Casein Kinase 2 Is Activated and Essential for Wnt/β-Catenin Signaling*

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Wnt/β-catenin signaling is essential to early development. Activation of Frizzled-1 by Wnts induces nuclear accumulation of β-catenin and activation of Lef/Tcf-dependent gene expression. Casein kinase 2 has been shown to affect Wnt/β-catenin signaling. How casein kinase 2 exerts an influence in Wnt signaling is not clear; casein kinase 2 has been reported to be constitutively active (i.e. not regulated). Herein we show to the contrary that casein kinase 2 activity is rapidly and transiently increased in response to Wnt3a stimulation and is essential for Wnt/β-catenin signaling. Chemical inhibition of casein kinase 2 or suppression of its expression blocks Frizzled-1 activation of Lef/Tcf-sensitive gene expression. Treatment with pertussis toxin or knock down of Gs blocks Frizzled-1 activation of Lef/Tcf-sensitive gene expression. Thus, casein kinase 2 is shown to be regulated by Wnt3a and essential to stimulation of the Frizzled-1/β-catenin/Lef-Tcf pathway.

Wnt signaling regulates essential aspects of early development, including cell fate determination, embryonic patterning, and cell proliferation (1, 2). Wnt ligands are palmitoylated glycoproteins (3) that are secreted and bind to cell surface hepthelical receptors termed “Frizzleds” (4, 5). Frizzleds are members of the superfamily of G protein-coupled receptors (6, 7). For the “canonical” Wnt pathway in mammalian cells, Wnt3a binds to Frizzled-1, activating heterotrimeric G proteins (e.g. Gq and Gs) and the phosphoprotein Dishevelled (Dvl) (6). In Drosophila, activation of the canonical pathway or the planar cell polarity pathway likewise requires Wnt activation of Frizzled-1, activation of Gs and downstream Dishevelled (Dsh) (8). Activation of Dsh/Dvl inhibits glycogen synthase kinase 3β and its ability to phosphorylate and thereby destabilize β-catenin. The Wnt3a-stimulated elevation of intracellular β-catenin leads to nuclear accumulation of β-catenin and to activation of Lef/Tcf-dependent gene expression (9).

Casein kinase 2 (CK2), a family of protein kinases highly conserved in nature, has been reported to regulate many cellular processes, including gene expression, protein synthesis, cell proliferation, and apoptosis (10–13). Several key components of the Wnt/β-catenin/Lef-Tcf pathway are known substrates of CK2 in vitro, including Dsh/Dvl (14), the product of the adenomatous polyposis coli gene (APC) (15), transcription elements Lef/Tcf (16, 17), Engrailed (18), and β-catenin (19). CK2 has been shown to act as a positive modulator of Wnt/β-catenin/Lef-Tcf pathway, suppressing β-catenin degradation and β-catenin binding to APC (19). Overexpression of CK2 in ventral blastomeres of Xenopus embryos stimulates dorsal axis formation, whereas expression of a kinase-dead CK2 mutant blocks ectopic axis formation in response to Xwnt8 (20).

Reported as “constitutively active” in cells, CK2 has been suggested to play an important, but passive, role in signaling (21). The ability of overexpression or inhibition of CK2 to affect aspects of early development suggests a pivotal role of CK2 in Wnt/β-catenin/Lef-Tcf pathway (20) but provides no insight as to whether the effects of CK2 are the result of “activation.” Our hypothesis is that the role of CK2 is not “passive” and that of a constitutively active protein kinase but rather that of a kinase regulated by upstream signaling. In support of this hypothesis are the observations that CK2 activity may be sensitive to phosphatidylinositol turnover (22) and to phosphorylation by non-receptor tyrosine kinases (23). These data piqued our interest in investigating whether or not CK2 was activated by Wnt stimulation. The results show that CK2 is activated by stimulation of Wnt3a operating via Frizzled-1, heterotrimeric G proteins, and Dishevelled-2 in the canonical Wnt/β-catenin/Lef-Tcf pathway of mammalian cells.

