p38 mitogen-activated protein kinase regulates canonical Wnt–β-catenin signaling by inactivation of GSK3β

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Accepted 5 August 2008
Journal of Cell Science 121, 3598-3607 Published by The Company of Biologists 2008
doi:10.1242/jcs.039954

Summary
The Wnt–β-catenin canonical signaling pathway is crucial for normal embryonic development, and aberrant expression of components of this pathway results in oncogenesis. Upon scanning for the mitogen-activated protein kinase (MAPK) pathways that might intersect with the canonical Wnt–β-catenin signaling pathway in response to Wnt3a, we observed a strong activation of p38 MAPK in mouse F9 teratocarcinoma cells. Wnt3a-induced p38 MAPK activation was sensitive to siRNAs against Gαq or Gαi, but not against either Gαs or Gα11. Activation of p38 MAPK is critical for canonical Wnt–β-catenin signaling. Chemical inhibitors of p38 MAPK (SB203580 or SB239063) and expression of a dominant negative-version of p38 MAPK attenuate Wnt3a-induced accumulation of β-catenin, Lef/Tcf-sensitive gene activation, and primitive endoderm formation. Furthermore, epistasis experiments pinpoint p38 MAPK as operating downstream of Dishevelleds. We also demonstrate that chemical inhibition of p38 MAPK restores Wnt3a-attenuated GSK3β kinase activity. We demonstrate the involvement of G-proteins and Dishevelleds in Wnt3a-induced p38 MAPK activation, highlighting a critical role for p38 MAPK in canonical Wnt–β-catenin signaling.

Key words: Wnt, β-catenin, p38 MAPK, Frizzled, Dishevelled, Canonical pathway, Lef/Tcf, Primitive endoderm, G-protein

Introduction
Wnt signaling is critical for normal embryonic development, patterning, cellular proliferation and homeostasis, and its aberrant activity is linked to many forms of prevalent human carcinomas (Logan and Nusse, 2004; Moon et al., 2002; Moon et al., 2004; Polakis, 2000). Wnt ligands initiate intracellular signaling pathways by binding to the G-protein-coupled receptors Frizzleds (Fzs) (Bhanot et al., 1996; Liu et al., 2001). The Wnt-sensitive pathways include the canonical (Wnt–β-catenin), and the non-canonical (planar cell polarity and Wnt-cGMP-Ca2+) pathways (Bhanot et al., 1996; Liu et al., 1999). In the canonical pathway, in the absence of Wnt, cellular β-catenin is subjected to proteasome-mediated degradation by the destruction complex, which includes among other proteins Axin and the product of the adenomatous polyposis coli gene (APC). These proteins facilitate the phosphorylation of β-catenin by the Ser/Thr protein kinase glycogen synthase kinase 3β (GSK3β). This ultimately leads to ubiquitinylation and proteasome-mediated degradation of β-catenin. Wnt3a binding to Fz1 leads to activation of the G-proteins Gαq and Gαi and of the phosphoprotein Dishevelled, resulting in reduced GSK3β activity and increased accumulation of β-catenin. Nuclear accumulation of β-catenin follows the stimulatory activation of lymphoid-enhancer factor/T-cell factor (Lef/Tcf)-sensitive transcription of developmentally related genes (Behrens et al., 1996; Molenaar et al., 1996). Aberrant accumulation of β-catenin contributes to tumorigenesis and therefore requires strict regulation.

Recent advances suggest a possible intersection of other major signaling pathways, including mitogen-activated protein kinase (MAPK) pathways with the Wnt–β-catenin signaling pathway (Bikkavilli et al., 2008; Caverzasio and Manen, 2007; Gao et al., 2002; Hildesheim et al., 2005; Keren et al., 2005). In NIH3T3 fibroblasts, Wnt3a activates the ERK pathway through Ras, Raf and MEK and plays an important role in cellular proliferation (Yun et al., 2005). In mesenchymal stem cells (MSCs), Wnt4-mediated activation of p38 MAPK was found to be crucial for enhancing osteogenic differentiation of MSCs (Chang et al., 2007). Similarly, in C3H10T1/2 mesenchymal cells, Wnt3a also induced transient activation of p38 and ERK, which regulate alkaline phosphatase activity and nodule mineralization, suggesting an important role for the MAPKs in the development of mesenchymal cells into osteoprogenitors (Caverzasio and Manen, 2007). Therefore, in addition to the canonical β-catenin pathway and non-canonical pathways, Wnts also activate MAPK pathways that play important roles in proliferation, myogenesis and osteogenesis (Caverzasio and Manen, 2007; Chang et al., 2007; Keren et al., 2005).

Our earlier studies highlighted a probable role for p38 MAPK in canonical Wnt–β-catenin signaling (Bikkavilli et al., 2008) but the mechanism remains unclear. Here, we reveal that p38 MAPK is activated upon stimulation by Wnt3a and is crucial for Wnt3a-induced accumulation of β-catenin, Lef/Tcf-sensitive gene activation and primitive endoderm formation. We also show that Wnt3a-induced activation of p38 MAPK is sensitive to depletion of Gαq, Gαi, or Dishevelled3 (Dv13) and reveal an important role for p38 MAPK in regulating GSK3β activity.

