Wnt-5a induces Dishevelled phosphorylation and dopaminergic differentiation via a CK1-dependent mechanism

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Summary

Previously, we have shown that Wnt-5a strongly regulates dopaminergic neuron differentiation by inducing phosphorylation of Dishevelled (Dvl). Here, we identify additional components of the Wnt-5a-Dvl pathway in dopaminergic cells. Using in vitro gain-of-function and loss-of-function approaches, we reveal that casein kinase 1 (CK1) δ and CK1ϵ are crucial for Dvl phosphorylation by non-canonical Wnts. We show that in response to Wnt-5a, CK1ϵ binds Dvl and is subsequently phosphorylated. Moreover, in response to Wnt-5a or CK1ϵ, the distribution of Dvl changed from punctate to an even appearance within the cytoplasm. The opposite effect was induced by a CK1ϵ kinase-dead mutant or by CK1 inhibitors. As expected, Wnt-5a blocked the Wnt-3a-induced activation of β-catenin. However, both Wnt-3a and Wnt-5a activated Dvl2 by a CK1-dependent mechanism in a cooperative manner. Finally, we show that CK1 kinase activity is necessary for Wnt-5a-induced differentiation of primary dopaminergic precursors. Thus, our data identify CK1 as a component of Wnt-5a-induced signalling machinery that regulates dopaminergic differentiation, and suggest that CK1δ/ϵ-mediated phosphorylation of Dvl is a common step in both canonical and non-canonical Wnt signalling.

Key words: Casein kinase 1δ/ϵ, Dishevelled, Wnt-5a, Dopaminergic neurons, Non-canonical Wnt signalling, siRNA

Introduction

The Wnt signalling pathway is a highly conserved biochemical pathway that is involved in a vast array of processes in both embryonic development and adult tissue homeostasis (for reviews, see Huelsken and Birchmeier, 2001; Patapoutian and Reichardt, 2000; Yamaguchi, 2001). Moreover, key molecular players of the Wnt pathway have been found to regulate midbrain development (McMahon and Bradley, 1990; Pinson et al., 2000; Thomas and Capecchi, 1990; Wang et al., 2002) and various aspects of dopaminergic neuron (DN) development (McMahon and Bradley, 1990; Pinson et al., 2000; Thomas and Capecchi, 1990; Wang et al., 2002; Arenas, 2005; Castelo-Branco et al., 2003; Prakash et al., 2006).

We have shown that Wnt-5a – a non-canonical Wnt, classified by its inability to activate β-catenin (Shimizu et al., 1997) – plays a pivotal role in the ventral midbrain DN differentiation (Castelo-Branco et al., 2006; Castelo-Branco et al., 2003). To date, the underlying molecular mechanism of action of Wnt-5a and the signalling pathways activated in DN as well as in other mammalian cells is still largely unknown (Veeman et al., 2003). Several molecular players have been implicated, including the Wnt receptors of the Frizzled family and the downstream signalling phosphoprotein Dishevelled (Dvl) (Gonzalez-Sancho et al., 2004; Hsieh, 2004; Wallingford and Habas, 2005; Wharton, Jr, 2003).

Here, we examined the mechanism through which Wnt-5a activates Dvl in dopaminergic cells. We report the identification of casein kinase 1 (CK1) δ and CK1ϵ (hereafter referred to as CK1δ/ϵ) as kinases phosphorylating Dvl2 and Dvl3 in response to Wnt-5a. We show that Wnt-5a-induced CK1ϵ-mediated phosphorylation of Dvl2 results in changes of the cytoplasmic distribution of Dvl2. Finally, we report that activity of endogeneous CK1 is crucial for the pro-differentiation function of Wnt-5a in dopaminergic precursors. Thus, we hereby identify CK1 as a positive regulator of DN development.

Results

Wnt-5a phosphorylates Dvl in a dopaminergic neuronal cell line

To characterise the pathways activated by Wnt-5a in dopaminergic cells, we treated SN4741 cells with Wnt-5a and used Wnt-3a (a canonical Wnt) for comparison. Treatment with either Wnt form (at 100 ng/ml) lead to the phosphorylation of Dvl2 and Dvl3, as shown previously by a mobility shift of the protein on SDS-PAGE (Gonzalez-Sancho et al., 2004; Lee et al., 1999; Schulte et al., 2005; Bryja et al., 2007). Both Dvl2 and Dvl3 showed the first visible signs of phosphorylation at 30 minutes of treatment and a clear phosphorylation shift after 1 hour (Fig. 1A). Maximal effects were detected after 2 hours and, hence, this time point was used subsequently unless otherwise specified. Whereas both Wnt-3a and Wnt-5a induced Dvl phosphorylation (Fig. 1A), only Wnt-3a induced β-catenin activation (Fig. 1B), as assessed by an antibody recognizing

active β-catenin (ABC, the form of β-catenin dephosphorylated on Ser37 and Thr41) (van Noort et al., 2002). To confirm that the observed signalling was specific to Wnts, we treated SN4741 cells with the broad-spectrum inhibitor of Wnt signalling, soluble Fz8-CRD (the cysteine-rich domain of Frizzled 8 that competes with Frizzled receptors for Wnt binding) (Hsieh et al., 1999). Pre-treatment with Fz8-CRD blocked both basal and Wnt-induced Dvl phosphorylation and β-catenin activation (Fig. 1C), indicating that the effects observed were specifically induced by Wnts.

CK1δ/ε mediate Wnt-5a-induced phosphorylation of Dvl

A large number of kinases have been implicated in Wnt signalling; however, it remains unclear which kinase(s) are responsible for Dvl phosphorylation, leading to its electrophoretic mobility shift after Wnt-5a stimulation. To identify the relevant signalling pathways leading to the phosphorylation-dependent mobility shift of Dvl, we analysed a panel of small molecule compounds for their ability to block or reduce the Wnt-5a-induced mobility shift of Dvl2 that takes place after phosphorylation (Gonzalez-Sancho et al., 2004). Twenty five pharmacological compounds interfering with heterotrimeric G proteins, protein kinase C, protein kinase A, MEK1/2, PI3K, p38, JNK, CamKII, GSK3, cAMP signalling, EPAC, adenylyl cyclase, Src-like kinases, EGFR, phospholipase C, CK1 or Ser/Thr kinases were tested (see Table 1). Only D4476, an inhibitor of CK1 (Rena et al., 2004), was able to block the Wnt-5a-induced phosphorylation-dependent mobility shift of Dvl2. Interestingly, D4476 reduced both basal and also Wnt-5a-induced phosphorylation of Dvl2 and Dvl3 (Fig. 2A). It should be noticed that our