EXPERIMENTAL PROCEDURES

Cell Culture—The mouse F9 teratocarcinoma (F9) cells (from ATCC, Manassas, VA) were grown in Dulbecco’s modified Eagle’s medium supplemented with 15% heat-inactivated fetal bovine serum (Hyclone, South Logan, UT) at 37 °C in a 5% CO2 incubator. F9 clones stably expressing rat Frizzled-1 (Rfz1), β2-adrenergic receptor/rat Frizzled-1 (β2AR/Rfz1) chimera, or β2-adrenergic receptor/rat Frizzled-2 (β2AR/Rfz2) chimera were grown and selected in the presence of 100 ng/ml of the neomycin analogue G418 as previously described (24).

Immunoprecipitation and Immunoblotting—F9 cells grown in monolayer were serum starved for 8 h prior to stimulation by either Wnt3a (10 ng/ml) or isoproterenol (ISO, 10 μM) for the periods indicated. Cells were harvested in lysis buffer containing protease and phosphahte inhibitors (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 200 μM phenylmethylsulfonyl fluoride, 10 μg/ml of aprotinin, 10 μg/ml of leupeptin, 10 mM NaF, 1 mM Na3VO4, and 100 mM okadaic acid). Samples (2 mg of protein) of whole-cell lysates were incubated with 8 μl of CK2α antibody (Santa Cruz Biotechnology, Santa Cruz, CA) immobilized on protein A/G-Sepharose. Immunocomplexes were washed with lysis buffer three times and then solubilized in Laemmli’s solution (25). Whole-cell lysates and samples from immunoprecipitation were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred to nitrocellulose blots electro-
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RESULTS

CK2 Is Activated by Stimulation of the Wnt/β-Catenin/Lef-Tcf Pathway—Mouse F9 embryonic totipotent teratocarcinoma cells were stably transfected with the βAR/Rfz1 chimeric receptor, a chimera that can activate the Wnt/β-catenin/Lef/Tcf canonical pathway by activation of the receptor with the β-adrenergic agonist ISO (10 μM). The ability to quantify chimeric receptor expression and to activate the Frizzled-1 downstream pathway was the basis for selecting these clones for the initial examination of possible CK2 activation. Treating the F9 βAR/Rfz1 stable transfectants with isoproterenol provoked a sharp stimulation of Lef/Tcf-dependent transcription at 5–6 h poststimulation of the Frizzled-1 pathway. Activation of Frizzled-1 likewise failed to stimulate CK2 activity (Fig. 1B). This stimulation by Frizzled-1 provoked an increase in CK2 activity within 15 min of treatment with β-agonist. CK2 activity remained elevated (~50%) for 30 min and thereafter declined to baseline values within 60 min of Frizzled-1 stimulation. Immunoblotting of CK2 displays no corresponding changes in the cellular level of this protein kinase (Fig. 1B, lower panel).

Wild-type F9 cells and stably transfected F9 clones expressing either the βAR/Rfz1 (βAR/Rfz1) or the βAR/Rfz2 (βAR/Rfz2) chimeric receptors were stimulated for 30 min and the CK2 activity then measured (Fig. 1C). Only those clones expressing the βAR/Rfz1 chimera displayed activation of CK2 in response to stimulation of the Frizzled-1 pathway. Stimulation of the wild-type F9 cells, in contrast, failed to stimulate CK2 activity. Stimulation of the Frizzled-2 pathway that leads to changes in the intracellular levels of Ca2+ and cyclic GMP (29) likewise failed to stimulate CK2 activity.

