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Rapid, Wnt-Induced Changes in GSK3β Associations that Regulate β-Catenin Stabilization Are Mediated by Gα Proteins

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Summary

Background: In the absence of Wnt stimulation, the transcriptional cofactor β-catenin is destabilized via phosphorylation by protein kinase GSK3β in complex with Axin family members. In the "canonical" Wnt signaling pathway, Disheveled (Dvl) is required to functionally inhibit the activity of the GSK3β/Axin complex and thereby stabilize β-catenin. Yet, the mechanisms that underlie Wnt regulation of GSK3 and stabilization of β-catenin are still not fully appreciated.

Results: Here, we examine time-dependent changes in protein-protein interactions that occur in response to Wnt treatment. We show that GSK3β/Axin complexes are rapidly ($t_{1/2}$ < 3 min) disrupted upon Wnt stimulation and that changes in GSK3β/Axin association substantially precede both β-catenin stabilization and Axin degradation. We further demonstrate that depletion of $G\alpha_o$ or $G\alpha_q$ will inhibit, respectively, the Wnt-induced disruption of GSK3β/Axin2 and GSK3β/Axin complexes and diminish Wnt stabilization of β-catenin. We also show that direct activation of G proteins in vivo with GTPγS in the absence of exogenous Wnt will disrupt GSK3β/Axin2 complexes and stabilize β-catenin. Finally, we demonstrate an association of $G\alpha_o$ with Fz that is also very rapidly ($t_{1/2}$ < 1 min) perturbed upon Wnt-3a stimulation and that the Wnt-dependent effects on both GSK3β/Axin2 and $G\alpha_o$/Fz are pertussis-toxin sensitive. Collectively, these data implicate a role for G proteins in the regulation of Wnt-mediated protein-protein interactions and signaling to β-catenin.

Conclusions: We conclude that rapid disruption of GSK3β/Axin interactions in response to Wnt leads to the initial stabilization of β-catenin and that $G\alpha_o$ and $G\alpha_q$ signaling contributes to Wnt-mediated GSK3β/Axin disruption and the ultimate stabilization of β-catenin.

Introduction

β-catenin is a transcriptional cofactor with essential regulatory functions during development [1]. In complex with the scaffolding protein Axin, β-catenin is phosphorylated by CK1α, priming it for additional phosphorylations by GSK3β [2, 3]. Polyphosphorylated β-catenin is then subject to proteasomal destruction [4, 5]. The Wnts define a family of lipid-modified, glycoproteins [6] that can function as extracellular factors to regulate β-catenin stability [1]. In the "canonical" Wnt pathway, GSK3β is functionally inhibited, promoting the stabilization of β-catenin. Complexes of β-catenin and Lef/Tcf transcription factors serve as nuclear [7–9] activators of gene sets that promote specific developmental pathways or tumorigenesis [1].

Wnt signals are initially transduced by coreceptors of seven-transmembrane Frizzled (Fz) [10, 11] and single-pass, LDL receptor-related proteins (LRP) 5/6 [12–14]. Additional components are also involved (see [1]). Dishevelled (Dvl/Dsh) functions downstream of Fz but upstream of GSK3 to mediate β-catenin stabilization [15–17], whereas Adenomatous Polyposis Coli (APC) in complex with GSK3β/Axin facilitates β-catenin degradation [1].

The immediate molecular events that transmit Wnt signals are not well characterized. According to postulated models, GSK3β/Axin complexes become disassembled upon Fz activation (see [1]). Wnt stimulation thus decreases GSK3β phosphorylation of both Axin and β-catenin. Unphosphorylated Axin is subject to proteasomal degradation, in contrasted to β-catenin, thereby reducing GSK3β/Axin complex formation [18–20], and it has been argued that Axin degradation participates as a primary element of the Wnt response pathway for stabilizing β-catenin [20, 21].

The Fz receptors are structurally related to G protein-coupled receptors, leading to early speculation that heterotrimeric Gαβγ protein signaling may mediate Wnt response. Although Fz receptors may interact with G proteins for Ca^{+2} signaling [22–24], a noncanonical Wnt pathway, evidence linking G protein signaling and β-catenin regulation in the canonical pathway has been less clear. Studies using chimeric Fz receptors that are activated by β-adrenergic analogs, but that signal to β-catenin, implicate G proteins in Wnt signaling during development [25]. In mammalian cells, overexpression of constitutively active $G\alpha_o$ or $G\alpha_q$ will promote Tcf-dependent gene expression, a downstream component of the Wnt/β-catenin pathway [26]. Recent epistatic experiments in *Drosophila* also suggest that $G\alpha_o$ can function downstream of Fz but upstream of Dsh during Wnt regulation of GSK3/β-catenin signaling [27] and that activated $G\alpha_o$-GTP can bypass a requirement for Fz receptor activation in this pathway [27]. Still, definitive biochemical evidence for G protein-dependent regulation of β-catenin signaling in native cells has been elusive.

