-3 β and its upstream regulators. *Cell* 120, 123–135.
- Kato, T., Watanabe, N., Morishima, Y., Fujita, A., Ishizaki, T., and Narumiya, S. (2001). Localization of a mammalian homolog of diaphanous, mDia1, to the mitotic spindle in HeLa cells. *J. Cell Sci.* 114, 775–784.
- Khawaja, S., Gundersen, G. G., and Bulinski, J. C. (1988). Enhanced stability of microtubules enriched in detyrosinated tubulin is not a direct function of detyrosination level. *J. Cell Biol.* 106, 141–149.
- Kodama, A., Karakesisoglou, I., Wong, E., Vaezi, A., and Fuchs, E. (2003). ACF7, an essential integrator of microtubule dynamics. *Cell* 115, 343–354.
- Kreitzer, G., Liao, G., and Gundersen, G. G. (1999). Detyrosination of tubulin regulates the interaction of intermediate filaments with microtubules in vivo via a kinesin-dependent mechanism. *Mol. Biol. Cell* 10, 1105–1118.
- Krylova, O., Messenger, M. J., and Salinas, P. C. (2000). Dishevelled-1 regulates microtubule stability: a new function mediated by glycogen synthase kinase-3 β . *J. Cell Biol.* 151, 83–94.
- Kupfer, A., Dennert, G., and Singer, S. J. (1983). Polarization of the Golgi apparatus and the microtubule-organizing center within cloned natural killer cells bound to their targets. *Proc. Natl. Acad. Sci. USA* 80, 7224–7228.
- Kupfer, A., Louvard, D., and Singer, S. J. (1982). Polarization of the Golgi apparatus and the microtubule-organizing center in cultured fibroblasts at the edge of an experimental wound. *Proc. Natl. Acad. Sci. USA* 79, 2603–2607.
- Leung, T., Chen, X. Q., Manser, E., and Lim, L. (1996). The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the cytoskeleton. *Mol. Cell. Biol.* 16, 5313–5327.
- Liao, G., and Gundersen, G. G. (1998). Kinesin is a candidate for cross-bridging microtubules and intermediate filaments. *J. Biol. Chem.* 273, 9797–9803.
- Lin, S. X., Gundersen, G. G., and Maxfield, F. R. (2002). Export from pericentriolar endocytic recycling compartment to cell surface depends on stable, detyrosinated (glu) microtubules and kinesin. *Mol. Biol. Cell* 13, 96–109.
- Lovestone, S., Hartley, C. L., Pearce, J., and Anderton, B. H. (1996). Phosphorylation of tau by glycogen synthase kinase-3 beta in intact mammalian cells: the effects on the organization and stability of microtubules. *Neuroscience* 73, 1145–1157.
- Naranatt, P. P., Krishnan, H. H., Smith, M. S., and Chandran, B. (2005). Kaposi's sarcoma-associated herpes virus modulates microtubule dynamics via RhoA-GTP-diaphanous 2 signaling and utilizes the dynein motors to deliver its DNA to the nucleus. *J. Virol* 79, 1191–1206.
- Palazzo, A. F., Cook, T. A., Alberts, A. S., and Gundersen, G. G. (2001a). mDia mediates Rho-regulated formation and orientation of stable microtubules. *Nat. Cell Biol.* 3, 723–729.
- Palazzo, A. F., Eng, C. H., Schlaepfer, D. D., Marcantonio, E. E., and Gundersen, G. G. (2004). Localized stabilization of microtubules by integrin- and FAK-facilitated Rho signaling. *Science* 303, 836–839.
- Palazzo, A. F., Joseph, H. L., Chen, Y. J., Dujardin, D. L., Alberts, A. S., Pfister, K. K., Vallee, R. B., and Gundersen, G. G. (2001b). Cdc42, dynein, and

- dynactin regulate MTOC reorientation independent of Rho-regulated microtubule stabilization. *Curr. Biol.* **11**, 1536–1541.
