BMP2 induction of actin cytoskeleton reorganization and cell migration requires PI3-kinase and Cdc42 activity

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Summary

Bone morphogenetic proteins (BMPs) are potent regulators of several cellular events. We report that exposure of C2C12 cells to BMP2 leads to an increase in cell migration and a rapid rearrangement of the actin filaments into cortical protrusions. These effects required independent and parallel activation of the Cdc42 small GTPase and the α-isoform of the phosphoinositide 3-kinase (PI3Kα), because ectopic expression of a dominant-negative form of Cdc42 or distinct pharmacological PI3K inhibitors abrogated these responses. Furthermore, we demonstrate that BMP2 activates different group I and group II PAK isoforms as well as LIMK1 with similar kinetics to Cdc42 or PI3K activation. BMP2 activation of PAK and LIMK1, measured by either kinase activity or with antibodies raised against phosphorylated residues at their activation loops, were abolished by blocking PI3K-signaling pathways. Together, these findings suggest that Cdc42 and PI3K signals emanating from BMP receptors are involved in specific regulation of actin assembly and cell migration.

Key words: BMP, Cell migration, Actin cytoskeleton, PI3K, Cdc42

Introduction

Cellular motility processes are essential in physiological and pathological situations such as embryonic morphogenesis, cell recruitment during tissue repair and regeneration, angiogenesis and tumor metastasis. An early feature of migrating cells is their polarization and the extension of protrusions, such as lamellipodia or spike-like filopodia, driven by actin polymerization, providing the basis for exploration of the local environment and directional migration (Ridley et al., 2003). Different proteins participate in signal transduction events that modulate actin cytoskeleton reorganization in response to a migration promoter agent. Members of the Rho family of small GTPases Cdc42 and Rac are essential mediators in regulating cytoskeleton dynamics during protrusion formation (Etienne-Manneville and Hall, 2002; Ridley et al., 2003). Small G proteins are activated by the exchange of bound GDP for GTP by a specific activated guanine exchange factor (GEF). Binding of GTP to Cdc42 or Rac allow the activation of p21-activated protein kinase (PAK) family members through binding of the GTPase to a CRIB (Cdc42/Rac interaction-binding) domain, which causes a conformational change on the kinase, inducing autophosphorylation and an increase in its kinase activity (Zhao and Manser, 2005). Activation of PAK has been shown to result in peripheral actin reorganization by phosphorylating substrates such as LIM kinase 1 (LIMK1), which in turn phosphorylates and inactivates cofilin, a protein that promotes depolymerization of F-actin, leading to actin filament stabilization (Edwards et al., 1999). Both PAK activation and cofilin phosphorylation by LIMK1 have a key role in maintaining and extending protrusions at the leading edge of migrating cells (Cau and Hall, 2005; Dawe et al., 2003).

Directional migration is also controlled by the establishment of an intracellular gradient of phosphatidylinositol-(3,4,5)-trisphosphate [PtdIns(3,4,5)P_3 or PIP3] and PI(3,4)P_2 generated at the leading edge by Class I phosphoinositide 3-kinases (PI3Ks) (Ridley et al., 2003). Class I PI3Ks are divided into Class IA (PI3Kα, PI3Kβ and PI3Kδ) and Class IB (PI3Kγ). Class IA isoforms (p110α, p110β and p110δ) form heterodimers with a regulatory subunit known as p85 and are activated specifically in response to growth factor or cytokine receptors, through a tyrosine-kinase-dependent mechanism (Cantley, 2002). In contrast to class IA, PI3Kγ activation is driven by activation of pertussis-toxin-sensitive $G_{\alpha i}$-coupled receptors, such as chemokine or chemoattractant receptors (Suire et al., 2006). Expression of PI3Kα and PI3Kβ is ubiquitous, whereas expression of the PI3Kδ and PI3Kγ isoforms is restricted to the hematopoietic tissue (Wetzker and Rommel, 2004). The increased concentration of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 leads to the rapid subcellular relocalization and consequent activation of several effector proteins containing pleckstrin homology (PH) domains, such as Akt, modulating their availability at the leading edge (Chung et al., 2001; Merlot and Firtel, 2003). PIP3 generation also appears to be required for the relocalization and activation of the small GTPase protein Cdc42 activity (Li et al., 2003) and of different GTP-exchange factor (GEF) proteins by virtue of their PH domains (Merlot and Firtel, 2003), implying a network of positive-feedback loops between small GTPases, PI3K products and

effectors, such as PAK, working together to initiate and maintain the polarity of migrating cells.

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β (TGF-β) superfamily and have been shown to participate in patterning and specification of several tissues and organs during vertebrate development, and to regulate cell growth, apoptosis and differentiation in different cell types (Capdevila and Izpisua Belmonte, 2001; Massague, 2000). BMPs were originally identified by their ability to induce ectopic bone formation and BMP2, BMP4 and BMP7 have been characterized as key molecules for normal bone development in vertebrates (Wan and Cao, 2005), and can induce osteoblastic differentiation of C2C12 mesenquimal pluripotent cells (Katagiri et al., 1994). Early events in BMP signaling are initiated through the phosphorylation of specific receptor-regulated Smad proteins, namely Smad1, Smad5 or Smad8. After phosphorylation, R-Smads form heteromeric complexes with the common mediator Smad4. These Smad complexes migrate to the nucleus and activate the transcription of specific target genes (Massague, 2000). BMP activity has been also shown to be involved in cell migration. BMP2 signaling is required for migration of neural crest pluripotent population that generates craniofacial structures and enteric nervous system (Dudas et al., 2004; Goldstein et al., 2005; Kishigami and Mishina, 2005). Furthermore, BMP2 induces migration of bone marrow mesenchymal progenitors, osteoblasts and endothelial cells (Fiedler et al., 2002; Lind et al., 1996; Sotobori et al., 2006). Despite the known signaling events leading to the transcriptional activity induced by BMPs, little is known about the signaling pathways involved in BMP2-mediated cell migration. In this study, we report that in pluripotent C2C12 cells, BMP2 induces cell migration and a rapid rearrangement of the actin cytoskeleton, with extension of protrusions resembling filopodia. This effect requires parallel activation of the small GTPase Cdc42 and the class IA PI3Kα isoform. Moreover, we also demonstrate that BMP2 activates PAKs and LIMK1 following the same kinetics observed for BMP2 induction of cytoskeletal rearrangement, suggesting their participation in the observed effects. The results presented here provide new information on the signaling mechanisms involved in BMP-induced actin reorganization and cell migration.

Results

BMP2 induces cell migration

Several reports indicate that BMPs regulate cell migration during embryonic morphogenesis (Dudas et al., 2004; Goldstein et al., 2005; Kishigami and Mishina, 2005). To test the involvement of BMP2 on C2C12 cell migration, we performed an in vitro migration assay with a chemotaxis chamber. Cells were allowed to migrate for 2 hours in the presence or absence of BMP2 in the lower chamber. Quantification of cell migration revealed that in the presence of BMP2, 70% more cells migrated compared with the control (Fig. 1A), demonstrating that BMP2 induced chemotactic cell migration.

Wound-healing migration assays were performed to confirm the migratory behavior of C2C12 cells in response to BMP2. Cell monolayers were scrape-wounded and allowed to heal in the presence of BMP2. As shown in Fig. 1B, the wound was more efficiently invaded in the presence of BMP2. To quantify this effect, we determined the percentage of invaded area relative to the initial wound area, confirming that BMP2 stimulated cell migration. Furthermore, inhibition of protein synthesis by addition of 1 μg/ml cycloheximide 30 minutes before stimulation of the cells with BMP2, had no effect on wound closure (data not shown), suggesting that changes in gene transcription and protein synthesis were not required for BMP2-dependent effects. Similarly, when cells were pretreated with the inhibitor of DNA synthesis mitomycin C (10 μg/ml), BMP2 was still able to accelerate the wound closure, indicating that the increase in cell migration is not due to an increase in cell proliferation.