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A + ISO

B

ISO

CK2 Activity (fold)

0 15 30 60

IP: CK2x

IB: CK2x

C

D

Wnt3a

E

F

Wnt3a

WT

ATE9251

HEK293

WT

NIH3T3

The observations derived from the study of F9 clones expressing the βAR/Rfz1 chimera were tested further using F9 clones expressing bona fide rat Frizzled-1 (Fz1) and treated with purified, active Wnt3a (Fig. 1D). Treating the clones expressing authentic Fz1 with purified Wnt3a provokes a rapid increase in CK2 activity, ~60% increase within the first 30 min. The activation of CK2 activity in response to Wnt3a appears to peak at 15–30 min poststimulation, declining to near basal levels within 60 min. The ability of Wnt3a to stimulate CK2 activity was also measured in human embryonic kidney 293 and mouse NIH3T3 cells transiently transfected to express Fz1; both cell lines responded as do the F9 cells (Fig. 1, E and F). Thus, activation of Frizzled-1 with Wnt3a stimulates a rapid but transient increase in the activity of CK2, confirming and extending the data obtained in clones expressing the β2AR/Rfz1 chimera (compare Fig. 1, B and D).

CK2 Inhibitors Block Wnt/β-Catenin/Lef-Tcf Signaling—To test the linkage between Wnt activation of the Wnt/β-catenin/Lef-Tcf signaling and the role of CK2, we explored the effects of chemical inhibitors of CK2 on the Lef/Tcf-dependent transcriptional response to stimulation either of the β2AR/Rfz1 chimera or of Frizzled-1 (Fig. 2). Apigenin (4′,5,7-trihydroxyflavone) inhibits CK2 activity (30). Treating F9 clones with apigenin (20 μM) leads to a dramatic inhibition of Lef/Tcf-dependent transcription in response to activation of the β2AR/Rfz1 chimera by β-agonist (Fig. 2A). Measured over 7 h of activation of the β2AR/Rfz1 chimera, Lef/Tcf-dependent transcription remained at essentially baseline in the presence of this CK2 inhibitor. The inhibition of β2AR/Rfz1 chimera-stimulated Lef/Tcf-dependent transcription by apigenin was dose dependent, with 10 μM concentrations of this flavone derivative capable of inhibiting the response by ~50% (Fig. 2B). The Frizzled-1 chimera-mediated stimulation of the Lef/Tcf-dependent transcription was nearly abolished by 15–20 μM apigenin (Fig. 2B).

In F9 clones stably expressing the rat Frizzled-1, apigenin was found to suppress the ability of Wnt3a to activate Lef/Tcf-dependent transcription (Fig. 2C). Similar experiments with 2-dimethylamino-4,5,6,7-tetramethylbenzimidazole (DMAT), a highly selective and potent CK2 inhibitor (31), displayed the same ability to block Frizzled-1-dependent activation of the Lef/Tcf-dependent transcription (Fig. 2D). Chemical inhibitors for other signaling kinases (e.g. protein kinase C and protein kinase A) were without effect (data not shown), suggesting a critical role of CK2 in the Wnt/β-catenin/Lef-Tcf response. We tested further the effects of inhibition of CK2 activity on Wnt3a-stimulated β-catenin stabilization in F9 clones expressing rat Frizzled-1 (Fig. 2E). F9 clones treated with Wnt3a displayed a time-dependent stabilization and accumulation of intracellular β-catenin. Wnt3a stimulated a 10-fold increase in free β-catenin levels within 4 h of stimulation. The Wnt3a-stimulated accumulation of β-catenin was markedly attenuated in the clones treated with apigenin. Quantification of the levels of β-catenin in response to Wnt3a stimulation in the absence and presence of apigenin confirms the data obtained using Lef/Tcf-dependent transcription as a read-out (Fig. 2, compare panels A and F). These results suggest that CK2 acts upstream of β-catenin to activate Lef/Tcf-dependent transcription.