Results
Wnt3a activates p38 MAPK in mouse F9 cells
Totipotent mouse embryonic carcinoma F9 cells transfected to express rat Frizzled-1 (Rfz1 or FZD1) provide an optimal system for the biochemical analysis of Wnt/Fz1 signaling (Malbon, 2005). Upon Wnt3a stimulation, F9 cells differentiate into primitive endoderm, which is characteristic of early mouse development (Liu
Wnt3a-induced activation of p38 MAPK is G-protein dependent

Heterotrimeric G-proteins are obligate for Wnt–β-catenin signaling in mammalian cells, Xenopus and Zebrafish embryos, as well as in Drosophila (Katanaev et al., 2005; Malbon, 2005). Earlier studies revealed critical roles for the G-proteins Gαo and Gαq in canonical Wnt–β-catenin signaling (Liu et al., 2001; Liu et al., 1999). More recently, a possible role for Gαo in Axin–β-catenin signaling has been reported (Castellone et al., 2005). To evaluate the possible involvement of these G-proteins in Wnt3a-induced activation of p38 MAPK, we used small-interfering RNAs (siRNAs) that specifically suppressed the expression of individual G-protein α-subunits. Cells were treated for 48 hours with siRNAs designed specifically to suppress the α-subunits of Gαo, Gαq, Gα11 or Gαs. Under these conditions of siRNA treatment, suppression by more than ~75% of each targeted G-protein α-subunit was achieved (Fig. 2). Treatment of Rfz1-expressing cells with Gαq or Gαo siRNA attenuates Wnt3a-induced p38 MAPK activation by more than ~75% in case of Gαq knockdown and by ~50% in case of Gαo knockdown (Fig. 2). By contrast, treatment of Gαs siRNA, unlike those targeting Gαq or Gαo, had no effect on the Wnt3a-simulated p38 MAPK activation (Fig. 2). Similarly, treating the cells with siRNA targeting Gα11, a G-protein α-subunit unrelated to Wnt–β-catenin signaling, had no impact on the ability of Wnt3a to activate p38 MAPK (Fig. 2). Interestingly, Gαq or Gα11 siRNA treatment of cells provokes activation of p38 MAPK in the absence of Wnt3a (Fig. 2). Taken together, these observations demonstrate critical roles for Gαq and Gαs in Wnt3a-stimulated p38 MAPK activation.

p38 inhibitor blocks canonical Wnt–β-catenin–Lef/Tcf pathway

To test the link between Wnt3a-mediated activation of canonical Wnt–β-catenin signaling and Wnt3a-stimulated activation of p38 MAPK, we probed the effects of two p38 MAPK selective inhibitors, SB203580 (Cuenda et al., 1995) or SB239063 on canonical Wnt–β-catenin signaling. Treatment of Rfz1 cells with

et al., 1999). We first tested whether Wnt3a treatment of F9 cells expressing Rfz1 results in p38 MAPK activation by probing the phosphorylation status of ATF2, a prime substrate of activated p38 MAPK (Fig. 1A). Addition of purified Wnt3a to Rfz1-expressing cells resulted in a sharp activation of p38 MAPK. Wnt3a-stimulated p38 activation was rapid, reaching a peak within 15 minutes of Wnt3a treatment (Fig. 1A). In either F9 cells or F9 cells expressing Rfz2, Wnt3a failed to stimulate p38 activation (Fig. 1B). The ability of Wnt3a to stimulate p38 MAPK activity was also measured in human embryonic kidney 293 (HEK293) cells. Similar to Rfz1 cells, wild-type HEK293 cells also showed a sharp stimulation of p38 MAPK activity upon Wnt3a treatment (Fig. 1C). Similar activation of p38 MAPK by Wnt3a has also been reported in C3H10T1/2 mesenchymal cells (Caverzasio and Manen, 2007), C2C12 mesenchymal cells (Chang et al., 2007), and also during Xenopus development (Keren et al., 2005).

Fig. 1. Stimulation of Frizzled-1 by Wnt3a activates p38 MAPK in F9 and HEK293 cells. (A) F9 cells stably expressing Rfz1 were treated with Wnt3a (20 ng/ml) for indicated periods of time and the lysates were assayed for p38 activation as described in Materials and Methods. The upper panel represents mean values ± s.e.m. obtained from three independent experiments; the lower panel displays representative blots probed with either anti-ATF2-P (p-ATF2), upper panel or with anti-p38 antibody (p38), lower panel. (B) F9 cells alone and F9 cells expressing either Rfz2 or Rfz1 were treated with Wnt3a for 15 minutes and the lysates were assayed for p38 activation. Representative blots of three independent experiments that proved highly reproducible are shown. (C) Confluent HEK293 cells were treated with Wnt3a for 15 minutes and the lysates were assayed for p38 activation. Representative blots of three independent experiments that proved highly reproducible are displayed. *P<0.05, **P<0.01 versus control (+Wnt3a, 0 minutes).
Wnt3a displayed a time-dependent accumulation of β-catenin (Fig. 3A,B). Consistently, a strong β-catenin accumulation was observed after 2 hours of Wnt3a treatment, with an ~8- to 12-fold increase in free β-catenin levels after 4 hours of Wnt3a treatment (Fig. 3A,B). Interestingly, Wnt3a-induced β-catenin accumulation was markedly attenuated by SB203580 (6 μM) or SB239063 (10 μM) (Fig. 3A,B), suggesting a probable role for p38 MAPK pathway in canonical Wnt–β-catenin signaling. The time delay in the ability of SB203580-treated cells to attenuate β-catenin accumulation in response to Wnt3a at the 1 hour time point may reflect the presence of distinct, p38-dependent and independent pathways, or incomplete p38 MAPK inactivation (Fig. 3A).