BMP2 induces actin cytoskeleton reorganization

The control of actin filament assembly is likely to underpin directed cell migration in all cell types (Ridley et al., 2003). To analyze whether BMP2 was playing a role in the organization of the actin cytoskeleton, C2C12 cells were stimulated with BMP2, fixed at different time-points and filamentous actin was visualized (Fig. 2A). BMP2 induced the accumulation of cortical actin to the cell periphery. To emphasize and quantify the effect induced by BMP2, C2C12 cells were pretreated with cytochalasin D, a depolymerizing F-actin agent, to transiently disrupt the actin cytoskeleton. To analyze whether the disruption of the actin filaments affected the BMP2-signaling pathway, phosphorylation of Smad1 was visualized in cells treated with

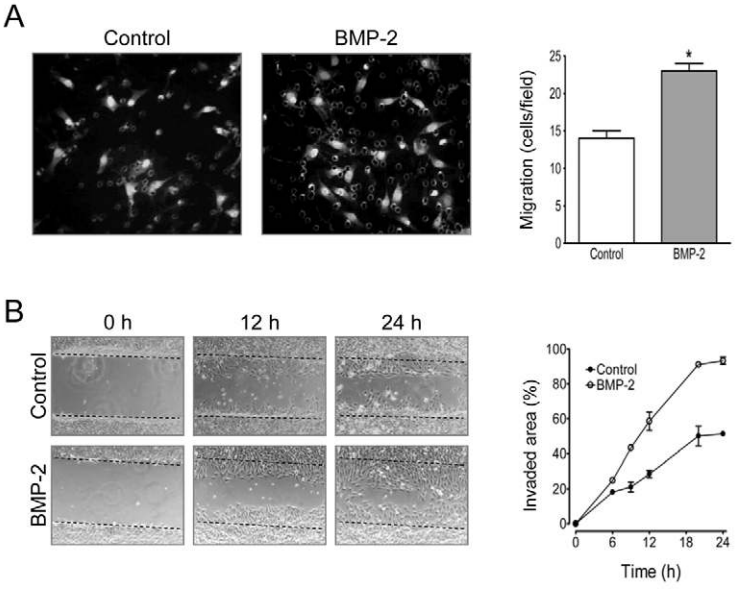

Fig. 1. BMP2 induces chemotactic C2C12 cell migration. (A) Representative images of propidium-iodide-stained C2C12 migrated cells are shown on the left. Quantitative analysis of eight random fields from three independent experiments is shown on the right (mean ± s.e.m.; **P*<0.001, paired *t*-test). (B) Wounded C2C12 cell monolayers were allowed to migrate in the presence or absence of 3 nM BMP2 for different times. A quantitative analysis of the invaded area was obtained from three photographed fields obtained in at least four independent experiments and represented as mean ± s.e.m.

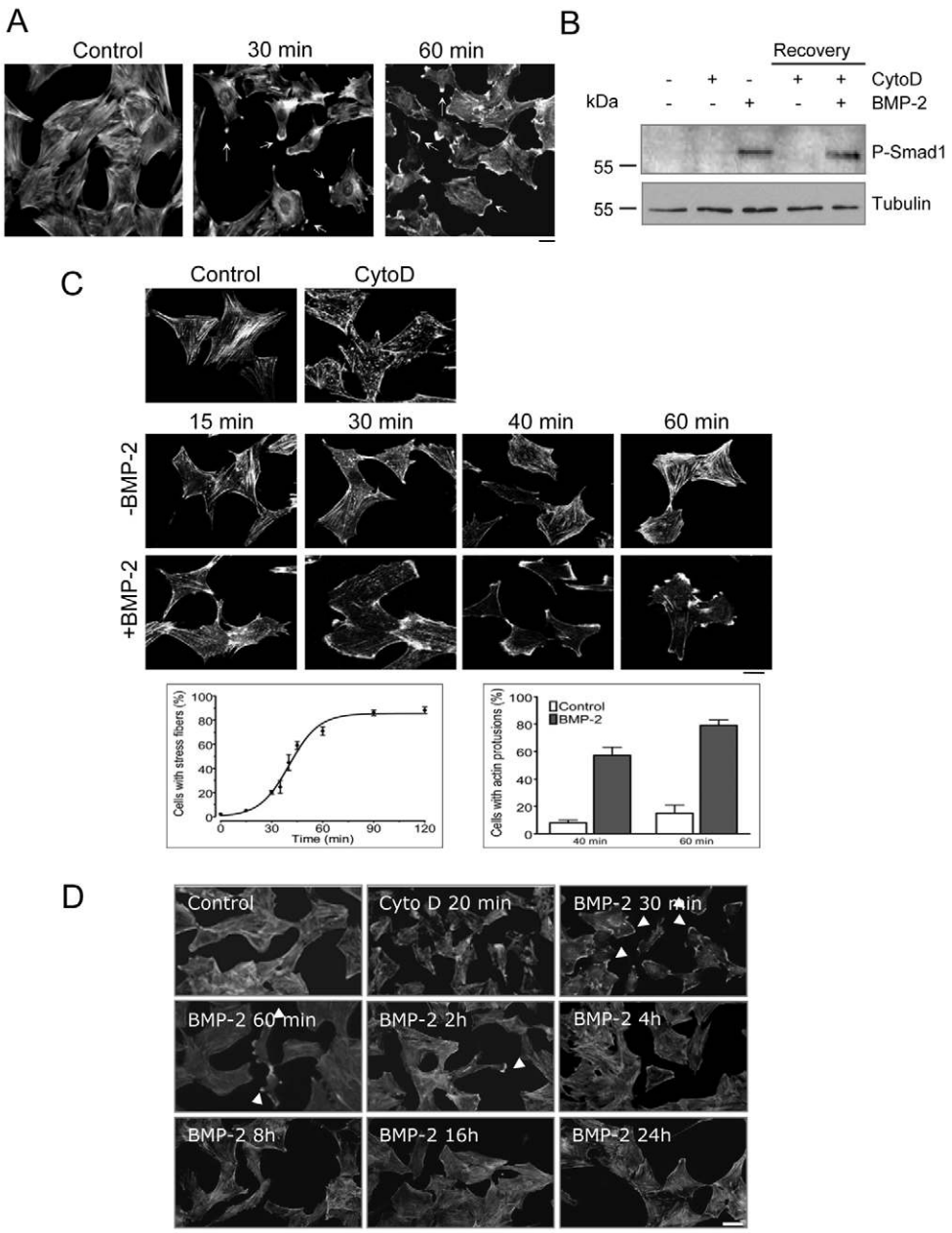

Fig. 2. BMP2 induces actin cytoskeleton reorganization. (A) C2C12 cells were stimulated with 3 nM BMP2 for the indicated times. Actin was visualized with TRITC-conjugated phalloidin using a confocal microscope. Arrows indicate actin cortical protrusions. Scale bar: 20 µm. (B) Serum-starved C2C12 cells were pretreated with 2 µM cytochalasin D (Cyto D) for 20 minutes and allowed to recover in the absence or presence of 3 nM BMP2 for 1 hour. Cell lysates were analyzed by immunoblotting with anti-phospho-Smad1 and reprobed with anti-α-tubulin antibodies. (C) Cells treated as above were allowed to recover in the absence or presence of 3 nM BMP2. Upper row shows cells before and after 2 µM Cyto D treatment. Middle and lower rows show recovery in the absence or presence of BMP2, respectively. Scale bar: 20 µm. A quantitative analysis of the formation of stress fibers in the absence of BMP2 or cells showing cortical actin protrusions as a percent of total in the presence of BMP2 are shown. Data are mean ± s.e.m. of at least 200 cells obtained in different fields from five independent experiments. (D) Serum-starved C2C12 cells (Control) were pre-treated with 2 µM cytochalasin D (Cyto D) for 20 minutes and after washing, were allowed to recover in the presence of 3 nM BMP2 for different times. Actin was visualized with TRITC-conjugated phalloidin using a confocal microscope. Arrows indicate actin cortical protrusions that were evident within the first 60 minutes and cells then recovered their initial aspect showing abundant stress fibers. Scale bar: 20 µm.

cytochalasin D. No significant changes were observed (Fig. 2B). As shown in Fig. 2C, whereas in control conditions cells recovered their initial aspect and had abundant stress fibers, in the presence of BMP2, the number of cells that presented cortical protrusions enriched in F-actin and without stress fibers was significantly increased (up to 70% more than in untreated cells). Cells pretreated with cytochalasin D were allowed to recover in the presence of BMP2 for longer periods. Cortical actin protrusions were evident until 2 hours and then cells recovered abundant stress fibers (Fig. 2D). Taken together, our findings indicate that BMP2 induced a rapid, significant and transient effect on the dynamics of the actin cytoskeleton.