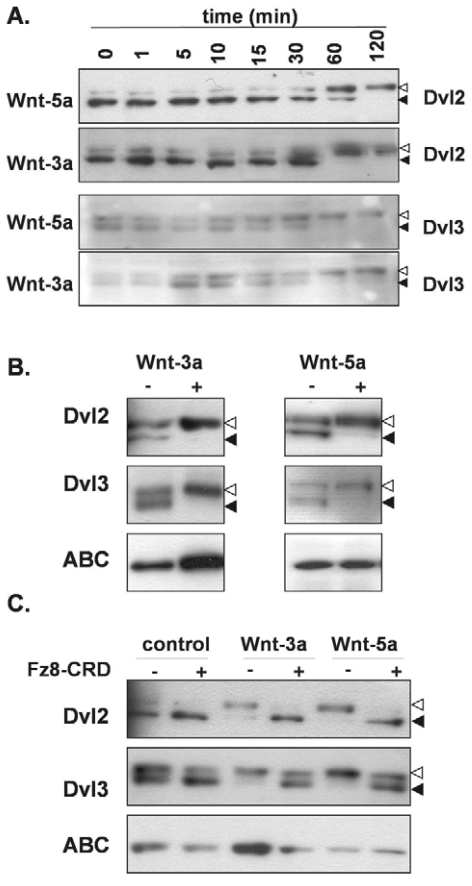

Fig. 1. Wnt-5a and Wnt-3a activate Dvl in dopaminergic cells. (A) Time course of Wnt-5a- and Wnt-3a-induced activation of Dvl2 and Dvl3. SN4741 cells, treated with Wnt-3a (100 ng/ml) or Wnt-5a (100 ng/ml), were lysed after 0, 1, 5, 10, 15, 30, 60 and 120 minutes. (B) Both Wnt-5a and Wnt-3a activated Dvl2 and Dvl3, but only Wnt-3a activated β-catenin after 2 hours of stimulation. (C) The effects of Wnt-5a or Wnt-3a (100 ng/ml) on Dvl were blocked by Fz8-CRD-conditioned medium. (A-C) Phosphorylation of Dvl isoforms were detected as phosphorylation-dependent mobility shifts of total Dvl2 and Dvl3. The position of dephosphorylated (◀) and phosphorylated Dvl (◁) is indicated. The activation of β-catenin was determined by western blotting using antibodies against active (Ser33-Thr41-dephosphorylated) β-catenin (ABC). Data are representative of at least three independent replicates.

Table 1. Screening of compounds for the ability to interfere with the Wnt-induced shift of Dvl

Compound	Target	Concn	Activity
PTX	$G_{i/o}$	100 ng/ml	No
PDBu	PKC activator	1 μM	No
Wortmannin	PI3K	50 nM	No
LY294002	PI3K	50 μM	No
PD98059	MEK1/2	10 μM	No
UO126	MEK1/2	10 μM	No
SB203580	p38	10 μM	No
JNKII inhib	JNK	6 μM	No
Genistein	PKC	50 μM	No
Chelerythrine	PKC	10 μM	No
Ro-31 8220	PKC	1 μM	No
BIM I	PKC	500 nM	No
KN93	CaMKII	10 μM	No
I3M	GSK-3	2 μM	No
Kenpaullone	GSK-3	6 μM	No
H89	PKA	10 μM	No
8-Br-cAMP	cAMP pathway activator	10 μM	No
8CPT-2Me-cAMP	EPAC activator	30 μM	No
SQ22536	Adenylyl cyclase	100 μM	No
MDL12330	Adenylyl cyclase	10 μM	No
PP2	Src-like	10 μM	No
AG1276	EGFR	10 μM	No
ET-18-OCH3	PLC	10 μM	No
D4476	Casein kinase 1	100 μM	Yes
Staurosporine	Ser/Thr kinases, PKC	2 μM	No

$G_{i/o}$, heterometric G protein (inhibitory); PTX, pertussis toxin; PDBu, phorboldibutyrate; PKC, Ca^{2+}-dependent protein kinase; PI3K, phosphatidylinositol-3′-kinase; LY294002, 2-(4-morpholino)-8-phenyl-4*H*-1-benzopyran-4-one; PD98059, 2′-amino-3′-methoxyflavone; MEK1/2, MAPK and ERK kinase1/2; UO126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene; SB203580, 4-[5-(4-fluorophenyl)-2-[4-(methylsulfonyl)phenyl]-1H-imidazol-4-yl]pyridine; p38, stress-activated protein kinase p38; JNK, c-jun N-terminal kinase; Ro-318220, bisindolylmaleimide IX; BIM I, bisindolmaleimide I; KN93, 2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine); CaMKII, Ca^{2+}/calmodulin-dependent kinase II; I3M, indirubin-3-monoxime; GSK-3, glycogen synthase kinase 3; H89, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; PKA, cAMP-dependent protein kinase; 8-Br-cAMP, 8-bromo-cyclic AMP; 8CPT-2Me-cAMP, 8-(4-chloro-phenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate; SQ22536, 9-(tetrahydro-2-furanyl)-9H-purin-6-amine; MDL12330, *cis*-N-(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine; PP2, 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine; AG1478, 4-(3-chloroanillino)-6,7-dimethoxyquinazoline; EGFR, epidermal growth factor receptor; ET-18-OCH_3, rac-2-methyl-1-octadecyl-glycero-(3)-phosphocholine; PLC, phospholipase C; D4476, 4-(4-(2,3-dihydrobenzo[1,4]dioxin-6-yl)-5-pyridin-2-yl-1H-imidazol-2-yl)benzamide.

results do not exclude the possibility that other phosphorylation events exist that were not detected in the mobility shift assay.

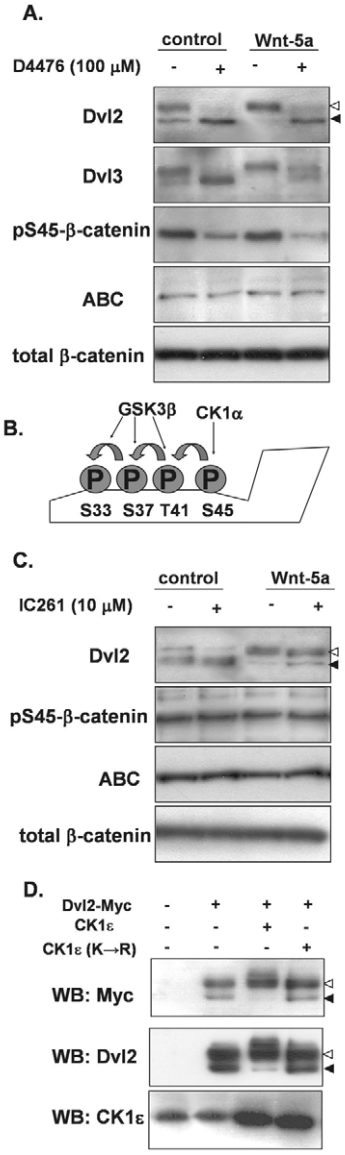

Fig. 2. CK1 inhibition blocks Wnt-5a-induced phosphorylation of Dvl2 and Dvl3. SN4741 cells were treated with vehicle (control) or Wnt-5a (100 ng/ml) for 2 hours in the presence or absence of D4476 (100 μM) (A) or IC261 (10 μM) (C). Western blot analysis was performed as in Fig. 1. The position of dephosphorylated (◀) and phosphorylated (◁) Dvl is indicated. Antibodies against phospho-Ser45-β-catenin (CK1α target site), against active (Ser37-Thr41-dephosphorylated) β-catenin (ABC) and total β-catenin were used. (B) Scheme of phosphorylation sites of β-catenin. Ser45 is a CK1α target site, which serves as a priming for sequential phosphorylation of Thr41, Ser37 and Ser33 by GSK3β. (D) SN4741 cells were transfected with plasmids encoding Dvl2-Myc and either CK1ε or kinase-dead CK1ε (K>R) mutant. Phosphorylation of Dvl2-Myc was detected as a phosphorylation-dependent mobility shift by Myc- and Dvl2-specific antibodies. CK1ε levels were monitored with a CK1ε specific antibody. Data in A, C and D are representative of at least three independent replicates.