Suppression of CK2α Subunits Attenuates Activation of Wnt/β-Catenin/Lef-Tcf Signaling—The CK2 holoenzyme is composed of two catalytic subunits (αα′, αα′′, or α′α′′) paired with two non-catalytic β subunits. CK2 catalytic subunits as well as their holoenzymes are enzymatically active. The functions of α and α′ are not completely redundant, because CK2α′ null mice are sterile and display a defect in spermatogenesis (35). In view of the ability of chemical inhibitors of CK2 to block Wnt3a signaling via the canonical pathway, we investigated the effects of suppressing expression of CK2 subunits. We adopted a knockdown (KD) strategy that makes use of unique siRNAs to suppress expression of individual subunits. Cells were treated
with siRNA targeting specific subunits CK2α, CK2α', and CK2β, and Wnt3a-stimulated Lef/Tcf-sensitive transcription was assayed (Fig. 3, Table 1). The siRNA designed for each subunit was found to be effective in suppressing the expression of the targeted subunit (Fig. 3B, Table 1). KD produced by siRNA treatment was effective as determined by immunoblotting.

TABLE 1

| siRNA     | Effect on transcription | Protein level |
|-----------|-------------------------|---------------|
|           | CK2α       | CK2α' | CK2β   |
| Control   | %          | %     | %      |
| CK2α      | 100        | 100   | 100    |
| CK2α'     | 35         | 36    | 118    | 45    |
| CK2β      | 64         | 103   | 54     | 93    |
| CK2α/α    | 99         | 100   | 92     | 42    |
| CK2α/α'   | 42         | 41    | 56     | 42    |
selective chemical inhibitors of CK2. Treating cells with siRNA targeting CK2β, in contrast, had no effect on the ability of Wnt3a to activate Lef/Tcf-sensitive transcription. These data demonstrate that CK2α subunits are essential in the signaling from Wnt3a to the canonical pathway as measured by activation of Lef/Tcf-sensitive gene expression by Wnt3a.

**Heterotrimeric G Proteins Mediate Wnt Activation of CK2**

We examined signaling downstream of Frizzled-1 in the activation of CK2. Frizzled-1 signaling has been shown previously to require activation of heterotrimeric G proteins in mammalian cells, Xenopus, and zebrafish embryos, as well as in Drosophila (32). Both Gαq and Gαo are required for stabilization of β-catenin as well as activation of Lef/Tcf-dependent transcription in canonical Wnt/β-catenin signaling pathway (6). Pertussis toxin catalyzes ADP ribosylation on the Gαi/o family of G proteins and inhibits their functions. To test whether G protein-mediated CK2 activation in Wnt/Frizzled-1 signaling, we pretreated F9 cells with pertussis toxin. Pertussis toxin completely blocked CK2 activation in βAR/Rfz1-expressing cells stimulated by β-agonist (Fig. 4A). The result indicated that activation of one or more members of the pertussis toxin-sensitive Gα family (Gαq, Gαo, Gα12) of G proteins was necessary for CK2 activation.

To test directly which G protein mediates CK2 activation in Wnt/Frizzled-1 signaling, we used antisense oligodeoxynucleotides (ODN) to knock down specific Gα subunit expression in F9 clones (6). F9 clones expressing βAR/Rfz1 chimera were treated with antisense ODN targeting Gαq, Gαo, or Gα12 for 72 h. Following the KD of individual Gα subunits, the Frizzled-1 chimera-expressing clones were stimulated with β-agonist for 15 min and CK2 activity assayed. Knock down of either Gαq, Gαo, or Gα12 blocks the activation of CK2 following stimulation of the Frizzled-1 pathway (Fig. 4A). KD of Gα12, in contrast, did not affect the ability of the Frizzled-1 chimera to activate CK2. We tested the ability of KD of Gαq, Gαo, and Gα12 to impact Frizzled-1 stimulation of Lef/Tcf-dependent transcription in these same cells (Fig. 4B). KD of Gαq or Gαo, but not Gα12, blocks not only CK2 activation but also the Lef/Tcf reporter gene response to stimulation (Fig. 4B). Immunoblot analysis of the G protein subunits demonstrates that antisense ODNs targeting specific G protein subunits were effective in selective suppression of the expression of Gαq, Gαo, and Gα12 (Fig. 4C). These results show that Gαq and Gαo are required for CK2 activation and Lef/Tcf-dependent transcription in response to Wnt.