BMP2-induced actin reorganization and cell migration is mediated by Cdc42

Actin filament organization is controlled by the Rho family of small GTPases, including Rho, Rac and Cdc42 (Raftopoulou and Hall, 2004). The participation of the Rho family of small GTPases in the organization of the actin cytoskeleton has been widely studied in Swiss3T3 fibroblasts, where Rho regulates the formation of contractile actin-myosin filaments to form stress fibers (Ridley and Hall, 1992), and Rac and Cdc42 regulate lamellipodia and filopodia formation, respectively (Nobes and Hall, 1999). We therefore tested the hypothesis that BMP2-induced membrane actin protrusion formation might be mediated through small GTPases in Swiss3T3 cells. As shown in Fig. 3A, BMP2 rapidly induced the appearance of long F-actin-rich spike-like filopodia, a phenotype associated with active Cdc42. As a control, cells were stimulated with EGF, which has been shown to activate Rho and to induce the formation of stress fibers (Mancini et al., 2003). To confirm the potential involvement of Cdc42 on BMP2-induced filopodia, we examined whether BMP2 activated Cdc42 in C2C12 cells. Treatment of C2C12 cells with BMP2 increased the endogenous levels of active

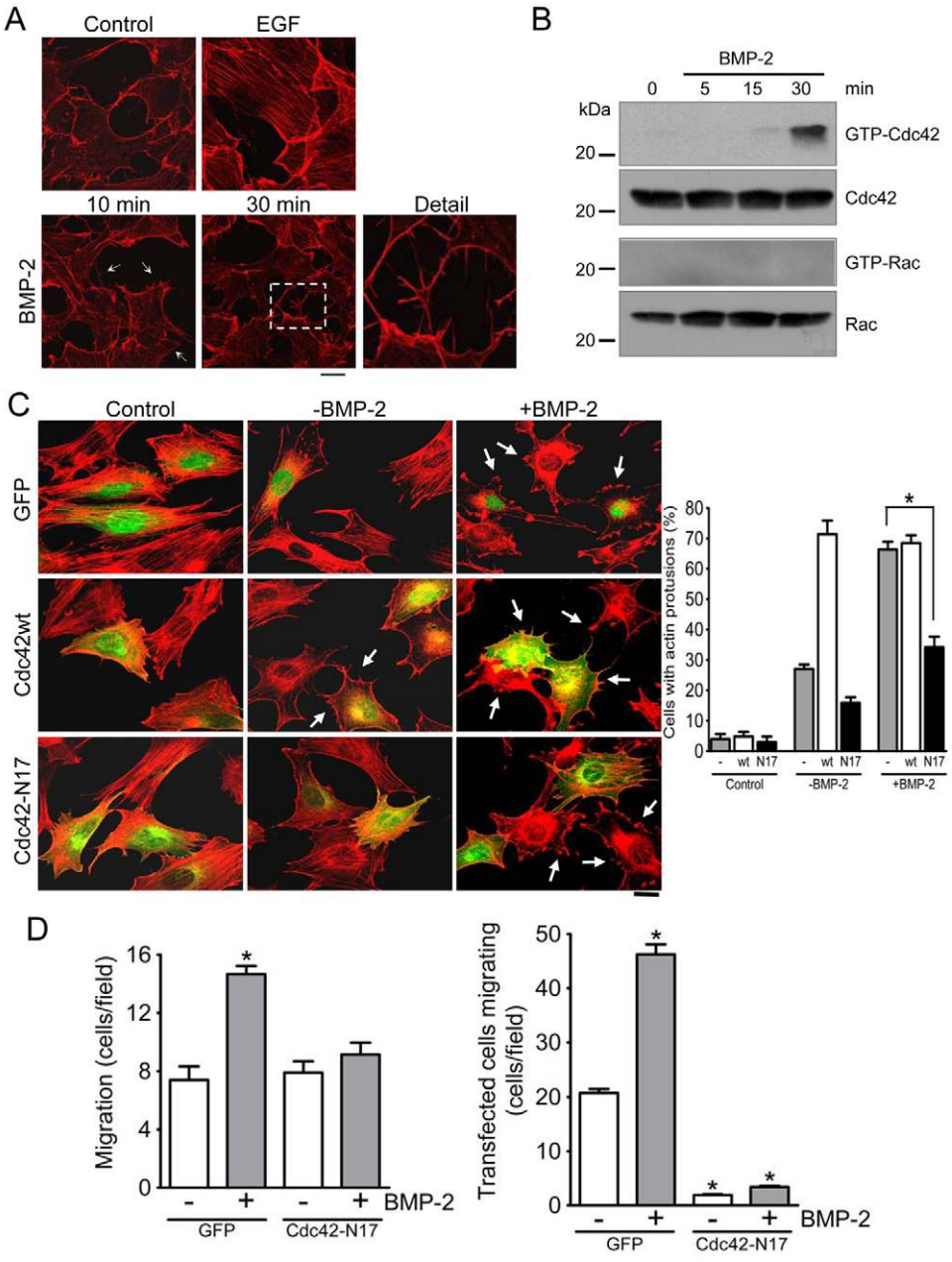

Fig. 3. BMP2-induced actin reorganization and cell migration is mediated by Cdc42. (A) Swiss 3T3 cells were serum-starved for 7 days and stimulated with 3 nM BMP2. Arrows indicate spike-like filopodia (see detail at 30 minutes). As a control, cells were stimulated with EGF 20 ng/ml for 10 minutes (upper row). Scale bar, 20 µm. (B) Serum-starved C2C12 cells were stimulated with 3 nM BMP2. Levels of active GTP-bound Cdc42 and Rac were determined using the PBD domain of PAK1 followed by immunoblotting with anti-Cdc42 and anti-Rac antibodies. (C) C2C12 cells were transfected with the indicated GFP-tagged expression vectors. Cells were serum-starved for 16 hours (control), pre-treated with cytochalasin D and allowed to recover for 1 hour in the absence or presence of 3 nM BMP2. Merged images of phalloidin (red) and GFP signal (green) are shown. Cells showing cortical actin protrusions (arrows) are indicated. Scale bar: 20 µm. Transfected cells were counted and those showing cortical actin protrusions represented as % of total (graph on right). Mean ± s.e.m. of at least 80 transfected cells obtained from three independent experiments (*P<0.001, paired t-test). (D) C2C12 cells were transfected with the GFP-tagged expression vectors indicated and analyzed by chemotaxis assay after 2 hours (left panel) and by wound-healing migration assay performed for 12 hours (right panel) in the presence or absence of BMP2. Mean ± s.e.m. of three independent experiments (*P<0.001 compared with the GFP-transfected control cells and in the absence of BMP2, One-way ANOVA followed by Bonferroni's multiple comparison test).

Cdc42 after 15 minutes of ligand addition and peaked at 30 minutes. By contrast, active GTP-bound endogenous Rac levels were not altered up to 30 minutes after BMP2 stimulation (Fig. 3B). To verify the requirement of the Cdc42 pathway in the observed morphological changes induced by BMP2, cells were transfected with the dominant-negative mutant Cdc42-N17-GFP and allowed to recover as described above (Fig. 2C). Microscopy examination showed that BMP2 prevented the formation of stress fibers and induced the accumulations of actin in cortical protrusions in control cells, whereas these effects were impaired in cells expressing Cdc42-N17. Expression of wild-type Cdc42 was sufficient for the induction of actin protrusions and reduction of stress fibers in the absence of BMP2, but did not further increase BMP2-induced actin protrusion formation (Fig. 3C).

We further addressed whether Cdc42 was also regulating the BMP2 effects on cell migration using chemotaxis and wound-induced migration assays. The results obtained in both types of assay indicated that in cells expressing Cdc42-N17, BMP2 was not able to induce migration when compared with the GFP-transfected control cells (Fig. 3D). Altogether, these data suggest that Cdc42 activation is involved in BMP2-induced rearrangement of the actin cytoskeleton and cell migration.

PI3K activity is required for BMP2-induced actin reorganization

PI3K activity has previously been shown to mediate the activation of small GTPases of the Rho family upon stimulation with several growth factors (Hawkins et al., 2006; Merlot and Firtel, 2003; Raftopoulou and Hall, 2004; Suire et al., 2006). We first confirmed the ability of BMP2 to activate PI3K by examining the phosphorylation status of Akt, because phosphorylation of Akt on residue Ser473 correlates with Akt activation by PI3K. We demonstrated that BMP2 activated PI3K activity in a time-

dependent manner. An increase in Akt phosphorylation was observed by 30 minutes and reached a plateau at 60 minutes. BMP2-induced PI3K activation was completely abolished by treatment with the inhibitor of PI3K activity LY294002 (Fig. 4A). We next characterized the effect of LY294002 on the observed BMP2 reorganization of the actin cytoskeleton. Pretreatment with LY294002 completely abolished BMP2-dependent induction of cortical actin protrusion formation, suggesting that an intact PI3K-signaling pathway was required for the BMP2-induced mobilization of the actin filament system (Fig. 4B). In a wound-induced migration assay, addition of BMP2 to serum-free medium accelerated the wound closure, whereas the presence of PI3K inhibitor strongly diminished the BMP2 migratory effects (Fig. 4C). These data suggest that PI3K is involved in BMP2-induced cell migration.