CK1 has previously been shown to be involved in various steps of Wnt signal transduction (Davidson et al., 2005; Kishida et al., 2001; Price, 2006; Zeng et al., 2005). Importantly, CK1α was shown to phosphorylate β-catenin at Ser45, priming β-catenin for subsequent phosphorylation by GSK3β (Fig. 2B) (Amit et al., 2002; Liu et al., 2002; Matsubayashi et al., 2004). By contrast, CK1δ and CK1ε lack the ability to phosphorylate β-catenin (Liu et al., 2002; Peters et al., 1999), but are known to bind and phosphorylate Dvl in the canonical Wnt signalling pathway (Cong et al., 2004; Kishida et al., 2001; McKay et al., 2001a; Peters et al., 1999; Swiatek et al., 2004). To test which CK1 subtype is inhibited by D4476, we analysed the effect of D4476 on the level of CK1α-mediated phosphorylation of the Ser45 residue of β-catenin. As shown by phosphorylation-specific antibodies, the phosphorylation of β-catenin at Ser45 was not affected by Wnt-5a but was significantly decreased following the application of D4476 (Fig. 2A), suggesting that CK1α can be inhibited by D4476. To elucidate which CK1 isoforms are responsible for Dvl2 phosphorylation, we used the more specific CK1 inhibitor IC261. IC261 has previously been reported to efficiently inhibit CK1δ/ε at low micromolar doses (in vitro IC_{50}=1 μM) but not CK1α (in vitro IC_{50}=16 μM) (Mashhoon et al., 2000). Despite being less efficient than D4476, IC261 (10 μM) inhibited Wnt-5a-induced Dvl2 phosphorylation (Fig. 2C). However, in contrast to D4476, IC261 did not reduce the levels of β-catenin phosphorylated at Ser45, indicating that CK1δ/ε, but not CK1α, kinase activity was inhibited. The levels of active β-catenin, as well as total β-catenin levels, were unchanged by Wnt-5a, D4476 or IC261 treatment (Fig. 2A). These experiments suggested that CK1δ/ε, rather than CK1α, phosphorylate Dvl2 and Dvl3 in response to Wnt-5a.

To confirm that CK1ε phosphorylates Dvl2, gain-of- and loss-of-function experiments were performed in SN4741 cells transiently transfected with plasmids encoding Dvl2-Myc (Lee et al., 1999), CK1ε or the CK1ε (K>R) mutant (a kinase-dead form of CK1ε) (Fish et al., 1995). We found that CK1ε, but not CK1ε (K>R), phosphorylated Dvl2-Myc (Fig. 2D). Similar data were obtained with Dvl2-GFP (not shown), demonstrating that the kinase activity of CK1ε is required for Dvl2 phosphorylation in the overexpression system.

To analyse the role of endogenous CK1 in the Wnt-5a-induced phosphorylation of Dvl2, we performed gene knockdown of CK1α, CK1δ and CK1ε using small interfering RNAs (siRNA). Three independent siRNAs, each designed against the various CK1 isoforms, were tested for their efficiency in silencing endogenous CK1 in SN4741 cells. Efficiency of gene knockdown was analysed by western blotting for CK1ε (Fig. 3A) and by quantitative reverse transcriptase (RT)-PCR for CK1α and CK1δ (not shown), where subtype-specific antibodies failed to detect endogenous CK1α and CK1δ. At least one siRNA against each CK1 isoform provided a strong gene knockdown of more than 50%, as assessed by western blotting (CK1ε, Fig. 3A) and quantitative RT-PCR for CK1δ and CK1α (data not shown). The most efficient siRNAs – CK1αIII, CK1δIII and/or CK1εII were then transfected into SN4741 that were stimulated with increasing doses of Wnt-5a. The compound knockdown of CK1δ and CK1ε resulted in a significant reduction of the Wnt-5a-induced phosphorylation of Dvl2, whereas the CK1α

siRNA had no effect compared with control (Fig. 3B). Although one cannot exclude the possibility that lack of the effect of CK1α siRNA was due to incomplete knockdown, our data suggest that CK1δ/ϵ, rather than CK1α, are responsible for Wnt-5a-mediated phosphorylation of Dvl2. Interestingly, knockdown of CK1ϵ alone, or together with less efficient CK1 (δI and δII) siRNAs, was not sufficient to reduce the effects of Wnt-5a on Dvl2 (not shown), suggesting that CK1δ and CK1ϵ are to some extent redundant in their function. To confirm that CK1δIII does not act by an off-target mechanism but rather that a joint knockdown of CK1δ and CK1ϵ is necessary, we treated SN4741 cells with control RNA, CK1δIII or CK1ϵII, or the combination of the siRNAs. As we show in Fig. 3C, CK1δIII or CK1ϵII alone were not able to block Wnt-5a-mediated phosphorylation of Dvl, whereas their combination blocked phosphorylation of endogenous Dvl2 very efficiently. Taken together, these experiments demonstrate that CK1δ and CK1ϵ, rather than CK1α, phosphorylate Dvl2 in response to Wnt-5a.

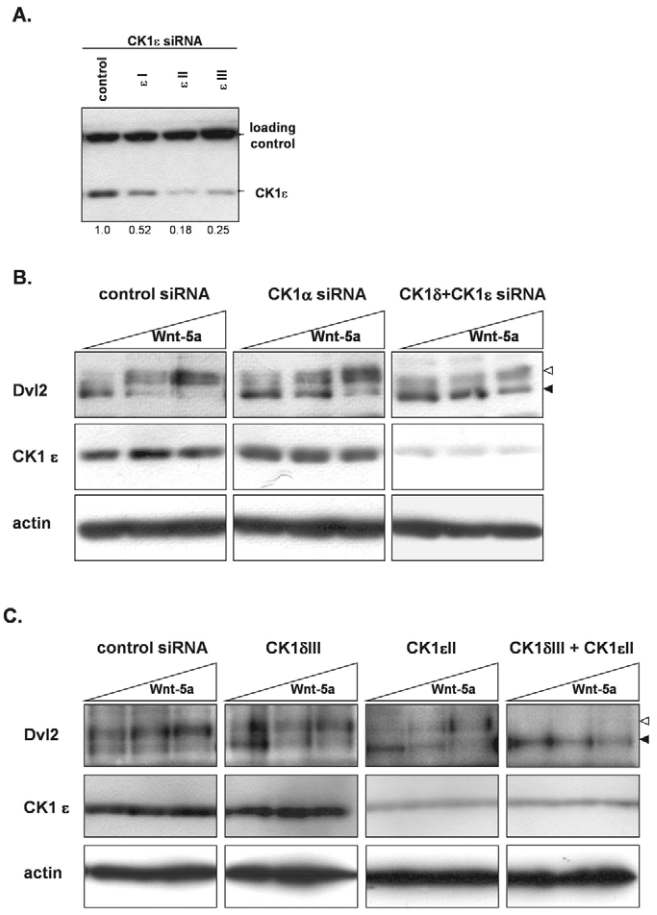

Fig. 3. Gene knockdown of CK1δ/ϵ reduces the phosphorylation of Dvl2 induced by Wnt-5a. (A) SN4741 cells were transfected with control siRNA and three independent siRNAs against CK1ϵ isoform. The efficiency of gene knockdown induced by individual siRNAs against CK1ϵ was quantified in western blots. A non-specific protein, resulting in a band recognised by anti-CK1ϵ antibody served as a loading control. Quantification (normalised to loading control) is shown below. (B,C) SN4741 cells were transfected with the indicated combinations of siRNAs, and treated with control, 50 and 100 ng/ml of Wnt-5a. The phosphorylation of Dvl isoforms was detected as phosphorylation-dependent mobility shift of total Dvl2. Data are representative of three (B) or two (C) independent experiments. The position of dephosphorylated (◀) and phosphorylated Dvl (◁) is indicated. The levels of CK1ϵ and β-actin were also determined to confirm the efficient gene knockout and the equal protein loading.