We next tested for the effects of inhibition of p38 MAPK activity on Wnt3a-stimulated Lef/Tcf-sensitive transcription in Rfz1 cells (Fig. 3C,D,E). Consistent with the effects on β-catenin accumulation, SB203580 or SB239063 dramatically attenuated the Lef/Tcf-sensitive transcription (Fig. 3C,D,E). Treatment with SB203580 (6 μM) resulted in a more than 75% decrease in Lef/Tcf-
p38 regulates Wnt–β-catenin signaling

**Fig. 3.** p38 inhibitors block canonical Wnt–β-catenin–Lef/Tcf pathway. Confluent F9 cells stably transfected with pRfz1 and pTOPFLASH (M50) luciferase reporter were treated with either vehicle (DMSO) or p38 MAPK selective inhibitors, SB203580 (6 μM) (A) or SB239063 (10 μM) (B) for 1 hour before addition of Wnt3a for indicated periods of time. After stimulation, the lysates were collected and cytosolic β-catenin levels were assayed. Upper panel displays mean values ± s.e.m. obtained from three independent experiments; the lower panel displays representative blots probed with anti-β-catenin antibody (β-catenin), immunoblots probed with anti-actin antibody (actin) were used as loading controls. Confluent F9 cells stably transfected with pRfz1 and pTOPFLASH (M50) luciferase reporter were treated with either vehicle (DMSO), SB203580 (6 μM) (C,D) or SB239063 (10 μM) (E) for 1 hour before addition of Wnt3a for indicated periods of time (C) or 7 hours (D,E). Activity of the luciferase reporter was monitored. The data represent mean values ± s.e.m. obtained from three independent experiments; the lower panel displays representative blots probed with anti-β-catenin antibody (β-catenin), immunoblots probed with anti-actin antibody (actin) were used as loading controls. (F) HEK293 cells were treated with Wnt3a for 4 hours and the lysates were assayed for cytosolic β-catenin levels. Upper panel displays mean values ± s.e.m. obtained from three independent experiments; the lower panel displays representative blots probed with anti-β-catenin antibody (β-catenin), immunoblots probed with anti-actin antibody (actin) were used as loading controls. (G) HEK293 cells were transfected with pTOPFLASH (M50, 10 ng/well) and phRl-CMV Renilla luciferase control vector (5 ng/well) for 48 hours followed by stimulation with Wnt3a for 7 hours. Lef/Tcf-sensitive transcription was determined. The data represents mean values ± s.e.m. from a single experiment performed in triplicate and is representative of three separate experiments whose results were highly similar. (H) F9 cells stably transfected with pRfz1 and pTOPFLASH luciferase reporter were treated with vehicle (DMSO, control), p38 inhibitor (SB203580, 6 μM), JNK inhibitor (SP600125, 0.4 μM) or MEK inhibitor (PD98059, 20 μM) for 1 hour prior to stimulation with Wnt3a for 4 days. Subsequently, the cells were prepared for immunocytochemistry and stained with a monoclonal antibody to the cytookeratin endoA (TROMA1) marker protein for primitive endoderm. Alexa Fluor 488-conjugated secondary antibodies were used with indirect epifluorescence to detect the immune complexes. Typical phase-contrast images (PC) and the indirect immunofluorescence images (IIF) are shown from a single experiment, representative of three independent experiments. *P<0.05 and **P<0.01 versus Wnt3a control; #P<0.05 and ##P<0.01 versus Wnt3a control.

Sensitive transcription after 8 hours of Wnt3a treatment (Fig. 3C,D,E). The effect of SB203580 or SB239063 on Lef/Tcf-sensitive transcription was dose dependent (Fig. 3D,E), with 0.1 μM of this compound capable of inhibiting the response by ~50%. Wnt3a-induced Lef/Tcf-sensitive transcription was nearly abolished by treatment with 20 μM SB203580 or SB239063 (Fig. 3D,E). Consistent with the effects on F9 cells expressing Rfz1, SB203580 also showed strong attenuation of Wnt3a-induced β-catenin accumulation (Fig. 3F) and Lef/Tcf-sensitive transcription (Fig. 3G) in HEK293 cells.