To further characterize BMP2-mediated activation of PI3K, we analyzed the contribution of the individual PI3K isoforms to BMP2-induced PI3K activation. Since LY294002 is not selective among the members of the class I PI3Ks (Hawkins et al., 2006), we used a set of recently developed isoform-selective class I PI3K inhibitors (Camps et al., 2005; Jackson et al., 2005; Pomel et al., 2006; Sadhu et al., 2003). Cells were treated with either the PI3Kγ-selective inhibitor AS252424 (Pomel et al., 2006), the PI3Kβ-selective inhibitor TGX-155 (Jackson et al., 2005), the PI3Kδ-selective inhibitor IC87114 (Billottet et al., 2006; Sadhu et al., 2003) or the PI3Kα-selective inhibitor AS702630 (Ruckle et al., 2004), and analyzed phosphorylation of Akt after stimulated with BMP2 for different times. BMP2-mediated Akt phosphorylation was abolished only in cells pretreated with AS702630, but not with any other of the selective inhibitors used, suggesting that, in C2C12 cells, BMP2 induced Akt phosphorylation through the specific activation of the PI3Kα isoform (Fig. 5A). Furthermore, the wound-healing assay was performed in the presence of the class I PI3K isoform-selective inhibitors mentioned above and only in the presence of AS702630, was BMP2 unable to induce cell migration (Fig. 5B). Together, these data suggest that PI3Kα is required for BMP2-induced actin cytoskeleton rearrangement and cell migration in C2C12 cells.

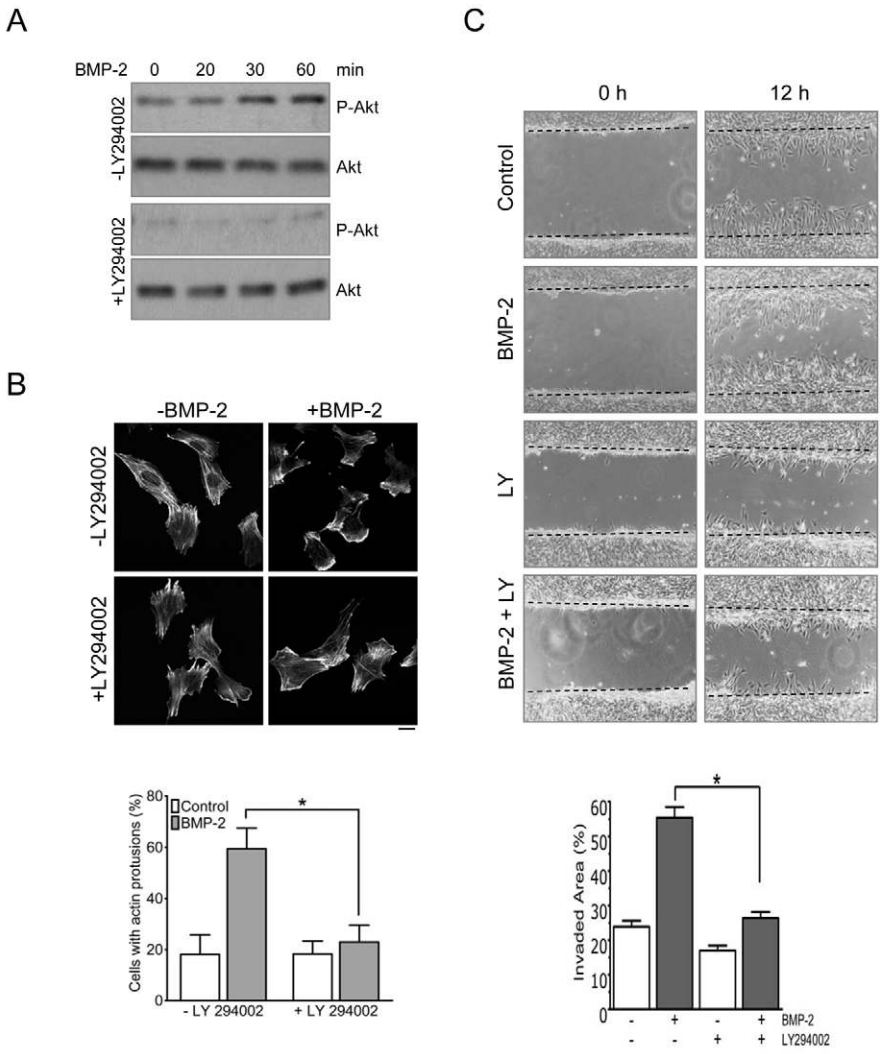

Fig. 4. PI3K activity is required for BMP2-induced actin reorganization and cell migration in C2C12 cells. (A) Cells were serum-starved for 16 hours prior to preincubation with 15 μM LY294002 for 60 minutes and then stimulated with 3 nM BMP2 for the indicated times. Cell lysates were analyzed by immunoblotting with anti-Akt-*P*(Ser473) and reprobed with anti-Akt total antibody. (B) Cells treated as above were fixed and stained with TRITC-conjugated phalloidin. Scale bar: 20 μm. Cells with actin protrusions were quantified as shown in graph below. Mean ± s.e.m. from four independent experiments (**P*<0.001, paired *t*-test). (C) Cell monolayers were wounded and allowed to migrate for 12 hours in the presence or absence of 3 nM BMP2 and/or LY294002. The graph in lower panel indicates the invaded area. Mean ± s.e.m. from three independent experiments (**P*<0.001, paired *t*-test).

These observations indicate that BMP2 activates Ccd42 and PI3K pathways independently and that both signaling pathways are required for the BMP2-induced mobilization of the actin filaments system.

BMP2 induces LIMK1 activation through a PI3K-dependent mechanism

Since previous results indicate that other members of the BMP family are able to induce LIMK1 activity (Foletta et al., 2003; Lee-Hoeflich et al., 2004), we tested whether LIMK1 was regulated by BMP2. As shown in Fig. 7A, BMP2 enhanced the phosphorylation of LIMK1 at their activation loop in Thr508 (Scott and Olson, 2007) with maximal effect 40 minutes after ligand addition. We next examined the requirement of PI3K on BMP2-induced LIMK1

We next investigated the relationship between Cdc42 and PI3K in this process. We first checked whether PI3K was implicated in the activation of Cdc42 by measuring the activation of the endogenous Cdc42 by BMP2 in the presence of the PI3K inhibitor LY294002. Under these conditions, we did not observe differences in the activation of Cdc42 (Fig. 6A). Similarly, the expression of a dominant-negative form of Cdc42 did not modify the ability of BMP2 to induce PI3K activity and to phosphorylate Akt (Fig. 6B).

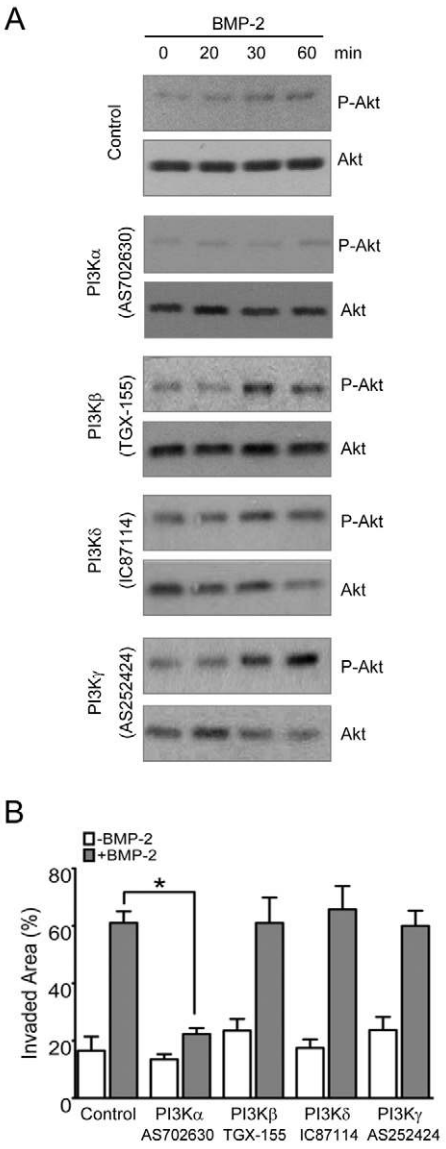

Fig. 5. PI3Kα is required for BMP2-induced cell migration. (A) Cells were serum-starved for 16 hours. The isoform-selective inhibitors of class I PI3K were added to the medium 1 hour before stimulation of cells with BMP2 and used at a final concentration of 1 μM. Cell lysates were analyzed by immunoblotting with anti-Akt-*P*(Ser473) and membranes reprobed with anti-Akt total antibody. (B) Quantitative analysis of phase-contrast images of a wound-healing assay performed for 12 hours in the presence of 3 nM BMP2 and the PI3K-isoform-selective inhibitors. Mean ± s.e.m. from three independent experiments (**P*<0.001, paired *t*-test).

phosphorylation. As seen in Fig. 7B, complete inhibition of BMP2-induced LIMK1 phosphorylation was achieved by pretreatment with LY294002 inhibitor. Because LIMK1 has been described to directly activate cofilin, we also examined whether the BMP2-dependent phosphorylation of LIMK1 observed correlated with an increase of LIMK1 activity and/or with enhanced cofilin phosphorylation. BMP2 stimulation resulted in an ~1.9-fold increase in LIMK1-mediated cofilin in vitro phosphorylation. As expected, the effect of BMP2 on LIMK1 activity was abrogated in the presence of LY294002 inhibitor (Fig. 7C). These data suggest that BMP2 stimulates both LIMK1 activity and cofilin phosphorylation and that these effects require PI3K activity.