Wnt-5a induces the activation of endogenous CK1ϵ kinase that interacts with Dvl2

To examine whether Wnt-5a induces the kinase activity of endogenous CK1ϵ, we tested and confirmed that CK1ϵ can be immunoprecipitated in SN4741 cells (Fig. 4A). Using myelin basic protein (MBP, a general substrate of Ser/Thr kinases) as a substrate in an in vitro CK1ϵ kinase assay, we found that treatment of SN4741 cells with either Wnt-5a or Wnt-3a clearly upregulates CK1ϵ activity (Fig. 4B), which is an effect previously shown only for canonical Wnts (Swiatek et al., 2004). This demonstrates that Wnt-5a, as well as Wnt-3a, activates endogenous CK1ϵ in dopaminergic SN4741 cells. Additionally, we examined whether Dvl2 and CK1ϵ physically interact in SN4741 cells, as it has been shown previously in other cellular models (Peters et al., 1999; Sakanaka et al., 1999), and found that Dvl2-Myc binds both overexpressed CK1ϵ (Fig. 4C, lane 2) and endogenous CK1ϵ (Fig. 4D). Please notice that endogenous CK1ϵ was only clearly detected when beads coupled to antibody where used to enhance the signal above background. Importantly, the lack of kinase activity in CK1ϵ (K>R) does not prevent the interaction with Dvl2 (Fig. 4C, lane 3). These results suggest that the kinase activity of CK1ϵ is not needed for binding to Dvl2, but it is required for the phosphorylation of Dvl2.

CK1ϵ and Wnt-5a change the subcellular localization of Dvl2

The subcellular distribution of Dvl was examined after transfection of SN4741 cells with a Dvl2-Myc plasmid. Dvl2-Myc was detected by immunocytochemistry in the cytoplasm and found either in a diffuse and even pattern, or in punctae that represent large multiprotein complexes (Schwarz-Romond et al., 2005; Smalley et al., 2005). Based on the presence of Dvl2-Myc punctae and their size, cells were sorted into four different categories (Fig. 5A): (1) even distribution (no punctae detected), (2) punctae of small size (small dot-like punctae on the background of still detectable even cytoplasmic staining), (3) punctae of intermediate size (distinct punctae strongly contrasting with the negative staining of the remaining cytoplasm) or, (4) punctae of large size (individual punctae already fused that form large doughnut-like structures). Interestingly, the distribution of Dvl2-Myc was dramatically regulated by transfection of the CK1ϵ constructs (Fig. 5B). Both CK1ϵ and CK1ϵ (K>R) showed a homogeneous cytoplasmatic distribution when transfected alone and stained with an anti-CK1ϵ-specific antibody (not shown). Upon coexpression of CK1ϵ with Dvl2-Myc, the distribution of Dvl2-Myc changed from punctate 70% in the control to an even distribution in 65% of the cells (Fig. 5B,C); a finding similar to what has previously been shown in HEK293 cells (Cong et al., 2004). Conversely, CK1ϵ (K>R) promoted the punctate localization of Dvl2-Myc in 95% of the cells by predominantly increasing intermediate puncta (Fig. 5B,C). In all cases, we found that CK1ϵ (wt or K>R) colocalises with Dvl2-Myc, suggesting that CK1ϵ controls the distribution of Dvl.

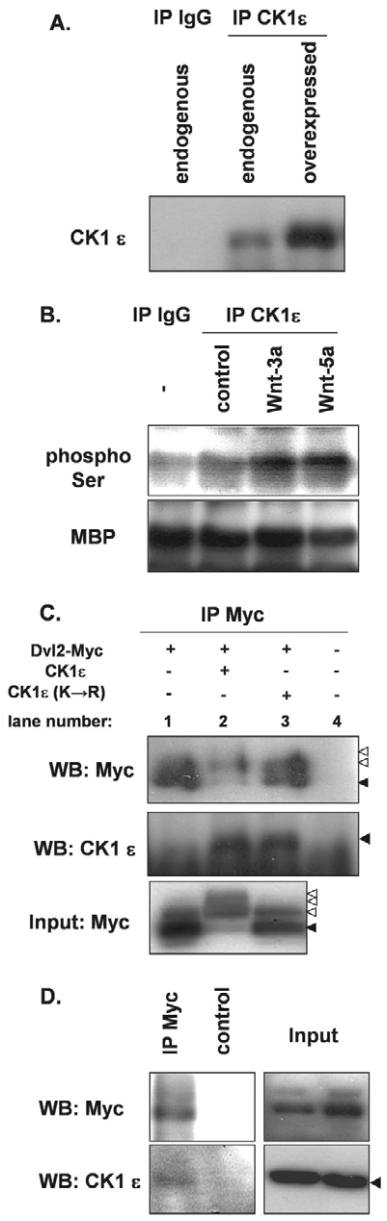

Fig. 4. CK1ϵ is activated by Wnt-5a and binds Dvl2 in dopaminergic cells. (A) SN4741 cell lysates were immunoprecipitated with a control IgG or a CK1ϵ-specific antibody and endogenous or overexpressed CK1ϵ and detected by western blotting. (B) Wnt-3a and Wnt-5a increased the phosphorylation of MBP as detected by western blotting using a specific antibody against phosphorylated serine. Cell lysates from control (1% CHAPS), Wnt-3a or Wnt-5a treated (100 ng/ml) SN4741 cells were immunoprecipitated with a CK1ϵ-specific antibody and subjected to kinase assay using MBP as a substrate. (C) Dvl2-Myc interacts with either CK1ϵ (lane 2) or a kinase-dead CK1ϵ (K>R) (lane 3) mutant in SN4741 cells, as assessed by immunoprecipitation and western blotting with Myc- and CK1ϵ-specific antibodies. Co-precipiated CK1ϵ by the Myc antibody is indicated by ◀. For all other panels: ◁, phosphorylated Dvl2-Myc; ◀, unphosphorylated Dvl2-Myc. (D) Dvl2-Myc interacts with endogenous CK1ϵ as assessed by immunoprecipitation of SN4741 cells transfected with Dvl2-Myc only, by using Myc antibody-conjugated agarose beads (control, G-protein-conjugated beads). Data are representative of three independent replicates.

When the CK1 inhibitor D4476 was added after transfection, it significantly reduced CK1ϵ-mediated phosphorylation of Dvl2-Myc (Fig. 5D, lanes 6, 8 and 10) confirming that this CK1 inhibitor specifically blocks the action of CK1ϵ on Dvl2 in a dose-dependent manner. Interestingly, when the cells were transfected with Dvl2-Myc alone and treated with the CK1 inhibitor D4476, Dvl2-Myc phosphorylation was prevented (Fig. 5D, lane 4) and the localization of Dvl2-Myc changed from an even distribution in 50% of the cells to a punctate pattern in 85% of the cells (Fig. 5E). Similar results were obtained using the CK1δ/ϵ-specific inhibitor IC261 (Fig. 5F). To directly test whether activation of endogenous CK1δ/ϵ by Wnt-5a resulted in a relocalization of Dvl2-Myc similar to the one induced by overexpressed CK1, we treated Dvl2-Myc-overexpressing SN4741 cells with Wnt-5a. Wnt-5a treatment resulted in a statistically significant increase in the number of cells with even distribution of Dvl2-Myc at the expense of the cells with Dvl2-Myc in punctae (Fig. 5G). When using 100 ng/ml of Wnt-3a and an identical experimental setup as for Wnt-5a, we failed to detect similar changes in cytoplasmic distribution of Dvl2-Myc induced by Wnt-3a (not shown), suggesting that Wnt-induced relocalization of Dvl is an effect specific for non-canonical Wnts. In summary, these results demonstrate that Wnt-5a has an effect similar to that of CK1ϵ and suggest that endogenous CK1δ/ϵ regulate the phosphorylation and cellular localization of Dvl2 in response to Wnt-5a. Combined, our results indicate that active CK1ϵ, either overexpressed or endogenous (activated by Wnt-5a), phosphorylates Dvl2 and induces a diffuse cytoplasmic distribution of phosphorylated Dvl2 that can be blocked by either CK1 inhibition or the kinase-dead CK1ϵ (K>R).