To further probe the role of Gαq and Gαo in CK2 activation, constitutively active forms of G proteins were expressed in the F9 clones. Q209LGαq (QLGαq), Q205LGαo (QLGαo), and Q209LGα12 (QLGα12) mutants are deficient in the GTPase activity intrinsic to α subunits of heterotrimeric G proteins that is responsible for “turning off” Gα subunit activity. The G protein mutant versions were expressed transiently in F9 cells, and the CK2 activity was measured (Fig. 4D). Expression of Q209LGαq stimulates constitutive activation of CK2 in the absence of Frizzled-1 activation (Fig. 4D). Expression of Q205LGαo also stimulates constitutive activation of CK2. Expression of constitutively active Q209LGα11, in sharp contrast to expression of the Gαq and Gαo mutants, had no such effect on CK2 activity. The expression of mutant Gαo subunits, which contain internal glutamate-glu- mate epitope (EE tagged), was essentially equivalent as determined by immunoblotting of whole-cell lysates by using antibody against EE tag (data not shown). Expression of Q209LGαq or Q205LGαo, but not Q209LGα11, constitutively activated not only CK2 but also Lef/Tcf-dependent transcription as determined with the luciferase reporter gene (Fig. 4E). The constitutive activation of Lef/Tcf-dependent transcription observed in response to expression of either the constitutively active Q209LGαq or Q205LGαo mutant was abolished by treatment with the CK2 inhibitor apigenin (Fig. 4E). Constitutive activation of CK2 itself, observed in response to expression of the constitutively active Q209LGαq, or Q205LGαo mutants, also was blocked by treatment with apigenin (data not shown). These results demonstrate that Wnt activation of CK2 is mediated by Gαq and Gαo.

**Role of Dvl-2 in CK2-dependent Activation of Wnt/β-Catenin Signaling**

Dvl is a downstream element of the Wnt3a/Frizzled/G protein pathway to activation of Lef/Tcf-dependent transcription (8). As observed earlier (14, 37), Dvl-2 is phosphorylated in F9 cells stimulated with Wnt3a (Fig. 5A). Treated with the CK2 inhibitor DMAT.
Phosphorylation of Dvl-2 was abolished in the presence of DMAT. A representative immunoblot for Dvl-2 was performed on the lysates. A major phosphorylated Dvl-2 band migrating slightly more slowly than Dvl-2 was observed in response to Wnt3a stimulation. After stimulation with Wnt3a for the indicated times, cells were lysed and immunoblots were stained with antibodies against Dvl-2, CK2α, and actin level in the cell lysate were subjected to SDS-PAGE and immunoblotting. The means values from three separate experiments. The knock down of Dvl-1, Dvl-2, or none control for Wnt3a stimulation by treatment with siRNA was determined in whole-cell lysates by SDS-PAGE and immunoblotting (lower panel). Immunoblotting of β-catenin in whole-cell lysates was performed as a loading control.