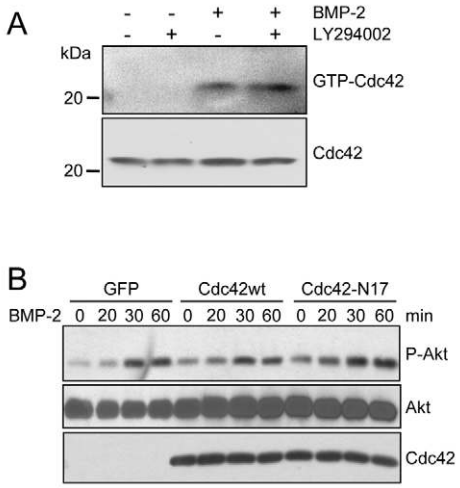

Fig. 6. BMP2 activates Cdc42 and PI3K pathways independently. (A) C2C12 cells treated with 3 nM BMP2 and/or LY294002. Levels of GTP-bound Cdc42 were determined using GST pull-down assay. (B) C2C12 cells were transfected with GFP-tagged plasmids for wild-type Cdc42 (GFP-Cdc42wt) or a dominant-negative form (GFP-Cdc42N17). Cells were stimulated with 3 nM BMP2 and cell lysates were analyzed by immunoblotting with anti-Akt-*P*(Ser473), and reprobed with anti-Akt antibody and anti-Cdc42 antibody.

BMP2 stimulates PAK1 and PAK4 kinase activity through PI3K- and Cdc42-dependent mechanisms

The PAK family of protein kinases have been implicated in activation of LIMK1 (Dan et al., 2001; Edwards et al., 1999). Thus, we analyzed whether PAK proteins were also regulated by BMP2. Cells were stimulated with BMP2 and phosphorylation of either PAK1 or PAK4 was analyzed. Increased phosphorylation was detected 30 minutes after stimulation, which peaked at 40 minutes in both cases and decreased thereafter (Fig. 8A). Incubation with LY294002 blocked BMP2-induced PAK1 and PAK4 phosphorylation (Fig. 8B). As further support for PI3K involvement in PAK1 and PAK4 activation by BMP2, in vitro kinase assays were carried out. Whereas BMP2 stimulated both PAK1 and PAK4 kinase activity to more than twofold basal levels after 40 minutes, LY294002 completely inhibited this activation (Fig. 8C). These results thus indicate that BMP2 activates PAK1 and PAK4 in a time-dependent manner and that this activation depends on PI3K activity.

We next determined whether the BMP2 activation of PAK depended on Cdc42 activity. C2C12 cells were transfected with the dominant-negative mutant Cdc42-N17 and, after BMP2 stimulation, the phosphorylation of either PAK1 or PAK4 was analyzed. In contrast to control cells transfected with GFP, expression of the dominant-negative form of Cdc42 prevented BMP2-induced phosphorylation of PAK1 and PAK4 (Fig. 9A). These results thereby indicated that BMP2 activation of PAK1 and PAK4 depended on Cdc42. We also analyzed the involvement of PAK on the activation of LIMK1 associated with the tail of BMPRII. We generated C2C12 cells stably expressing His-tagged BMPRII under a tetracycline-responsive promoter (Fig. 9B, left panel). These cells were transfected with a constitutive active form of PAK1 (PAK-H83,86L) and a dominant-negative form (PAK-PID) and analyzed for activation of LIMK1 associated with BMPRII after purification of His-tagged BMPRII with Ni^{2+}-NTA-beads. Addition of BMP2 slightly increased association of LIMK1 to the tail of BMPRII and more importantly, increased the level of phosphorylated LIMK1

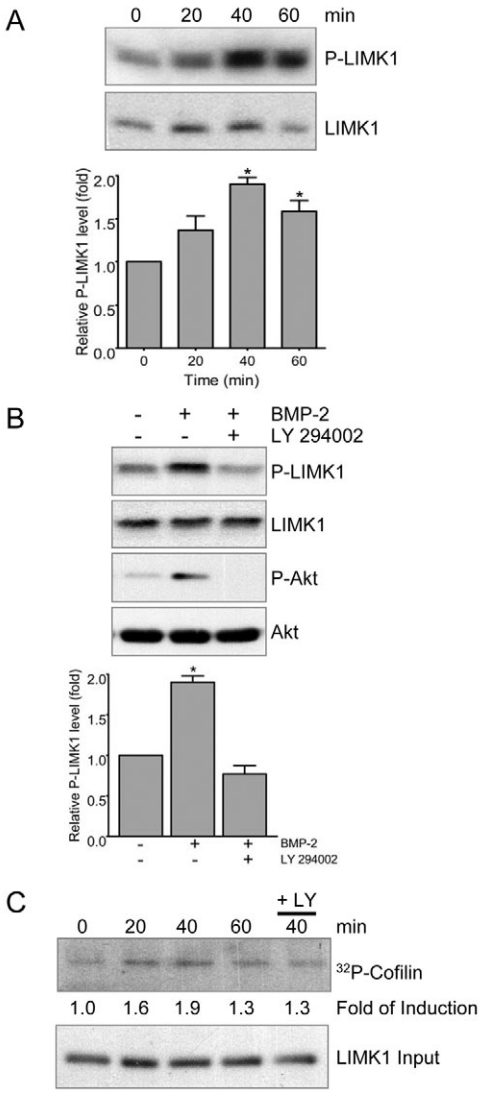

Fig. 7. BMP2 stimulates LIMK1 activity. (A) Serum-starved C2C12 cells were stimulated with 3 nM BMP2 and cell lysates analyzed by immunoblotting with anti-LIMK1-*P* and anti-LIMK1 antibody. The bottom panel indicates the relative LIMK1-*P* levels. Mean ± s.e.m. from three independent experiments (**P*<0.05, paired *t*-test). (B) C2C12 cells were treated as above for 40 minutes but in the absence or presence of LY294002. Cell lysates were analyzed by immunoblotting with the indicated antibodies. The bottom panel indicates the relative LIMK1-*P* levels. Mean ± s.e.m. from three independent experiments (**P*<0.05, paired *t*-test). (C) C2C12 cells were treated with BMP2 and LY294002 as indicated. Endogenous LIMK1 was subjected to an in vitro kinase assay with GST-cofilin as a substrate. Values of cofilin phosphorylation with respect to the LIMK1 present in cell extracts are shown.

(LIMK1-*P*) at Thr508 (Fig. 9, right panel). Furthermore, expression of the active form of PAK1 increased the levels of LIMK1-*P* even in the absence of BMP2, whereas expression of PAK-PID partially prevented the phosphorylation of LIMK1 induced by BMP2. These data suggest that PAK activity is involved in the activation of LIMK1 interacting with the BMPRII tail.

It has been shown that although BMP2 binds preferentially to BMPRII, BMP6 and BMP7 signal preferentially through ActRII, which lack the cytoplasmic tail present in BMPRII (Ebisawa et al., 1999; Macias-Silva et al., 1998). Furthermore, in BMPRII-deficient cells BMP2 can signal through ActRII in conjunction with a set of type I receptors distinct from those used by BMPRII (Yu et al., 2005). Taking this into account, we also analyzed the migration and invasive effects of addition of BMP7 as well as the effects of BMP2 in C2C12 cells in which BMPRII expression was knocked down (Fig. 10C). Addition of BMP7 led to similar effects as observed with BMP2 in chemotaxis and wound-healing assays (Fig. 10A,B), and reduction of BMPRII levels did not significantly modify cytoskeletal and migratory responses to BMP2 (Fig. 10D,E). These data suggest that, although LIMK physically interacts with the C-terminal tail of BMPRII, the C-terminal tail is not absolutely required for the cytoskeletal and migratory effects of BMPs.

Discussion

Cell responses to morphogens include actin cytoskeletal reorganization and cell migration, which are crucial not only during embryogenesis or bone turnover but also during tumor progression and invasion (Dormann and Weijer, 2003; Kishigami and Mishina, 2005). Here, we describe that BMP2 stimulation induces the accumulation of actin in cortical protrusions and migration of mesenchymal cells. The data presented indicate that, in C2C12 cells, BMP2-induced cytoskeletal rearrangements depend on the activities of both Cdc42 and the α-isoform of PI3K, which are activated independently by BMP2. These data also demonstrate that BMP2 activates group I and group II PAKs as well as LIMK1 in a PI3K-dependent manner, suggesting that they have a role integrating the signals from both pathways and in the control of BMP-induced actin reorganization and cell migration.