Wnt-5a cooperates with Wnt-3a in the phosphorylation of Dvl, but antagonises Wnt-3a in the activation of β-catenin

Given that D4476 is a reversible competitive inhibitor of the ATP binding site in CK1, we examined whether the inhibition of Dvl2 phosphorylation by D4476 is modulated by increasing doses of Wnt-5a. Increased amounts of Wnt-5a dose dependently overcame the D4476-mediated inhibition and lead to a phosphorylation-dependent mobility shift of Dvl2 (Fig. 6A). We next explored whether Wnt-5a and Wnt-3a phosphorylates Dvl by similar mechanisms and, if so, whether their effects are additive. SN4741 cells were pre-treated with Wnt-5a (100 ng/ml) or Wnt-3a (20 ng/ml), the lowest doses leading to the efficient phosphorylation of Dvl2 and Dvl3 (data not shown); then, increasing doses of Wnt-3a (50, 100 and 200 ng/ml) or Wnt-5a (100, 200 and 500 ng/ml) were applied. The results showed that both Wnt-3a and Wnt-5a activate Dvl phosphorylation (Fig. 5B and C, respectively). Moreover, Wnt-3a and Wnt-5a cooperated in the phosphorylation of Dvl in the absence of D4476 (as monitored by the disappearance of the non-shifted band of Dvl2). When the additive effects of Wnts were tested in the presence of D4476, Wnt-3a rescued the D4476-mediated block of Wnt-5a-induced phosphorylation of Dvl2 and vice versa (Fig. 6C,D). Not surprisingly, active β-catenin was induced when Wnt-3a was added to Wnt-5a pre-treated cells (Fig. 6B,D). By contrast, when Wnt-5a was added to cells pre-treated with Wnt-3a, it significantly and dose dependently reduced the activation of β-catenin, irrespective of

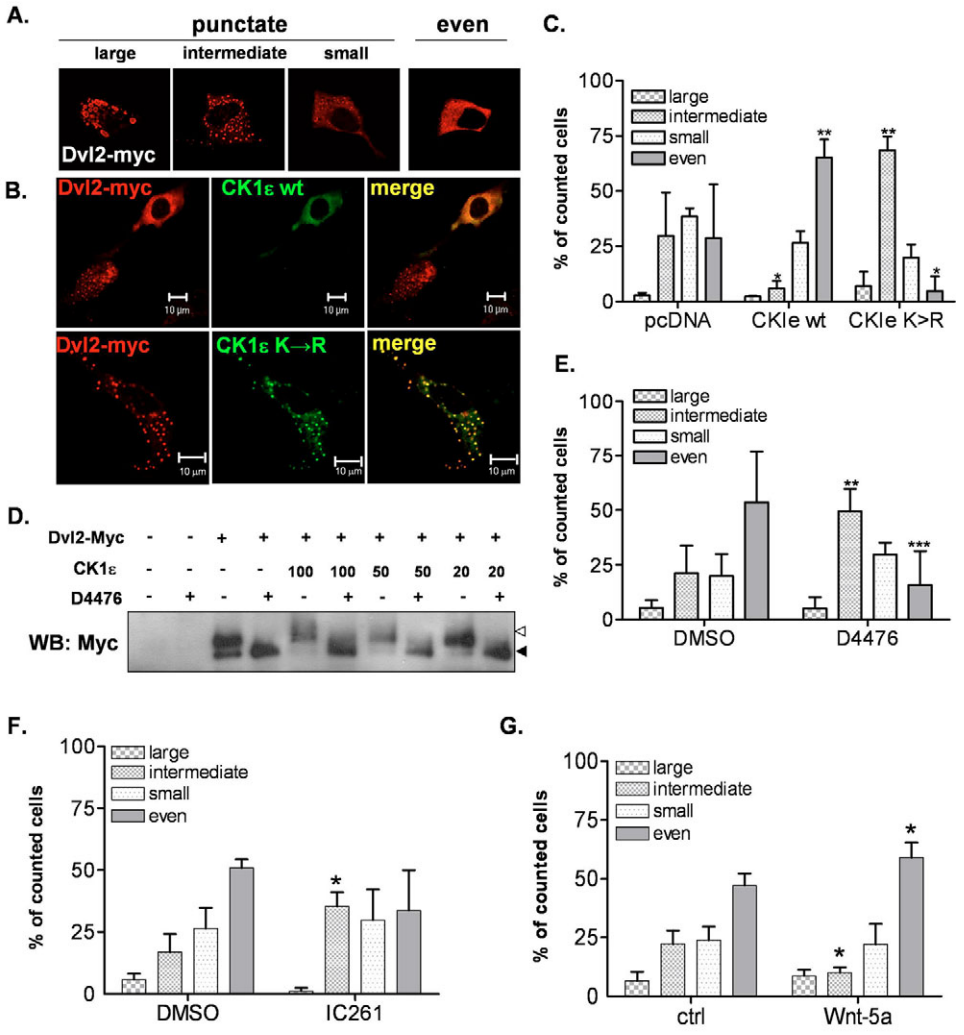

Fig. 5. CK1ϵ and Wnt-5a mediate the changes in phosphorylation and cytoplasmic distribution of Dvl2. (A) The subcellular distribution of Dvl2-Myc in transfected SN4741 cells was assessed through an anti-Myc antibody by confocal microscopy. Four patterns were detected: (1) even distribution, and punctae of (2) small, (3) intermediate or, (4), large size. For a detailed description see the results section. (B) Co-transfection of Dvl2-Myc and CK1ϵ or the kinase-dead CK1ϵ (K>R) mutant resulted in a predominant even or punctate distribution of Dvl2-Myc, respectively. The arrow points to a cell transfected with Dvl2-Myc (Cy3, red) with no or low levels of exogenous CK1ϵ (Cy2, green), serving as an internal control of the experiment. (C) Quantification of changes in the intracellular localization of Dvl2-Myc upon coexpression of CK1ϵ or CK1ϵ (K>R) as described in A and B. (D) D4476 (100 μM) blocks the phosphorylation dependent shift of Dvl2-Myc induced by different amounts (ng/well) of transfected CK1ϵ in SN4741 cells. The position of dephosphorylated (◀) and phosphorylated (◁) Dvl2 is indicated. Data are representative of at least three independent replicates. (E-G) SN4741 cells were transfected with Dvl2-Myc and treated as indicated: (E) The general CK1 inhibitor D4476 (100 μM) and (F) the CK1δ/ϵ-specific inhibitor IC261 (20 μM) changed the subcellular localization of Dvl2-Myc to a predominant punctate distribution. This is in contrast with Wnt-5a treatment (100 ng/ml) (G), which leads to a more even distribution of Dvl2-Myc than in control. Data in C,E,F,G ($n \geq 3$) were statistically evaluated, Data represent the mean ± s.d., one-way ANOVA with Bonferroni post-tests (*P<0.05, **P<0.01, ***P<0.001).

the presence of D4476 (Fig. 6D,E). Combined, these data suggests that Wnt-3a and Wnt-5a phosphorylate Dvl in SN4741 cells by a similar or even identical mechanism involving the activation of CK1δ/ϵ. Moreover, we show that, although Wnt-3a and Wnt-5a cooperate in the phosphorylation of Dvl, Wnt-5a can antagonise Wnt-3a-mediated induction of β-catenin. These results suggest that phosphorylated Dvl serves different functions when recruited to pathways activated by Wnt-3a or Wnt-5a.