Previous studies reveal that activation of the canonical pathway leads to primitive endoderm formation in F9 cells (Liu et al., 2002; Liu et al., 1999). In the present study, experiments were performed to determine whether the Wnt3a-p38 MAPK pathway also participates in primitive endoderm formation of F9 cells. F9 cells stably expressing Rfz1 were treated with MAPK inhibitors and the ability of Wnt3a to promote formation of primitive endoderm was determined by positive staining of cytookeratin endoA, a hallmark protein for primitive endoderm, with the TROMA1 antibody (Liu et al., 2002; Liu et al., 1999). Treatment of cells with p38 MAPK inhibitor (SB203580, 6 μM) abolished the Wnt3a-induced primitive endoderm formation (Fig. 3H), whereas JNK inhibitor (SP600125, 0.4 μM) and MEK inhibitor (PD98059, 20 μM) had no impact on the Wnt3a-induced primitive endoderm formation (Fig. 3H). Taken together, these results suggest that p38 MAPK operates upstream of β-catenin stabilization and regulates Lef/Tcf-sensitive transcription and primitive endoderm formation.

**Suppression of p38α or expression of dominant-negative mutant of p38 blocks canonical Wnt–β-catenin–Lef/Tcf pathway** To investigate the specific involvement of p38 MAPK in canonical Wnt/β-catenin signaling, we utilized two approaches: (1) small interfering RNAs (siRNAs) specific to p38α and (2) expression of a dominant-negative mutant of p38. We suppressed the expression of p38α by ~75% using siRNA (Fig. 4A,B,C). Treatment of F9 cells expressing Rfz1 with p38α siRNAs for 48 hours significantly attenuated Wnt3a-stimulated β-catenin stabilization (Fig. 4A). By contrast, suppression of Jun N-terminal kinase (JNK) MAPK showed no significant effect on β-catenin stabilization (Fig. 4A). Consistent with the effects of p38α-specific siRNAs on β-catenin stabilization, there was a significant attenuation of Wnt3α-induced Lef/Tcf-sensitive transcription by p38α-specific siRNAs (Fig. 4B), strongly suggesting the importance of p38 MAPK in regulating canonical Wnt–β-catenin signaling. To test the selectivity of the p38α siRNA for p38 MAPK as well as the possible existence of ‘off-target’ effects of the siRNAs on canonical Wnt–β-catenin signaling, we performed a ‘rescue’ experiment in the knocked down cells, using an expression construct harboring the entire coding region of the p38 MAPK. The expression of exogenous p38 MAPK rescued the ability of Wnt3a to activate Lef/Tcf-sensitive transcription in those cells in which the response had been attenuated by knockdown of p38 MAPK (Fig. 4C). Thus the ability of the knockdown of p38 MAPK to attenuate canonical Wnt–β-catenin signaling appears to reflect loss of the targeted p38 MAPK and not some ‘off-target’ effect. To further test the role of p38 MAPK in canonical Wnt–β-catenin signaling, we used an expression construct harboring the dominant-negative mutant of p38α MAPK (Flag-tagged p38) and examined the effects of expression of this construct on Wnt3a-stimulated β-catenin accumulation and Wnt3a-stimulated Lef/Tcf-sensitive transcription. Expression of the dominant-negative p38 mutant not only abolished the Wnt3a-induced β-catenin stabilization (Fig. 4D), but also dose-dependently attenuated the Wnt3α-induced Lef/Tcf-sensitive transcription (Fig. 4E). Interestingly, chemical inhibition of p38 MAPK could eliminate ~80% of the canonical Wnt–β-catenin signaling (Fig. 4F). Combination of either dominant-negative p38 or p38 siRNA with SB203580 to inhibit p38 MAPK activity could not eliminate the remaining Wnt3α-induced Lef/Tcf-sensitive transcription signal (Fig. 4F), even with complete loss of function of p38 MAPK (Fig. 4G) under those conditions. Taken together, it appears that the canonical Wnt–β-catenin signaling pathway is regulated by both p38-MAPK-dependent and p38-MAPK-independent pathways.

**Role of Dvl3 in Wnt3α-induced activation of p38 MAPK** Dishevelled is a cytoplasmic phosphoprotein that plays critical roles in both canonical Wnt–β-catenin pathway and non-canonical pathways (Bikkavilli et al., 2008; Lee et al., 2008; Sheldahl et al., 2003). As observed earlier, Dvl3 has an important role in both the canonical Wnt–β-catenin pathway (Lee et al., 2008) and the planar cell polarity (PCP) pathway (Bikkavilli et al., 2008). We were keen to investigate whether Dvl3 also contributes to Wnt3α-induced p38 MAPK activation. Depletion of Dvl3 by ~75% or more was achieved by utilizing siRNAs specifically targeted against Dvl3 (Fig. 5A). Under these knockdown conditions, complete loss of Wnt3α-induced p38 MAPK activation (Fig. 5A) was observed, suggesting...
that Dvl3 is critical for p38 MAPK activation and also that Dvl3 operates upstream of p38 MAPK activation. If our hypothesis that Dvl3 mediates activation of p38 MAPK induced by Wnt3a is correct, overexpression of Dvl3 would be expected to activate p38 MAPK, even in the absence of Wnt3a. Transient expression of Dvl3 indeed stimulates p38 MAPK activity, mimicking the effect of Wnt3a stimulation (Fig. 5B). Dvl3 siRNA also showed marked attenuation of Wnt3a-induced β-catenin accumulation (Fig. 5C).