BMPs have been shown to promote chemotactic migration of mesenchymal cells in vitro, as well as during skeletal development (Fiedler et al., 2002; Sotobori et al., 2006). Our results indicate that the rapid appearance of cortical actin protrusions did not require protein synthesis, suggesting it is independent of transcriptional activity. Previous data also suggest a role for the inhibitory Smad7 in the delayed activation of Cdc42 (12-24 hours) by TGFβ in prostate carcinoma cells (Edlund et al., 2004). However, overexpression of Smad7 in mesenchymal C2C12 cells did not increase either basal or BMP-induced appearance of actin-enriched membrane protrusions (data not shown). These results are in agreement with data obtained in Swiss3T3 fibroblasts (Vardouli et al., 2005) suggesting that Smad7 is not involved in the rapid cytoskeletal rearrangements induced by BMP2 in mesenchymal versus epithelial cells.

We also show that BMP2 activates Cdc42 and PI3K, confirmed by the appearance of GTP-bound Cdc42 and Akt phosphorylated on Ser473, respectively. We propose that both signaling pathways, acting in parallel but independently, are required for the BMP-induced cytoskeletal effects. We base this conclusion on the following observations: the temporal profiles of activation of both Cdc42 and PI3K correlated with the formation of BMP2 induced cortical actin protrusions in C2C12 cells and filopodia in Swiss 3T3 fibroblasts. Moreover, expression of a dominant-negative form of Cdc42 or pretreatment with the PI3K inhibitors LY294002 or AS702630, both completely suppressed the BMP-induced appearance of actin protrusions. Although the pathway that leads to BMP2 activation of Cdc42 is still unknown, several reports indicate its requirement for cytoskeletal changes induced by BMPs or TGFβ (Edlund et al., 2002; Edlund et al., 2004; Lee-Hoeflich et al., 2004; Ricos et al., 1999). Depending on the cell type or stimuli, activation of Cdc42 has been described to be upstream or downstream of PI3K activity (Hawkins et al., 2006; Jimenez et al.,

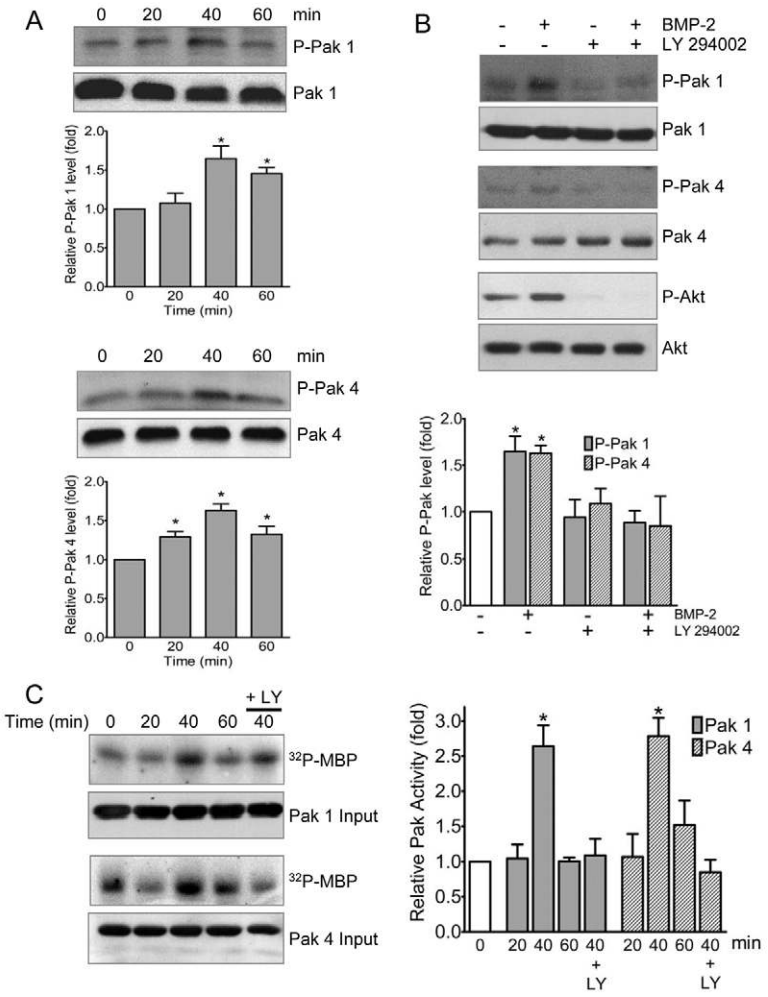

Fig. 8. BMP2 stimulates PAK1 and PAK4 activity. (A) C2C12 cells were stimulated with 3 nM BMP2 for different times and cell lysates were analyzed with the indicated phospho-specific antibodies, and membranes reprobed with anti-PAK1 and anti-PAK4 antibodies. Graphs indicate the relative PAK1-*P* and PAK4-*P* levels. Mean ± s.e.m. from three independent experiments (**P*<0.05, paired *t*-test). (B) C2C12 cells were serum-starved, incubated with LY294002 and stimulated with 3 nM BMP2 for 40 minutes. Cell lysates were analyzed as above. Graph indicates the relative PAK1-*P* and PAK4-*P* levels. Mean ± s.e.m. from three independent experiments (**P*<0.05, paired *t*-test). (C) C2C12 cells stimulated with 3 nM BMP2 and LY294002 were analyzed for PAK1 or PAK4 in vitro kinase activity using MBP as a substrate. The graph indicates the relative PAK1 and PAK4 activity. Mean ± s.e.m. from three independent experiments (**P*<0.05, paired *t*-test).

2000; Merlot and Firtel, 2003; Raftopoulou and Hall, 2004; Ridley et al., 2003). Importantly our results indicate that, although Cdc42 and PI3K pathways are both required for the migratory effects, BMP2 is able to activate both routes independently, because the block of one pathway does not alter the ability of BMP2 to stimulate the other. Although cell migration in response to several stimuli, and in a vast majority of cell types, has been unequivocally associated to PIP3 formation (and therefore to class I PI3K activation), the contribution of specific PI3K isoforms to this cellular function has only been reported for PI3Kγ and PI3Kδ, in response to chemoattractants or to PI3Kδ and PI3Kβ, in response to growth factors (Camps et al., 2005; Puri et al., 2004; Sadhu et al., 2003; Vanhaesebroeck and Waterfield, 1999). However, no role of class IA PI3Kα in cell migration has been reported so far. Our data strongly involve class I_A, and specifically p110α, in BMP2-induced cytoskeletal effects and cell migration, as well as in the activation of Akt-signaling pathway in C2C12 cells.

Members of the PAK family have been shown to regulate a wide variety of cytoskeletal changes, usually in response to small GTPases. Upon activation, PAKs redistribute from the cytosol into cortical actin structures including lamellae, the leading edge of polarized cells and membrane ruffles. PAKs can be categorized into two subgroups: the group I (PAK1, PAK2 and PAK3) share high sequence homology throughout the protein, whereas group II (PAK4, PAK5 and PAK6) have highly related kinase domains but are more divergent in other domains of the protein (Bokoch, 2003; Zhao and Manser, 2005). Our results are the first to show that BMP2 is able to activate members of both classes of PAKs. Activation takes place following similar kinetics (30-60 minutes) observed for BMP2 induction of cytoskeletal rearrangement. Furthermore, we also demonstrate that activation of PAKs by BMP2 is abolished by either inhibition of the PI3K pathway or expression of dominant-negative Cdc42. Thus, it could be hypothesized that PAKs act as integrative signaling modules where the signals from both pathways converge. It has been shown that class I PAKs are not only stimulated by GTP-bound forms of Rac and Cdc42 through binding to an autoinhibitory N-terminal region (Parrini et al., 2002), but also by a variety of GTPase-independent mechanisms. For example, although full catalytic activity is achieved by autophosphorylation of Thr423 in the catalytic domain of PAK1 (equivalent to Thr402 of PAK2), other kinases activated in response to PI3K stimulation, such as the PH-containing, 3-phosphoinositide-dependent kinase-1 (PDPK1), can also activate PAK1 through phosphorylation at the same site (King et al., 2000). In addition, Akt, through phosphorylation of Ser21, and PI3K, through physical association, have been shown to activate PAK1 independently of small GTPase binding (Chung and Firtel, 1999; Papakonstanti and Stournaras, 2002; Zhou et al., 2003). In contrast to group I PAKs, group II PAKs lack this autoinhibitory domain and are not activated by Cdc42/Rac binding, and the exact mechanisms that regulate their kinase activity are still unclear. However, it has been shown that PI3K regulates not only kinase activity but also its subcellular localization (Wells et al., 2002) and that full kinase activity requires phosphorylation of the corresponding residues in the catalytic domain (Abo et al., 1998). We therefore suggest that different PAKs, with slightly different modes of activation, might integrate the different signals emanating from the BMP receptors into specific cytoskeletal rearrangements.