**FIGURE 5.** Wnt activation of CK2 requires Dishevelled-2. A, Wnt3a stimulation of Dvl-2 phosphorylation is blocked by inhibition of CK2 with DMAT. F9 cells expressing rat Frizzled-1 were pretreated with DMAT or not (control) for 30 min, followed by Wnt3a stimulation. After stimulation with Wnt3a for the indicated times, cells were lysed and immunoblots were performed on the lysates. A major phosphorylated Dvl-2 band migrating slightly more slowly than Dvl-2 was observed in response to Wnt3a stimulation. Phosphorylation of Dvl-2 was abolished in the presence of DMAT. A representative image is presented. B, overexpression of Dvl-2 stimulates Lef/Tcf-dependent transcription, mediated by CK2α. Cells were transfected with either Dvl-2 (pTAP-Dvl-2) and Lef/Tcf-sensitive luciferase reporter (pSuper8XTOPFLASH) or the reporter pSuper8XTOPFLASH alone. The activity of luciferase reporter gene was determined 24 h after the transfection. C, TAP-Dvl-2, CK2α, and actin level in the cell lysate were subjected to SDS-PAGE and immunoblotting. The means values from three separate experiments. The knock down of Dvl-1, Dvl-2, or none control for Wnt3a stimulation by treatment with siRNA was determined in whole-cell lysates by SDS-PAGE and immunoblotting (lower panel). D, knock down of Dvl-2 expression by siRNA treatment for 24–48 h decreased Dvl-2 phosphorylation (Fig. 5A) as well as Lef/Tcf-sensitive transcription (Fig. 2D) in response to stimulation by Wnt3a. Overexpression of Dvl was shown to activate the transcription (33, 34). We wonder whether Dvl plays a similar role in CK2-dependent activation of Wnt/β-catenin signaling. Transient expression of a TAP-tagged Dvl2 activates Lef/Tcf-dependent transcription (Fig. 5B). Knock down of CK2α in F9 cells by siRNA treatment resulted in a sharp decrease in Dvl2-stimulated Lef/Tcf-sensitive transcriptional activation (Fig. 5B), indicating that CK2 is downstream of Dvl and essential for Dvl to activate Lef/Tcf-dependent transcription. Knock down of Dvl-2 expression by siRNA treatment for 24–48 h decreased Dvl-2 >90% (Fig. 5C). The Wnt3a-stimulated CK2α activation was blocked by knocking down Dvl-2 (Fig. 5C). Knock down of Dvl-1, in contrast, had no effect on the ability of Wnt3a to stimulate CK2 activity (Fig. 5D). Thus, Wnt3α activation of CK2 requires Frizzled-1, Gαq/11, and Dvl-2 in a vectorial arrangement.

**DISCUSSION**

CK2 has been shown to induce dosalization and axis formation in Xenopus embryos, mimicking the effects of XWnt8, Dvl, and β-catenin stimulation, and is hypothesized to play a role in Wnt/β-catenin pathway (20). CK2 can phosphorylate β-catenin at Thr393 in the armadillo repeat region of β-catenin in mammalian cells in vitro, leading to stabilization and accumulation of β-catenin and to co-transcriptional activation of Lef/Tcf-sensitive gene expression (19). How CK2 exerted its role upon Wnt stimulation, however, was insufficiently understood because of its ubiquitous expression and results suggesting CK2 to be
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“constitutively active”, i.e. an “unregulated” enzyme (11). Our study is the first report that CK2 activity is regulated acutely in response to stimulation of Wnt3a. We have demonstrated that Wnt stimulates transient activation of CK2 and that the CK2 response is obligate for Wnt-stimulated accumulation of β-catenin and activation of Lef/Tcf-dependent transcription in canonical pathway (Fig. 6). The CK2 activation is mediated by the heterotrimeric G proteins Gq and Go and requires the downstream signaling phosphoprotein Dishevelled-2. Suppression of the expression of CK2α subunits, Gαq or Gαo, or of Dvl2, like chemical inhibition of this kinase, inhibited the activation of CK2 activity as well as β-catenin accumulation and co-activation of transcription in response to Wnt3a stimulation. Whether the change in CK2 activity reflects a change in intrinsic activity or the removal of negative regulators of CK2 activity remains an open question.

It has been proposed that β subunit of CK2 confers the substrate specificity (11). The α and β subunits of CK2 have been reported to be necessary in tandem to induce dual axis formation in Xenopus (36). Microinjection of either CK2 subunit alone did not affect axis development (36). Our results from siRNA treatments to specifically transient activation of CK2 and that the CK2 response is obligate for Wnt-stimulation of Wnt3a. We have demonstrated that Wnt stimulates tran-

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