Fig. 4. See next page for legend.
Fig. 4. Suppression of p38 MAPK by siRNA treatment or expression of dominant-negative mutant of p38 blocks canonical Wnt–β-catenin–Lef/Tcf pathway. (A) F9 cells stably transfected with pRfz1 and pTOPFLASH luciferase reporter were treated with 100 nM siRNA specific to mouse p38α or JNK for 48 hours, and the lysates were assayed for cytosolic β-catenin levels. Upper panel displays mean values ± s.e.m. obtained from three independent experiments; the lower panel displays representative blots probed with anti-β-catenin antibody (β-catenin); immunoblots probed with anti-actin antibody (actin) were used as loading controls. (B) F9 cells stably transfected with pRfz1 and pTOPFLASH luciferase reporter were treated with 100 nM siRNA specific to mouse p38α for 48 hours followed by stimulation with Wnt3a for 7 hours. Lef/Tcf-sensitive transcription was determined. The data represent mean values ± s.e.m. from a single experiment performed in triplicate and is representative of three separate experiments whose results were in high agreement. (C) Rescue experiment performed by transfection of hp38 into F9 cells stably transfected with pRfz1 and pTOPFLASH luciferase reporter in which p38 MAPK was knocked down by siRNA treatment. Lef/Tcf-sensitive transcription was determined. The data represents mean values ± s.e.m. from a single experiment performed in triplicate and is representative of three separate experiments whose results were in high agreement. (D–E) F9 cells stably transfected with pRfz1 and pTOPFLASH luciferase reporter were either transfected with empty vector (–) or with indicated amounts of Flag-tagged dominant-negative mutant (DN) of p38 MAPK [p38α (AGF)] for 24 hours and the lysates were assayed either for β-catenin stabilization after 4 hours of Wnt3a treatment (D) or luciferase reporter activity after 7 hours of Wnt3a treatment (E). Upper panel displays mean values ± s.e.m. obtained from three independent experiments; the lower panel displays representative blots probed with anti-β-catenin antibody (β-catenin), anti-β-catenin antibody (p38α) and the immunoblot with anti-actin antibody (actin) were used as loading controls. (F,G) F9 cells stably transfected with pRfz1 and pTOPFLASH luciferase reporter were treated with either 100 nM siRNA specific to mouse p38α or dominant-negative mutant (DN) of p38 MAPK for 48 hours. The cells were then treated with SB203580 (6 μM) for 1 hour followed by stimulation with Wnt3a for 7 hours. Lef/Tcf-sensitive transcription (F) and p38 MAPK activation (G) was determined as described in Materials and Methods.

*P<0.05 and **P<0.01 versus –Wnt3a control; #P<0.05 versus +Wnt3a control.

Overexpression of Dvl3 induces Lef/Tcf-sensitive transcription (Lee et al., 2008). To confirm that p38 MAPK operates downstream of Dvl3 in Wnt–β-catenin signalling, we compared the ability of overexpressed HA-Dvl3-GFP2 to activate Lef/Tcf-sensitive transcription in the absence versus the presence of the p38 MAPK inhibitor (SB203580) (Fig. 5D). Overexpression of HA-Dvl3-GFP2 induced a robust stimulation of Lef/Tcf-sensitive transcription (~70-fold), which was attenuated more than twofold in the presence of SB203580 (Fig. 5D). Taken together, these observations suggest that in the canonical Wnt–β-catenin signalling pathway, Dvl3 is critical for Wnt3a-induced p38 MAPK activation and p38 MAPK operates downstream of Dishevelleds.

p38 MAPK suppresses GSK3β activity

To identify the likely targets of p38 MAPK in regulating canonical Wnt–β-catenin signaling, we explored the effects of SB203580 and dominant-negative p38 on GSK3β activity. GSK3β is the key enzyme that regulates canonical Wnt–β-catenin signaling by phosphorylating β-catenin, ultimately leading to proteasome-mediated degradation. We tested the possible regulation of GSK3β by p38 MAPK experimentally by using two approaches: (1) probing Wnt3a-induced GSK3β Ser9 phosphorylation in the absence or presence of SB203580. (2) In vitro GSK3β kinase assay in the absence or presence of SB203580. GSK3β is highly active under basal conditions. Akt-mediated phosphorylation of GSK3β at Ser9 leads to GSK3β inactivation (Cross et al., 1995). Whether Ser9 phosphorylation is also a major mechanism of GSK3β inactivation in Wnt–β-catenin signaling is still under debate. However, earlier studies demonstrate an increase in GSK3β Ser9 phosphorylation upon Wnt stimulation (Cook et al., 1996; Yokoyama et al., 2007). Therefore, we tested the effects of p38 MAPK inhibitor, SB203580, on Wnt3α-induced Ser9 phosphorylation of GSK3β (Fig. 6A). Treatment of Rfz1 cells with Wnt3a stimulated a sharp increase in GSK3β Ser9 phosphorylation, as revealed by immunoblotting with antibodies specific to GSK3β Ser9-P (Fig. 6A). A time-dependent increase in GSK3β Ser9 phosphorylation was observed, with a peak activity at ~1 hour after Wnt3a treatment (Fig. 6A). Interestingly, Wnt3a-induced Ser9 phosphorylation was abolished by SB203580 (Fig. 6A). Similar to the effects with SB203580, overexpression of dominant-negative p38 also abolished Wnt3α-induced GSK3β Ser9 phosphorylation (Fig. 6B). To validate our finding, we performed an in vitro GSK3β kinase assay. Treatment of Rfz1 cells with Wnt3a suppressed the GSK3β kinase activity (Fig. 6C). Treatment of cells with p38 MAPK inhibitor, SB203580, prior to stimulation with Wnt3a abolished the ability of Wnt3a to inhibit GSK3β kinase activity (Fig. 6C), similar to the effects of this inhibitor on β-catenin stabilization and Lef/Tcf-sensitive transcription. Taken together, these data suggest that p38 MAPK regulates canonical Wnt–β-catenin signaling by inhibiting GSK3β activity.