To date, major substrates for PAKs identified are LIMK1 and LIMK2 (Dan et al., 2001; Edwards et al., 1999). LIM kinases are implicated in the regulation of actin cytoskeletal dynamics through their ability to phosphorylate cofilin at Ser3. This has been identified as the missing link that couples PAK activation to cytoskeletal rearrangements. Active PAKs phosphorylate LIM kinases in the activation loop (Thr508 for LIMK1) increasing their activity towards cofilin (Edwards et al., 1999; Scott and Olson, 2007). Our results indicate that, in C2C12 cells, BMP2 activates both LIMK1 phosphorylation on Thr508 and its activity against exogenous substrates. Moreover, we demonstrate that LIMK1 activation by

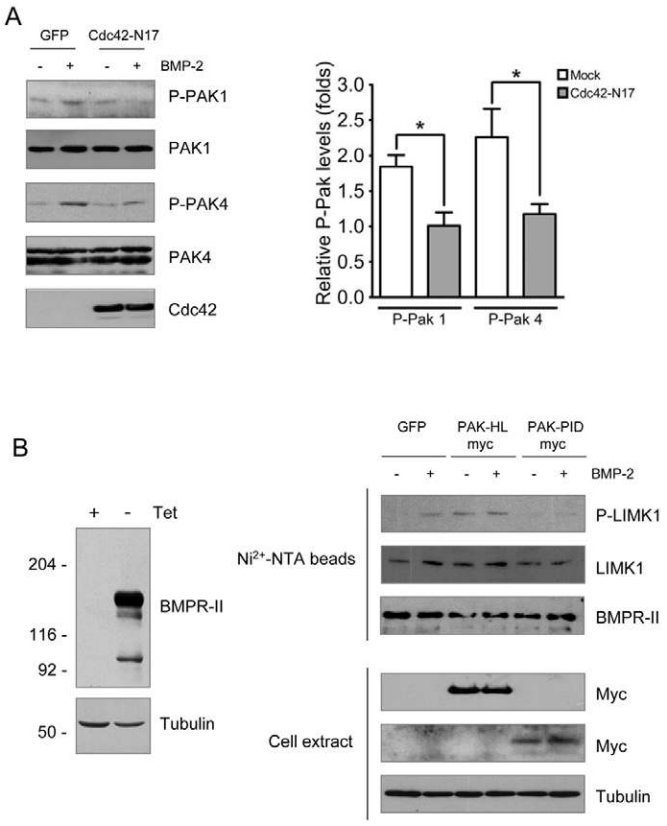

Fig. 9. Cdc42 is required for BMP2-dependent PAK1 and PAK4 phosphorylation. (A) C2C12 cells transfected with the indicated expression vectors were serum-starved for 16 hours and stimulated with 3 nM BMP2 for 40 minutes. Cell lysates were analyzed with the indicated phospho-specific antibodies, and membranes reprobed with anti-PAK1, anti-PAK4 and anti-Cdc42 antibodies. Graph indicates PAK1-*P* and PAK4-*P* levels of BMP2-treated cells relative to their respective untreated controls. Mean ± s.e.m. of three independent experiments (**P*<0.05, unpaired *t*-test). (B) PAK activity is required for BMP2 to phosphorylate the LIMK1 associated to the BMPRII tail. Immunoblot analysis of C2C12 cells overexpressing His-tagged BMPRII under a tetracyclin (Tet-off)-regulated promoter (left panel). Cells overexpressing His-tagged BMPRII were transfected with the expression constructs indicated, serum-starved for 16 hours and stimulated with 3 nM BMP2 for 40 minutes. Cell extracts were subjected to immunoblotting to detect the total and phosphorylated LIMK1 after Ni^{2+}-NTA-agarose purification (right panel).

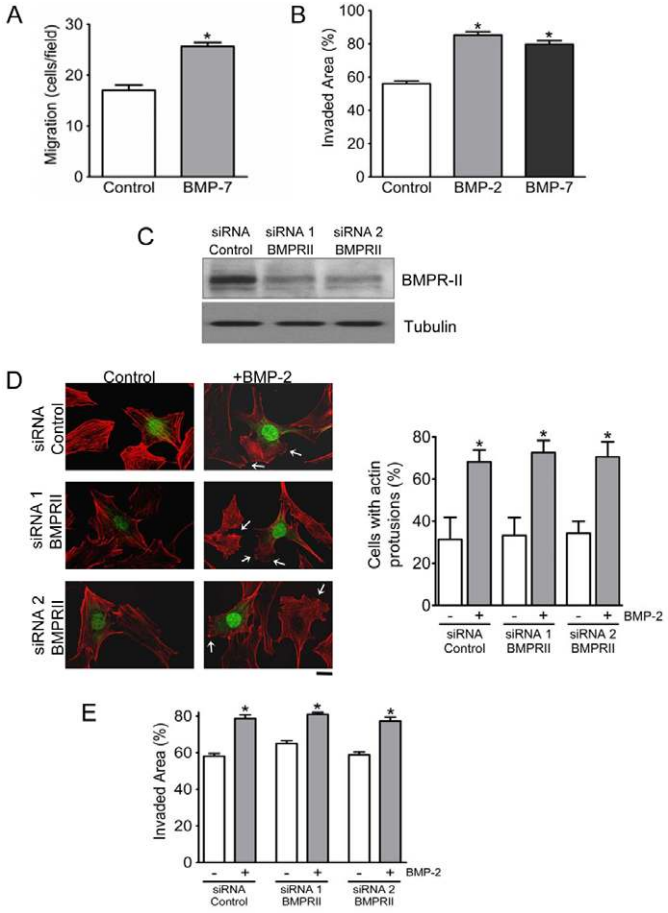

Fig. 10. Role of BMPRII in BMP2-dependent actin cytoskeleton reorganization and cell migration. (A) Chemotaxis assay. Serum-starved C2C12 cells were allowed to migrate for 2 hours in the presence or absence of 30 pM BMP7 as described in Materials and Methods. Quantitative analysis of eight random fields from three independent experiments (Mean ± s.e.m.; **P*<0.0001, paired *t*-test). (B) Wound-healing migration assay. Serum-starved wounded C2C12 cell monolayers were allowed to migrate in the presence or absence of 3 nM BMP2 or 3 nM BMP7 for 24 hours. The graph indicates the invaded area. Mean ± s.e.m. of three independent experiments (**P*<0.001, paired *t*-test). (C) Immunoblot analysis of BMPRII expression in C2C12 cells transfected with the indicated siRNA for 48 hours. (D) C2C12 cells were transfected with the indicated siRNA for 48 hours and serum-starved for 16 hours. Then, cells were pretreated with 2 μM cytochalasin D for 20 minutes and allowed to recover in the absence or presence of 3 nM BMP2 for 1 hour. Merged images of phalloidin and GFP signals are shown. Cells showing cortical actin protrusions (arrows) are indicated. Scale bar: 20 μm. Transfected cells were counted and those showing cortical actin protrusions represented as % of total (graph on right). Mean ± s.e.m. of at least 80 transfected cells obtained from three independent experiments (**P*<0.05, paired *t*-test compared with the condition in the absence of BMP2). (E) C2C12 cells were transfected with the indicated siRNA for 48 hours and serum-starved for 16 hours. Cell monolayers were wounded and allowed to migrate for 24 hours in the presence or absence of 3 nM BMP2. The graph indicates the invaded area. Mean ± s.e.m. from three independent experiments (**P*<0.001 compared with the condition in the absence of BMP2, one-way ANOVA followed by Bonferroni's multiple comparison test).

BMP2 is dependent on PI3K and PAK activities. Previous reports indicate a direct interaction and activation of LIMK1 by the long cytoplasmic tail of the BMP receptor type II and a further synergism with BMP-activated Cdc42 (Foletta et al., 2003; Lee-Hoeflich et al., 2004). Although these studies reached different conclusions about the mechanism by which BMP binding to its receptors increases LIMK1 activity, it seems clear that BMP signaling in dendritogenesis and synaptic stability requires LIMK1 activity downstream of BMP receptors (Eaton and Davis, 2005; Lee-Hoeflich et al., 2004).