CK1 inhibitors block the biological effects of Wnt-5a on dopaminergic precursors

To determine whether CK1 is also part of the signalling machinery mediating the pro-differentiation activity of Wnt-5a on dopaminergic precursors (Castelo-Branco et al., 2003; Schulte et al., 2005), we analysed the consequences of CK1 inhibition in rat embryonic day14.5 (E14.5) primary ventral midbrain precursor cultures. Cells were treated with Wnt-5a (100 ng/ml) with or without increasing concentrations of D4476. D4476 had no effect on the total cell number (not shown) and, in agreement with our previous results (Schulte et al., 2005), the number of tyrosine-hydroxylase-positive (TH^+) cells per field increased after Wnt-5a treatment. Importantly, we found that this effect was reduced in a dose-dependent manner upon addition of D4476 (Fig. 7). Thus, our results suggest that CK1 activity is necessary for the biological effects of Wnt-5a on primary dopaminergic cells.

Discussion

We have previously shown that Wnt-5a induces the differentiation of dopaminergic progenitors (Castelo-Branco et al., 2003; Schulte et al., 2005). This present study identifies another component mediating the function of Wnt-5a in DA neurons (DN) development. After testing a panel of small-molecule drugs and performing loss-of-function (CK1δ/ϵ-specific inhibitors and siRNA) as well as gain-of-function experiments, we identified CK1δ/ϵ as the relevant kinases hyperphosphorylating Dvl in response to Wnt-5a. Our findings place CK1δ/ϵ in a signalling pathway activated by a non-canonical Wnt and show for the first time that CK1 activity is

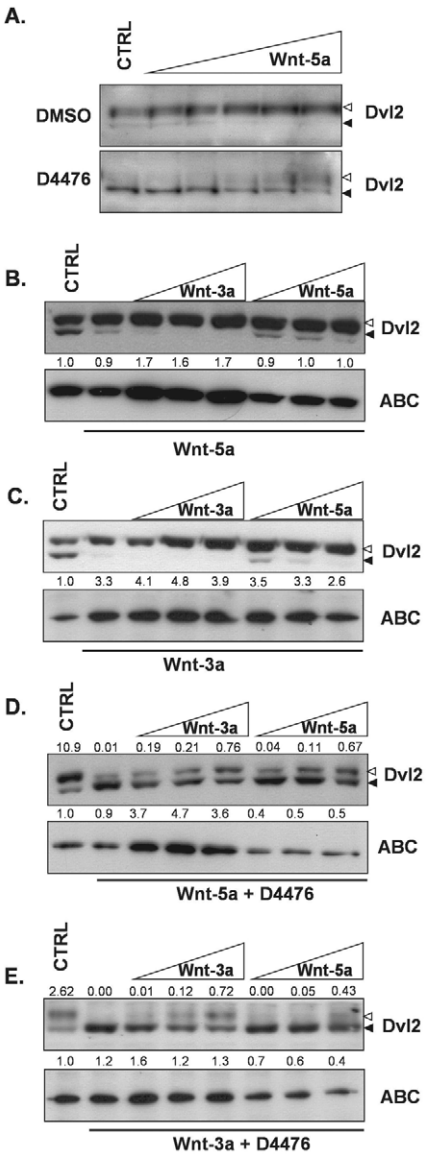

Fig. 6. Wnt-5a cooperates with Wnt-3a in the phosphorylation of Dvl2, but inhibits Wnt-3a-induced activation of β-catenin. (A) Increasing doses of Wnt-5a (50, 100, 300, 500, 1000 ng/ml) increase Dvl2 phosphorylation in vehicle (DMSO)-treated SN4741 cells and compete in the blockade of CK1 by D4476. (B-E) SN4741 cells were pretreated with 100 ng/ml of Wnt-5a (B,D) or 20 ng/ml of Wnt-3a (C,E) in the presence (D,E) or absence (B,C) of D4476 (100 μM) for 5 minutes. Subsequently, Wnt-3a (50, 100 and 200 ng/ml) or Wnt-5a (100, 200 and 500 ng/ml), was added for 2 hours. In A-E the phosphorylation of Dvl2 was detected by western blotting as phosphorylation-dependent mobility shift (dephosphorylated, ◀; phosphorylated, ◁). In B-E the activation of β-catenin (ABC) was determined using an antibody against Ser33/37-dephosphorylated β-catenin. Results shown in B-E were quantified using densitometry and either normalised to untreated control for ABC or shown as a ratio of phosphorylated:dephosphorylated for Dvl2. Duplicate experiments showed comparable results.

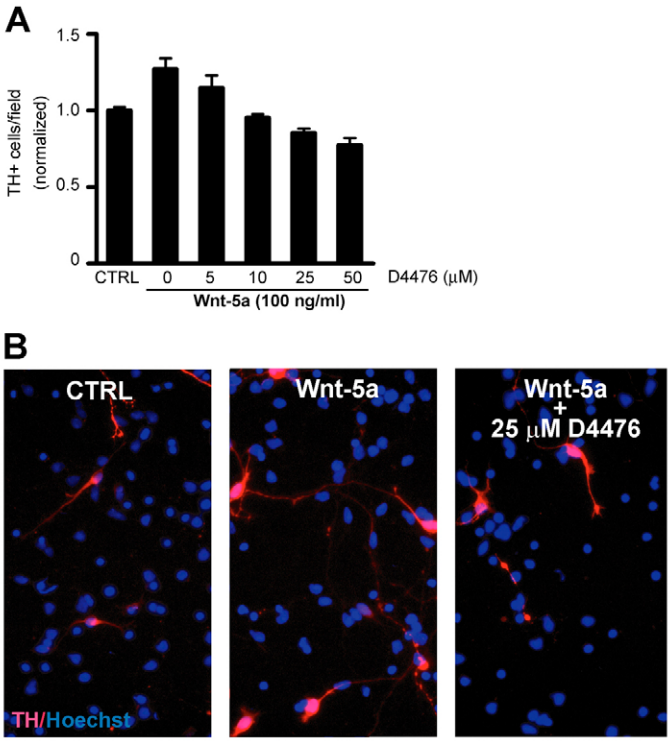

Fig. 7. CK1 inhibitors block the effects of Wnt-5a on dopaminergic differentiation. (A) Wnt-5a (100 ng/ml) increased the number of tyrosine-hydroxylase-positive (TH^+) neurons in rat E14.5 ventral midbrain precursor cultures. However, addition of increasing doses of the chemical inhibitor of CK1 (D4476) reduced the numbers of TH^+ neurons. (B) Double TH-Hoechst33258 immunostaining shows an increase in the number of TH immunoreactive neurons after treatment with Wnt-5a (100 ng/ml). Addition of 25 μM D4476 to Wnt-5a-treated cultures decreases the number of TH^+ neurons after 3 days. Bar, 25 μm.

required for a biological process induced by a non-canonical Wnt.

CK1δ/ε have previously been reported to be a Dvl-phosphorylating kinase acting in the β-catenin pathway (Gao et al., 2002; Kishida et al., 2001; McKay et al., 2001a; Peters et al., 1999; Swiatek et al., 2004). A recent report (Cong et al., 2004) describes that overexpression of CK1ε potentiates canonical Wnt signalling and diminishes JNK activation induced by Dvl1 overexpression. This finding led to the suggestion that CK1ε modulates the signalling specificity of Dvl towards β-catenin (Cong et al., 2004). Here, we report that CK1δ/ε also mediate non-canonical signalling, suggesting that canonical or non-canonical specificities are not determined by CK1ε but rather by the ligand. Moreover, the findings reported by Cong et al. could be alternatively explained by the fact that CK1ε-mediated phosphorylation diminishes the activity of axin in the MEKK1-JNK pathway at the expense of its function in canonical Wnt signalling (Zhang et al., 2002). Although the involvement of CK1δ/ε in the signal transduction of a non-canonical Wnt has not been demonstrated to date, a role of CK1δ/ε in other biological processes driven by non-canonical Wnts has been described. These include convergent extension movements in *Xenopus* (McKay et al., 2001b) and functions regulated by planar cell polarity pathway in *Drosophila* (Klein et al., 2006; Strutt et al., 2006). Thus, our data, together with published reports, support the notion that CK1ε mediates non-canonical Wnt signalling. Interestingly, a recent report by

Takada et al. suggests that, in *Drosophila* cells another CK1 isoform, CK1α is playing a similar role to the one described here for CK1δ/ε in Wnt-5a-driven phosphorylation of Dvl (Takada et al., 2005). Thus, it remains to be investigated whether the involvement of individual CK1 isoforms in Dvl phosphorylation differs among species.