- Prekeris, R., Mayhew, M. W., Cooper, J. B., and Terrian, D. M. (1996). Identification and localization of an actin-binding motif that is unique to the epsilon isoform of protein kinase C and participates in the regulation of synaptic function. *J. Cell Biol.* **132**, 77–90.
- Ridley, A. J., and Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70**, 389–399.
- Shi, S. H., Cheng, T., Jan, L. Y., and Jan, Y. N. (2004). APC and GSK-3beta are involved in mPar3 targeting to the nascent axon and establishment of neuronal polarity. *Curr. Biol.* **14**, 2025–2032.
- Soh, J. W., Lee, E. H., Prywes, R., and Weinstein, I. B. (1999). Novel roles of specific isoforms of protein kinase C in activation of the c-fos serum response element. *Mol. Cell. Biol.* **19**, 1313–1324.
- Tominaga, T., Sahai, E., Chardin, P., McCormick, F., Courtneidge, S. A., and Alberts, A. S. (2000). Diaphanous-related formins bridge Rho GTPase and Src tyrosine kinase signaling. *Mol. Cell* **5**, 13–25.
- Tzima, E., Kiosses, W. B., del Pozo, M. A., and Schwartz, M. A. (2003). Localized cdc42 activation, detected using a novel assay, mediates microtubule organizing center positioning in endothelial cells in response to fluid shear stress. *J. Biol. Chem.* **278**, 31020–31023.
- Wagner, U., Utton, M., Gallo, J. M., and Miller, C. C. (1996). Cellular phosphorylation of tau by GSK-3 beta influences tau binding to microtubules and microtubule organisation. *J. Cell Sci.* **109**(Pt 6), 1537–1543.
- Waller, B. J., and Alberts, A. S. (2003). The formins: active scaffolds that remodel the cytoskeleton. *Trends Cell Biol.* **13**, 435–446.
- Watanabe, N., Madaule, P., Reid, T., Ishizaki, T., Watanabe, G., Kakizuka, A., Saito, Y., Nakao, K., Jockusch, B. M., and Narumiya, S. (1997). p140mDia, a mammalian homolog of *Drosophila diaphanous*, is a target protein for Rho small GTPase and is a ligand for profilin. *EMBO J.* **16**, 3044–3056.
- Webster, D. R., Gundersen, G. G., Bulinski, J. C., and Borisy, G. G. (1987). Differential turnover of tyrosinated and detyrosinated microtubules. *Proc. Natl. Acad. Sci. USA* **84**, 9040–9044.
- Wen, Y., Eng, C. H., Schmoranz, J., Cabrera-Poch, N., Morris, E. J., Chen, M., Waller, B. J., Alberts, A. S., and Gundersen, G. G. (2004). EB1 and APC bind to mDia to stabilize microtubules downstream of Rho and promote cell migration. *Nat. Cell Biol.* **6**, 820–830.
- Wittmann, T., and Waterman-Storer, C. M. (2005). Spatial regulation of CLASP affinity for microtubules by Rac1 and GSK3beta in migrating epithelial cells. *J. Cell Biol.* **169**, 929–939.
- Yang, M., Zhong, W. W., Srivastava, N., Slavin, A., Yang, J., Hoey, T., and An, S. (2005). G protein-coupled lysophosphatidic acid receptors stimulate proliferation of colon cancer cells through the β -catenin pathway. *Proc. Natl. Acad. Sci. USA* **102**, 6027–6032.
- Yoshimura, T., Kawano, Y., Arimura, N., Kawabata, S., Kikuchi, A., and Kaibuchi, K. (2005). GSK-3beta regulates phosphorylation of CRMP-2 and neuronal polarity. *Cell* **120**, 137–149.
- Zigmond, S. H. (2004). Formin-induced nucleation of actin filaments. *Curr. Opin. Cell Biol.* **16**, 99–105.
- Zumbrunn, J., Kinoshita, K., Hyman, A. A., and Nathke, I. S. (2001). Binding of the adenomatous polyposis coli protein to microtubules increases microtubule stability and is regulated by GSK3 beta phosphorylation. *Curr. Biol.* **11**, 44–49.