Our results also indicate that addition of BMP7 led to similar effects as with BMP2 in chemotaxis and wound-healing assays, and reduction of BMPRII levels did not significantly modify formation of protrusions or migration in wound-healing assays in response to BMP2. This evidence, and the fact that LIMK1 activation by BMP2 is dependent on PI3K and PAK activities, suggests that in addition to the interaction of LIMK1 with the BMPRII cytoplasmic tail, BMP2 is able to stimulate additional pathways that lead to full activation of LIM kinases. In line with these findings, several reports indicate that TGFβ, whose receptor lacks the LIMK1-interacting cytoplasmic tail, regulates the actin cytoskeleton in mesenchymal but not in epithelial

cell types through activation of distinct PAK and LIMK family members (Vardouli et al., 2005; Wilkes et al., 2005; Wilkes et al., 2003). Further studies will be required to understand how different cell types use these pathways separately or synergistically in the spatial regulation of the actin cytoskeleton by BMPs.

Materials and Methods

Plasmids, reagents and antibodies

Vectors encoding Cdc42wt-GFP and Cdc42-T17N-GFP were kindly provided by Xosé Bustelo (CSIC, Salamanca, Spain). The expression vectors encoding myc-tagged full-length activated PAK (PAK1-H83,86L) and the deletion mutant PAK-PID (aa83-149) were provided by Gary Bokoch (The Scripps Research Institute, La Jolla, CA). BMP2 was a generous gift from Wyeth (Cambridge, MA) and BMP-7 was obtained from R&D Systems (Minneapolis, MN). The inhibitor LY294002 (Sigma, St Louis, MO) was added to medium 1 hour before stimulation of cells with BMP2 and used at a final concentration of 15 μM. The isoform-selective inhibitors of class I PI3Ks were obtained from Merck-Serono (Geneva, Switzerland) and used at a final concentration of 1 μM. Purified MBP was obtained from Sigma and purified cofilin 1 from Upstate Biotechnology (Lake Placid, NY). Antibodies used were Smad1-*P*(Ser463/465), Rac1 (Upstate); Tubulin (Sigma); Cdc42 (BD Biosciences, San Jose, CA); Akt, BMPR2, myc (Santa Cruz Biotechnology, Santa Cruz, CA); Akt-*P*(Ser473), PAK1-*P*(Thr423)/PAK2-*P*(Thr402), PAK4-*P*(Ser474)/PAK5-*P*(Ser602)/PAK6-*P*(Ser560), PAK1, PAK4, LIMK1-*P*(Thr508)/LIMK2-*P*(Thr505) and LIMK1 (Cell Signaling, Beverly, MA).

Cell culture and transfection

C2C12 and Swiss 3T3 cell lines were maintained in DMEM supplemented with 10% FBS, antibiotics and glutamine. Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) or FuGENE6 (Roche, Indianapolis, IN). We generated C2C12 cells with inducible expression of His-tagged BMPRII following the Tet-Off protocol as described (Chambard and Pognonec, 1998). First, we used two distinct vectors that encode a tetracycline-regulated transactivator (tTA) and puromycin resistance under the control of a tTA-responsive promoter (tetO-CMV) to generate C2C12 cells that stably expressed tTA. After selection of puromycin-resistant clones, we transfected BMPRII-His under the control of the tTA-responsive promoter. The concentration of tetracycline was 100 ng/ml in all experiments.

RNA interference assays

To knockdown BMPRII expression, two siRNA duplexes against murine BMPRII mRNA were purchased from Dharmacon (Lafayette, CO). Sense sequences of the siRNA used were (5′ to 3′): BMPRII siRNA 1, GCACAUAGGUCCCAAGAAAtt; BMPRII siRNA 2, GGGAGCACGUGUUAUGGUCtt (Yu et al., 2005). A scrambled control siRNA was transfected under the same conditions. 40 pmol siRNA duplexes (and GFP plasmid to control the transfection efficiency) were added to subconfluent C2C12 cells in 24-well plates in a mixture of Lipofectamine 2000 and OptiMEM in the absence of serum and antibiotics. After 6 hours, fetal calf serum was added to cultures at a final concentration of 10%. Assays to measure BMPRII levels were performed 48 hours after transfection. Assays to measure BMP2-mediated cell migration and actin cytoskeleton reorganization were performed after an additional 16 hours of serum starvation.

Immunoblotting, immunoprecipitation and protein kinase assays

Protein extracts were subjected to SDS-PAGE and immunoblotted as previously described (Lopez-Rovira et al., 2002; Vinals et al., 2004) or used for in vitro kinase assay. For kinase assays, cells were grown to confluence, starved in serum-free medium for 16 hours and stimulated with 2 nM BMP2. Cells were washed twice in cold PBS and lysed on ice with 500 μl per 10 cm dish of ice-cold lysis buffer (40 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.2% NP-40, 10% glycerol, 50 mM NaF, 40 mM β-glycerophosphate, 200 μM Na_3VO_4, 100 μM phenylmethilsulfonyl fluoride, 1 μM pepstatin A, 1 μg/ml leupeptin, 4 μg/ml aprotinin). Lysates were pre-cleared by centrifugation at 15,000 ***g*** for 10 minutes at 4°C and equivalent protein amount (500-700 μg) was incubated overnight at 4°C with the specific antibody. Immune complexes were collected with protein-A-Sepharose and protein-G-Sepharose (Sigma) and washed four times in lysis buffer and twice in kinase buffer (50 mM HEPES pH 7.4, 150 mM NaCl. 1 mM $MgCl_2$, 10 mM NaF, 1 mM Na_3VO_4, 5% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) prior to incubation in 50 μl kinase buffer containing 5 μM ATP, 5 μCi [γ-^{32}P]ATP per reaction and 5 μg of either myelin basic protein (Sigma) for endogenous PAK1 and PAK4 activity assays or GST-cofilin for endogenous LIMK1 activity assay. After 20 minutes at 30°C, reactions were stopped by addition of SDS sample buffer and boiled for 10 minutes. The reaction mixture was separated by SDS-PAGE and analysed by autoradiography. Quantification was performed using the Bio-Rad Molecular Imager software.

F-actin staining

Cells were grown on glass coverslips in 12-well plates, starved in serum-free medium for 16 hours and then stimulated with 3 nM BMP2. Cells were fixed in 3% paraformaldehyde in PBS for 30 minutes at room temperature, washed twice in PBS and permeabilized for 4 minutes in PBS containing 0.2% Triton X-100, and then blocked in TBS containing 2% BSA for 45 minutes. To visualize F-actin, cells were incubated with 1 μM TRITC-conjugated phalloidin (Sigma) and washed three times with PBS before mounting on slides. Images were acquired using Leica TCS-SL Spectral confocal microscope. For some experiments, cells were incubated with 2 μM cytochalasin D (Sigma) for 20 minutes, washed five times with medium and allowed to recover in the presence or absence of 3 nM BMP2.

Chemotaxis assay

Chemotaxis assays were performed in 24-well Transwell plates using 8 μm pore-size polycarbonate filters of 6.5 mm diameter. Filters were coated with 1% gelatin (from porcine skin, Type A, Sigma) for 2 hours at 37°C, washed in PBS and blocked in PBS containing 5% BSA for 16 hours at 4°C. C2C12 cells were trypsinized, and 5×10^4 cells were loaded onto the upper well and left for 2 hours at 37°C to allow the adhesion of the cells to the filter before the lower well was filled with DMEM-1% BSA or 30 pM BMP2. After 2 hours at 37°C, non-migrated cells in the top chamber were removed with a cotton swab and migrated cells were fixed and stained with 5 μg/ml propidium iodide for 5 minutes. Images were acquired using Leica DM IRB2 microscope linked to a Olympus DP50 camera and cell migration was quantified by counting the total number of cells in eight systematically sampled microscopic fields at ×100 magnification.

Wound-healing migration assay

C2C12 cells grown to confluence in 24-well plates and serum-starved for a minimum of 16 hours to establish quiescence. Cells were incubated with 10 μg/ml mitomycin C for 2 hours to eliminate the effect of proliferation, and cell monolayers were wounded with a plastic tip and washed with medium to remove detached cells. The wound was allowed to close in the presence or absence of 3 nM BMP2. The wound was photographed in a phase-contrast microscope (Leica DM IRB2 microscope linked to a OLYMPUS DP50 camera) and the rate of cell migration was measured as the percentage of invaded area with respect to the initial wound area.

GST pull-down assay

C2C12 cells were grown to confluence and serum-starved for 16 hours before stimulation with BMP2. Cells were lysed in MLB buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 1% NP-40, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM Na_3VO_4). Lysates were clarified by centrifugation and incubated with 20 μg of the bacterially produced PAK-PBD-GST fusion protein. Bound proteins were purified with Glutathione-Sepharose beads (Amersham Biosciences, Piscataway, NJ) and immunoblotted with antibodies against Rac1 and Cdc42. An aliquot of the total lysate used for precipitation was run alongside to quantify total Rac1 and Cdc42.

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