Our results clearly show that both canonical Wnt-3a and non-canonical Wnt-5a induce the phosphorylation of Dvl by a common mechanism, involving the activation of CK1δ/ε. This conclusion is based on the following lines of evidence: (1) the position of hyperphosphorylated Dvl bands in Wnt-3a- and Wnt-5a-treated samples is indistinguishable; (2) the time course of Dvl phosphorylation is identical when induced by either Wnt-3a or Wnt-5a; (3) the phosphorylation of Dvl by both Wnt-5a (this study) and Wnt-3a (Bryja et al., 2007) can be blocked by CK1δ/ε siRNAs; (4) both Wnt-3a- and Wnt-5a-induced phosphorylation of Dvl is CK1 inhibition sensitive; (5) the block of Wnt-3a-induced phosphorylation of Dvl by CK1 inhibitors can be rescued by Wnt-5a and vice versa; and (6) both Wnt-5a and Wnt-3a directly induce activation of CK1ε kinase. Thus, our results comply with the possibility that Wnt-3a- and Wnt-5a-induced Dvl phosphorylation are mediated by activation of similar or identical signalling complex(es) including CK1δ/ε. The CK1δ/ε-mediated phosphorylation of Dvl is necessary for Dvl to interact with other pathway specific components – as demonstrated for the interaction of Dvl with Frat-1 in the canonical Wnt signalling (Hino et al., 2003). This view is supported by our findings, demonstrating the effects of Wnt-5a and CK1ε on the localization of Dvl2. On the basis of previous studies (Schwarz-Romond et al., 2005; Smalley et al., 2005) one can expect that Dvl2 puncta are formed predominantly by Dvl multimers. The ability of Wnt-5a and CK1ε to promote a more even localization or, in other words, to dissolve the puncta may then reflect a decrease in affinity of Dvl-Dvl interaction (Angers et al., 2006) following CK1δ/ε-mediated phosphorylation of Dvl. Such release of monomeric Dvl from Dvl aggregates might be a necessary step for the interaction of phosphorylated Dvl with other downstream components of Wnt pathway(s).

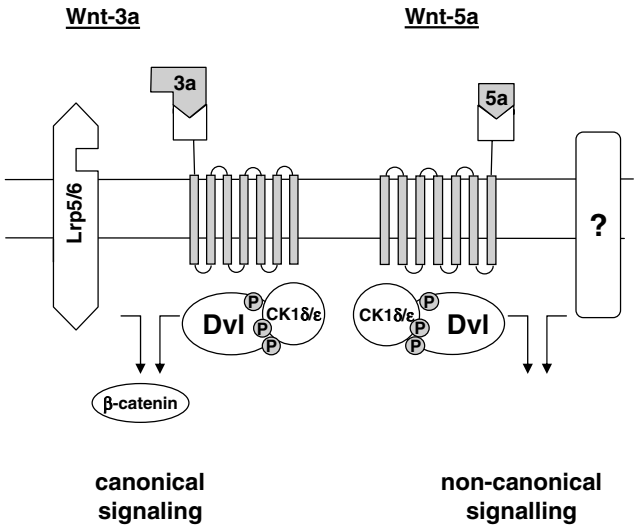

Fig. 8. Schematic model illustrating the role of CK1δ/ε in the activation of Dvl by Wnt-3a and Wnt-5a. Both Wnt-3a and Wnt-5a bind Frizzled that by unknown mechanism activates CK1δ/ε, which in turn phosphorylates Dvl. The interaction of Dvl with additional pathway-specific signalling components (including co-receptors), would direct downstream signalling. In case of Wnt-3a the co-receptor might be Lrp5/6, whereas Ror2 or Knypek might be the co-receptors for Wnt-5a. This model predicts that, when both Wnt-3a and Wnt-5a are present, Frizzled and phosphorylated Dvl will compete for binding and activation of additional signalling components. The prevalent signalling direction would thus be determined by the recruitment of the additional signalling units by phosphorylated Dvl.

It is important to notice that, although Wnt-3a and Wnt-5a cooperate in Dvl phosphorylation, Wnt-5a diminished Wnt-3a-induced activation of β-catenin. Previously, Wnt-5a has been shown to antagonise canonical signalling and different mechanisms were implicated in this process (Maye et al., 2004; Topol et al., 2003; Weidinger and Moon, 2003; Westfall et al., 2003). Our data support this concept but leave the question open of how the signal is redirected from Dvl to the final targets of canonical and non-canonical Wnt signalling pathways. It has been suggested that specific co-receptors play a role in directing the signals into different pathways and our data, demonstrating common signalling unit of canonical and non-canonical Wnt signalling, are well compatible with the crucial role of co-receptors (schematised in Fig. 8). In addition to their common cognate receptors of the Frizzled family, canonical Wnts bind to low-density lipoprotein receptor (LDLR)-related protein 5/6 (Lrp5/6) (Liu et al., 2003; Tamai et al., 2000), whereas non-canonical Wnts interact with the atypical receptor kinase Ror2 (Hikasa et al., 2002; Oishi et al., 2003) or membrane proteoglycan Knypek (Topczewski et al., 2001). A very recent report, showing that a Wnt-5a-Dkk2 CRD fusion can bind Lrp5/6 and activate canonical signalling (Liu et al., 2005), strongly supports a key role of co-receptors in directing canonical versus non-canonical Wnt-signalling.

Our results in primary precursor cultures further confirm the importance of CK1 activity for the biological effects of non-canonical Wnts. We demonstrate that CK1 activity is required for the effect of Wnt-5a on the differentiation of dopaminergic precursors into DNs. Thus, our findings argue that CK1 is an essential component of the Wnt-5a-induced signalling pathway not only in a suitable cell line but also in a more complex and biologically relevant system. This finding might also have implications in other areas of biology, such as tumour biology. Wnt-5a is known as a factor promoting cell migration, epithelial mesenchymal transition and increased cancer invasiveness (Taki et al., 2003; Weeraratna et al., 2002). Our findings, linking Wnt-5a to activation of CK1δ/ε and showing that CK1δ/ε mediate the effects of Wnt-5a, correlate well with the emerging role of CK1δ/ε in tumour development (for a review, see Knippschild et al., 2005). The generation and analysis of CK1δ- and/or CK1ε-deficient mice will certainly help to define how widespread the involvement of CK1δ/ε is in vertebrate non-canonical Wnt-signalling.

Materials and Methods

Cell culture and transfection

SN4741 cells were generously provided by J. H. Son (Son et al., 1999) and grown in DMEM, 10% FCS, L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 U/ml), glucose (0.6%) (all purchased from Invitrogen). For transfections, 40,000-60,000 cells/well were seeded in 24-well plates and grown overnight. Cells were transfected under serum-free conditions using Lipofectamine 2000 (Invitrogen)

according to manufacturer's instructions. In total, 1 μg of DNA encoding Dvl2-Myc, CK1ε or CK1ε (K>R), or their combinations, and 2 μl of Lipofectamine 2000 were used per well. Medium was changed 4 hours post transfection and cells were grown in complete culture medium for another 24 hours prior to analysis by western blot or immunocytochemistry.