Discussion

The p38 MAPKs are a MAPK subfamily that is conserved from yeast to mammals. They are activated in response to many extracellular stimuli, including growth factors, cytokines and environmental stress (Martin-Blanco, 2000). Recently, the p38 MAPK pathway has been shown to be important for mineralization and development of osteoprogenitors (Caverzasio and Manen, 2007), bone regeneration of mesenchymal stem cells (Chang et al., 2007) and in myogenesis during Xenopus development (Keren et al., 2005). In the present study, we demonstrate for the first time that p38 MAPK is transiently activated upon Wnt3a stimulation and this activation is dependent on both G-protein and Dishevelleds (Fig. 7). The activation of Wnt3a/Fz1, which signal through G-proteins, leads to activation of the Wnt–β-catenin pathway (Katanaev et al., 2005; Liu et al., 2001; Liu et al., 1999; Malbon, 2005). By utilizing siRNA, we show that GqG11 and Gq11 are obligate for Wnt3a-induced p38 MAPK activation. By contrast, Gq11, which is obligate for Wnt3a-induced JNK activation, was found to be dispensable for Wnt3α–Fz1–p38 MAPK signaling (Bikkavilli et al., 2008). Thus, there is a divergence between G-protein function in the Wnt3α–Fz1–p38 MAPK pathway compared with that in the planar cell polarity pathways.

Epistasis experiments reveal that Dishevelleds operate downstream of G-proteins in the fly (Katanaev et al., 2005), as well as in mammalian cells (Bikkavilli et al., 2008). Dishevelleds are cytosolic phosphoproteins that play crucial roles in canonical Wnt–β-catenin pathways (Lee et al., 2008) and also in the planar cell polarity pathway (Bikkavilli et al., 2008). We demonstrate that Dvl3 is obligate for Wnt3α-stimulated p38 MAPK activation. Overexpression of Dvl3 stimulates Lef/Tcf-sensitive transcription, mimicking the effect of Wnt3a (Lee et al., 2008). Furthermore, the Dvl3-stimulated activation of Lef/Tcf-sensitive transcription was found to be sensitive to SB203580. This ability of SB203580 to attenuate the response to Dvl3, positions p38 MAPK downstream of Dishevelleds in the Wnt3α–Fz1–p38 MAPK pathway. Thus Wnt3α binding to Fz1 activates Gq11 and Dishevelled, which in turn leads to p38 MAPK activation (Fig. 7).

The current study reveals a novel role for p38 MAPK in canonical Wnt–β-catenin signaling. The p38 MAPK specific inhibitors (SB203580 or SB239063), p38 siRNA, as well as
expression of dominant-negative p38 strongly attenuate the Wnt3a-induced β-catenin accumulation, Lef/Tcf-sensitive transcription and primitive endoderm formation (Fig. 7). Although, inhibition of p38 MAPK interrupts canonical Wnt-β-catenin signaling, p38 MAPK activation does not appear to be an integral step in canonical Wnt-β-catenin signalling, because signaling in this pathway was observed to operate even upon complete loss of p38 MAPK activation (Fig. 4F,G). Our findings suggest that the Wnt3a–Fz1–p38-MAPK and the canonical Wnt–β-catenin pathways operate in a ‘parallel-input’ scenario (Fig. 7). The Wnt3a–Fz1–p38-MAPK pathway feeds into the canonical Wnt–β-catenin pathway at the level of GSK3β, a point upstream of Wnt3a-induced β-catenin accumulation (Fig. 7). p38 MAPK regulates canonical Wnt-β-catenin signaling at the level of GSK3β, a key regulatory enzyme in canonical Wnt-β-catenin signaling. Previously, the GSK3β-binding protein, GBP, was shown to be important for axis formation during Xenopus development by inhibiting GSK3β activity (Farr et al., 2000) and inositol pentakisphosphates have recently been shown to regulate Wnt-β-catenin activity by inactivating GSK3β activity (Gao and Wang, 2007). The nature of signaling intermediates between p38 MAPK and GSK3β inactivation awaits further study.

Materials and Methods

Cell culture

Mouse F9 teratocarcinoma cell stocks were obtained from ATCC (Manassas, VA) and cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% heat-inactivated fetal bovine serum (Hyclone, South Logan, UT) at 37°C in a 5% CO2 incubator. For generation of F9 clones stably expressing Rfz1, cells were transfected with pcDNA3.1-Rfz1 plasmid using Lipofectamine Plus reagents (Invitrogen, Carlsbad, CA). Fifteen independent clones resistant to G418 were isolated, and propagated, and the level of expression of Rfz1 mRNA was measured indirectly by reverse transcription, polymerase chain reaction (RT-PCR). The clone expressing the highest level of Rfz1 mRNA was propagated in culture medium supplemented with 100 μg/ml G418 and used for all the studies reported herein (Rfz1). F9 cells stably expressing Rfz1 as well as pTOPFLASH (M50) luciferase reporter were generated in a similar manner.
Fig. 6. p38 suppresses GSK3β activity. (A) Confluent F9 cells stably transfected with pRfz1 and pTOPFLASH luciferase reporter were treated with either vehicle (DMSO) or SB203580 (6 μM) for 1 hour prior to the addition of Wnt3a for indicated periods of time. After stimulation, the lysates were collected and subjected to immunoblot analysis with anti-GSK3β Ser9-P antibody. Upper panel represents mean values ± s.e.m. from three independent experiments and the lower panel represents representative blots probed with either anti-GSK3β Ser9-P (p-GSK3β Ser 9), anti-p38 (p38) or with anti-GSK3β (GSK3β) antibodies. (B) F9 cells stably transfected with pRfz1 and pTOPFLASH luciferase reporter were either transfected with empty vector (−) or with Flag-tagged dominant-negative (DN) mutant of p38 MAPK [p38ΔT (AGF)] for 24 hours. After Wnt3a stimulation for 1 hour, the lysates were collected and subjected to immunoblot analysis with anti-GSK3β Ser9-P antibody as described. Upper panel represents mean values ± s.e.m. from three independent experiments and the lower panel represents representative blots probed with either anti-GSK3β Ser9-P (p-GSK3β Ser 9), anti-p38 (p38) or with anti-GSK3β (GSK3β) antibodies. (C) Confluent F9 cells stably transfected with pRfz1 and pTOPFLASH luciferase reporter were treated with either vehicle (DMSO) or SB203580 (6 μM) for 1 hour prior to the addition of Wnt3a for 10 minutes. GSK3β was immunoprecipitated from whole cell lysates and its activity was measured by an in vitro kinase assay. The data represent mean values ± s.e.m. from two independent experiments that are highly reproducible. *P<0.05 and **P<0.01 versus −Wnt3a control; +P<0.05 and **P<0.01 versus +Wnt3a control.

Immunoblotting
F9 clones stably expressing Rfz1 were cultured in six-well plates. After the assay, the cells were lysed in 250 μl lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Na3P2O7, 1 mM β-glycerophosphate, 1 mM Na2VO4, 1 μg/ml leupeptin). The lysates were cleared by centrifugation at 20,000 g for 5 minutes. The supernatants were transferred into new tubes and protein concentrations were determined using the Lowry method. Total lysates (30-60 μg protein/lane) were subjected to electrophoresis in 10-12% SDS-PAGE gels. The resolved proteins were transferred electrophoretically to nitrocellulose membrane blots. The blots were incubated with primary antibodies overnight at 4°C and the immunocomplexes were made visible by use of a secondary antibody coupled to horseradish peroxidase and developed using the enhanced chemiluminescence method. The antibodies were purchased: anti-HA high affinity antibody (Roche Applied Science, Indianapolis, IN), anti-β-catenin and anti-β-actin antibodies (Sigma-Aldrich, St Louis, MO), anti-p38 MAPK and anti-phospho GSK3β Ser9 (Cell Signaling Technology, Danvers, MA) and anti-GSK3β (BD transduction laboratories, San Jose, CA).

p38 activity assay
F9 cells expressing Rfz1 were grown to confluence in six-well plates. The cells were then serum-starved by reducing the FBS supplement to 0.5% for 8 hours followed by reduction to 0.005% for a further 16 hours. The cells were then exposed to recombinant Wnt3a (20 ng/ml, R&D Systems, Minneapolis, MN) for 15 minutes at 37°C. The activity of the p38 MAPK was assayed using the p38 MAPK activation kit (Cell Signaling Technology, Danvers, MA) according to the manufacturer’s instructions. Briefly, the cells were lysed in 300 μl lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Na3P2O7, 1 mM β-glycerophosphate, 1 mM Na2VO4, 1 μg/ml leupeptin) and the lysates were cleard by centrifugation at 20,000 g for 5 minutes. The lysates (500 μg) were incubated with 10 μl immobilized phospho-p38 MAPK antibody overnight with rotation at 4°C. The immunocomplexes were washed twice with lysis buffer and twice with kinase buffer (25 mM Tris-HCl pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 M Na2VO4, 10 mM MgCl2). The immobilized phospho-p38 MAPK was incubated with 20 μl kinase buffer containing 1 μg phospho-p38 MAPK substrate peptide GST-ATF2 (19-96) and 20 μM ATP at 30°C for 30 minutes. The kinase reaction was stopped by addition of 10 μl of 4× SDS sample buffer. The samples were boiled for 5 minutes and immunoblotting was performed as described above. The p38 MAPK activity was determined by probing with anti-p-pATF2 antibody. For all the experiments, the supernatants after immunoprecipitates were collected and used for normalization by probing the blots with anti-p38 antibody.