Cell treatments

For analysis of intracellular signalling, 40,000 cells/well were seeded in 24-well plates, grown overnight without serum and subsequently stimulated with recombinant mouse Wnt-3a or Wnt-5a (R&D Systems) for 2 hours. Control stimulations were done with equivalent volumes of 0.1% BSA-1% CHAPS-PBS. To screen for compounds that reduce Dvl-mobility, the cells were treated with the various chemical inhibitors (Table 1) for 15 minutes and subsequently stimulated with Wnt. (Note: PTX and PDBu, were added overnight as a pre-stimulus.) Appropriate solvent was used as a control. All compounds were tested in duplicate. The cells were also exposed to FuGENE 6 transfection reagent (Roche, 1 μl FuGENE per 200 μl of culture media) to enhance the penetration of poorly cell-permeable compounds (D4476 and IC261) into SN4741 cells. Control (DMSO-treated) and experimental conditions were both treated with FUGENE. PTX, PDBu, wortmannin, genistein, chelerythrin, BIM I, SQ22536, MDL12330, AG1278, ET-18-OCH3 and staurosporine were purchased from Sigma; LY294002 and SB203580 from Tocris; PD98059 and UO126 from Cell Signaling Technology; Ro-31 8220, H89, 8-Br-cAMP, PP2, D4476 and IC261 from Calbiochem and KN93, I3M and kenpaullone from Alexis Biotechnology. 8CPT-2Me-cAMP was a kind gift from J. L. Bos (University of Utrecht, Netherlands).

Precursor cultures

Embryonic day 14.5 (E14.5) ventral mesencephala obtained from time-mated Sprague-Dawley rats (ethical approval for animal experimentation was granted by Stockholm Norra Djurförsöks Etiska Nämnd) were dissected, mechanically dissociated and plated on plates coated with poly-D-lysine (10 μM) at a final density of 1×10^5 cells/cm^2. Serum-free N2 medium was added, consisting of a mixture of F12 and MEM with N2 supplement, 15 mM HEPES buffer, 1 mM glutamine, 5 mg/ml Albumax (all purchased from Invitrogen) and 6 mg/ml glucose (Sigma). Recombinant Wnt-5a and D4476 (Calbiochem) were added and the cells were cultured for 3 days in a 37°C, 5% CO_2 incubator. Cells were fixed for immuncytochemistry in ice-cold 4% paraformaldehyde for 15-20 minutes and washed in PBS. The following primary and secondary antibodies were used: rabbit anti-tyrosine hydroxylase specific antibody (1:100 dilution, Pel-Freez Biologicals) and rhodamine-coupled goat anti-rabbit IgG (1:200; Jackson Laboratories). Cultures were subsequently incubated with Hoechst 33258 reagent for 10 minutes. Images were acquired from stained cells using a Zeiss Axioplan 100M microscope (LD Achroplan 40×, 0.60 Korr PH2 8 0-2) and collected with a Hamamatsu camera C4742-95 (with QED imaging software). TH-immunoreactive cells from two independent experiments, three wells per condition, nine non-overlapping fields per well were counted independently by two researchers. The numbers of TH^+ cells represent the mean values ± s.d. and are expressed as percentage change compared with control.

Western blotting

Western blot analysis and protein samples were prepared as described previously (Bryja et al., 2004). The antibodies used were: anti-Dvl2 (sc-13974), anti-Dvl3 (sc-8027) and anti-c-Myc (sc-40) (Santa Cruz Biotechnology); anti-β-catenin (BD Biosciences); anti-active-β-catenin (anti-ABC, Upstate Biotechnology); anti-phospho-β-catenin (S45, Biosource) anti-phospho-serin (AB1603, Chemicon International) and anti-MBP (Dako).

Immunoprecipitation and kinase assay

For kinase assays, total protein from the cells (cultured in 10-cm dishes) was extracted and processed using Protein G Sepharose fast-flow beads (Amersham Biosciences) as described previously (Bryja et al., 2005). Rabbit polyclonal antibody against Myc (sc-789), mouse agarose-conjugated antibody against Myc (sc-40 AC) and goat polyclonal antibody against CK1ε (sc-6471) were used in the immunoprecipitation studies (Santa Cruz Biotechnology). CK1ε kinase reactions were carried out for 15 minutes at room temperature in a 40-μl volume of kinase-assay buffer (50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 20 mM β-glycerolphosphate, 5 mM NaF) supplemented with 100 μg/ml MBP (M1831, Sigma) and 100 μM ATP. Reactions were terminated by addition of 5× Laemmli sample buffer. Each reaction mix was then subjected to SDS-PAGE.

RNA interference and quantitative RT-PCR

SN4741 cells were transfected with siRNA using neofection according to manufacturer's instructions (Ambion). In brief, siRNAs (0.75 μl of 20 μM siRNA) were mixed with Lipofectamine 2000 (2 μl; Invitrogen) and OptiMEM (47.25 μl; Gibco) and incubated for 20 minutes at room temperature. The transfection mixture (50 μl) was added to the 24-well plate and mixed with a suspension of freshly trypsinised SN4741 cells (25,000 cells/well in 500 μl of complete media) resulting in the final concentration of 30 nM siRNA. When a combination of two different siRNAs was used, each siRNA was used at 30 nM and the control siRNA at 60 nM. The transfection was terminated after 5 hours by changing culture media. At 48 hours post transfection, cells were stimulated with Wnt-5a and collected for further analyses. siRNAs against individual isoforms of mouse CK1 were purchased from Ambion; CK1α (I, cat. no. 176063; II, cat. no. 176062; III, cat. no. 176061), CK1δ (I, cat. no. 88202; II, cat. no. 88309; III, cat. no. 88298) and CK1ε (I, cat. no.188527; II, cat. no. 188528, III , cat. no. 188529). Silencer® Negative Control siRNA (cat. no. 4635, Ambion) was used as a negative control. The efficiency of the silencing was assessed by western blotting or real time RT-PCR. Quantitative RT-PCR was performed as described previously (Castelo-Branco et al., 2006) The following primers were used (DNA Technology A/S, Aarhus, Denmark): $CK1\alpha_{for}$ 5′-TTTGAGGAAGCTCCGGATTACAT-3′, $CK1\alpha_{rev}$ 5′-TCGTCCAATCAAACGTG-TAGTCAT-3′, $CK1\delta_{for}$ 5′-ACATCTATCTCGGTACGGACATTG-3′, $CK1\delta_{rev}$ 5′-GAGGATGTTTGGTTTTGACACATTC-3′.

Confocal imaging

SN4741 cells (20,000-40,000 cells/well) were grown overnight on glass coverslips and transfected with the indicated plasmids. Treatment with chemical inhibitors or Wnt-5a was performed at 4 hours or 9 hours post transfection. 24 hours post transfection cells were fixed in 4% paraformaldehyde for 15 minutes. For immunodetection cells were washed three times in PBS and blocked with 1% BSA, 0.1% Triton X-100 in PBS for 1 hour. The primary antibodies (see western blotting section) were incubated for 3 hours at room temperature and subsequently the appropriate secondary antibodies coupled to Cy3 or Cy2 (1:500, Jackson Immunoresearch) were applied for 2 hours at room temperature. After washing, coverslips were mounted on slides using glycerol gelatine-mounting medium (Sigma-Aldrich). Fluorescent labelling was examined using a Zeiss LSM510 confocal system including a Zeiss Axioplan2 microscope equipped with filters for the detection of Cy2 and Cy3.

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