Cytosolic β-catenin accumulation assay
To separate the cytosolic β-catenin from membrane-associated β-catenin, lysates were treated with concanavalin A Sepharose (Amersham Biosciences, Uppsala, Sweden), as described earlier (Aghib and McCrea, 1995). Briefly, confluent F9 cultures were treated with Wnt3a for different durations and lysed in lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 40 mM Na3P2O7, 50 mM K2HPO4, 10 mM Na2MoO4, 2 mM Na2VO4, 1 mM Triton X-100, 0.5% NP40, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μg/ml phenylmethylsulphonyl fluoride). The lysates were transferred into 1.5 ml Eppendorf tubes and rotated at 4°C for 15 minutes followed by centrifugation at 20,000 g for 15 minutes. The supernatants were transferred into new tubes, protein concentrations determined and reduced to 2.5 mg/ml with lysis buffer. 60 μl ConA Sepharose was added to each tube and rotated at 4°C for 1 hour. After a brief centrifugation, the supernatants were transferred to new tubes and 30 μl of ConA Sepharose was added to each tube and rotated again at 4°C for 1 hour. Finally, after a brief centrifugation, the supernatants were transferred to new tubes and their protein concentration determined. Immunoblotting was performed with the samples and β-catenin accumulation was analyzed by probing the blots with anti-β-catenin antibodies and normalized by probing with anti-actin antibodies.

Dual luciferase assay
F9 cells stably expressing Rfz1 and the pTOPFLASH (M50) luciferase reporter were seeded into 12-well plates. Following incubation for 24 hours at 37°C, cells were transfected with pRL-CMV Renilla luciferase control vector (5 ng/well) for 24 hours, followed by treatment with or without purified Wnt3a for 7 hours. Cells were then
Fig. 7. Schematic representation of role of p38 MAPK in canonical Wnt–β-catenin signaling pathway. Activation of Fz1 by Wnt3a leads to accumulation of β-catenin, Lef/Tcf-sensitive transcriptional response and primitive endoderm formation in mouse F9 cells through G-proteins and Dishevelleds. p38 MAPK plays a crucial role in canonical signaling by inactivating GSK3β and by operating downstream of Dishevelleds.

directly lysed on the plates by addition of 1× cell culture lysis reagent (Promega, Madison, WI). Lysates were collected into chilled microfuge tubes on ice and centrifuged at 20,000 g for 5 minutes. The supernatant was transferred into a new tube and directly assayed as described below. 20 μl lysate was added either to 100 μl of luciferase assay buffer (20 mM Tricine pH 7.8, 1.1 mM MgSO4, 4 mM MgSO4, 0.1 mM EDTA, 0.27 mM coenzyme A, 0.67 mM luciferin, 33 mM DTT and 0.6 mM ATP) or to 100 μl Renilla luciferase assay substrate (E2810, Promega, Madison, WI) and the luciferase activities were measured by use of a luminometer (Berthold Lumat LB 9507). The samples were assayed in triplicate and the firefly luciferase (M50, TOPFLASH) activities were normalized by the luciferase control vector (5 ng/well) for another 24 hours. The luciferase assay was performed as described previously (Van Lint et al., 2008) was published that shows 'phosphorylation by p38 MAPK as an alternative pathway for GSK3β inactivation'.

Indirect immunofluorescence studies

F9 cells stably expressing Rfz1 in 24-well plates were fixed with 3% glutaraldehyde and permeabilized with 0.1% Triton X-100 for 15 minutes, then washed three times with MSM-PIPES buffer, the cells were incubated with an anti-mouse antibody coupled to Alexa Fluor 488 (Invitrogen) at 37°C for 30 minutes. Finally, the cells were washed in blotting buffer (560 mM NaCl, 10 mM KH2PO4, 0.1% Triton X-100, 0.02% SDS) and images were captured using a Zeiss LSM510 inverted fluorescence microscope.

Data analysis

For all experiments, data were compiled from at least three independent, replicate experiments performed on separate cultures on separate occasions. In all cases, fold changes over the untreated control (set to one) were calculated and displayed. Comparisons of data among groups were performed using one-way analysis of variance (ANOVA) or Student’s t-test. Statistical significance (P<0.05) is denoted on graphs as * or #. The indirect immunofluorescence and phase-contrast images are of representative fields of interest.

We thank Randall Moon for the generous gift of reporter plasmid Super SXTOPFLASH, Roger Davis for pCMV-Flag-tagged dominant-negative mutant of p38 (AGF) and John Kyriakis for pMT3-p38 expression vector. We also thank Hsien-Yu Wang (Department of Physiology and Biophysics, SUNY, Stony Brook) for critical comments on the manuscript. This work was supported by USPHS Grant DK30111 from the NIDDK, National Institutes of Health (to C.C.M.).

Note added in proof

Following the submission of the final version of this manuscript, work by colleagues (Thornton et al., 2008) was published that shows 'phosphorylation by p38 MAPK as an alternative pathway for GSK3β inactivation'.
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