We have explored the immediate molecular responses of native, untransfected cells to Wnt stimulation and demonstrated a rapid ($t_{1/2}$ < 3 min) disruption of GSK3β/Axin complexes that significantly precedes β-catenin stabilization and degradation of Axin. We also observe an initial release of GSK3β to the cytosol, without an enhanced interaction with Dvl. These data allowed us to examine the dependency of an immediate Wnt response on heterotrimeric G protein signaling. We show that depletion of both $G\alpha_o$ and $G\alpha_q$ protein

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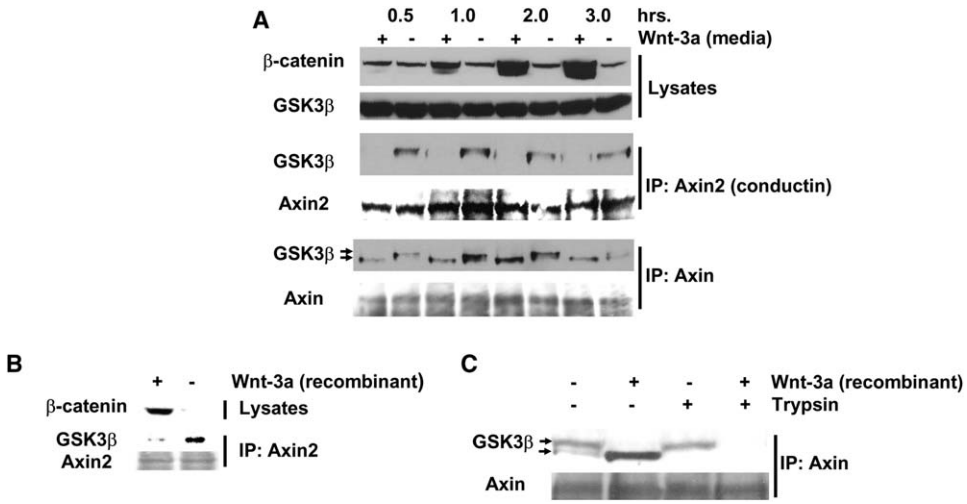

Figure 1. Wnt-3a Regulates Axin/GSK3 Complexes and β-Catenin Stability in L929 Cells

(A) L929 whole-cell lysates were prepared from cells cultured in conditioned media with or without Wnt-3a for various times. Total β-catenin and GSK3β levels were measured directly by Western blot assay. GSK3β levels were measured directly or following immunoprecipitation (IP) by α-Axin2 or α-Axin. Arrows indicate Wnt-regulated mobility differences for GSK3β in association with Axin. Immunoblot (IB) controls were performed for each IP. Data are representative of two separate experiments. (see also Figures 2 and 4–7).

(B) L929 whole-cell lysates were prepared from cells cultured in the presence or absence of recombinant Wnt-3a (200 ng/ml) for 1 hr. Total β-catenin levels were measured directly by Western blot assay. GSK3β levels were measured following immunoprecipitation by α-Axin2. Immunoblot (IB) controls were performed for each IP. Data are representative of three separate experiments.

(C) L929 whole-cell lysates were prepared from cells cultured in the presence or absence of recombinant Wnt-3a (200 ng/ml) for 1 hr. Cleared lysates were immunoprecipitated with α-Axin and immunoprecipitates analyzed for GSK3β and Axin levels directly (−) by immunoblot or following a very mild trypsin (25 μg/ml for 2 min at 20°C) treatment (+). Arrows indicate Wnt-regulated mobility differences for GSK3β in association with Axin. Immunoblot controls were performed for each IP. Data are representative of three separate experiments.

using siRNA technology significantly inhibits the ability of exogenous Wnt to disrupt GSK3β/Axin complexes and to stabilize β-catenin. In addition, we document the disruption of GSK3β/Axin2 complexes and the stabilization of β-catenin in the absence of Wnt stimulation by direct activation of G proteins in vivo with GTPγS. Finally, we show an association of $G\alpha_o$ with Fz that is very rapidly ($t_{1/2}$ < 1 min) altered upon Wnt-3a stimulation. Thus, we argue that both $G\alpha_o$ and $G\alpha_q$ may participate in Wnt signal regulation of β-catenin stabilization.

Results and Discussion

Wnt Regulation of GSK3β/Axin Association

Although overexpression of wild-type or mutated proteins can give essential and predictive information about intracellular signaling networks, such ectopic expression studies are also subject to artifact. We wished, therefore, to the examine the effects of G protein signaling on Wnt response in cells expressing endogenous levels of downstream components. To investigate native mechanisms of Wnt action, we alternatively used a modified tissue culture system that requires exogenous media containing Wnt-3a to stabilize β-catenin [28] or purified, recombinant Wnt-3a protein. Unstimulated mouse L929 cells have modest levels of total β-catenin (see Figure S1 in the Supplemental Data available online). However, media containing Wnt-3a induces a subtle rise in β-catenin levels within 30 min, a further increase by 60 min, and maximal levels at 3 hr (Figure S1 and Figure 1A).

We next examined native associations of GSK3β with Axin members following Wnt stimulation. Two Axin members are expressed in L929 cells: Axin and Conductin (Axin2). Antibodies specific to each were used for coimmunoprecipitation studies with GSK3β. No significant differences in total cellular GSK3β protein levels were observed with or without Wnt treatment (Figure 1A), and similarly, immunoprecipitable (Figure 1A) or total (see Figure 2A) levels of Axin and Axin2 were unchanged during the time course of the Wnt stimulation (see also [18]).

GSK3β is notably associated with Axin2 in the unstimulated L929 cells. However, Wnt-3a exposure disrupted these interactions throughout the time course (Figure 1A). Although conditioned media from cells expressing Wnt-3a have been used successfully to establish Wnt-specific signaling responses [28], media from cells expressing Wnt-3a contain a myriad of other factors that are absent from control media. Thus, it may be argued that it is these additional factors, and not Wnt-3a per se, that are required to promote changes in protein associations. Nonetheless, identical results were obtained using recombinant Wnt-3a (Figure 1B; vide infra Figure 7B).

The GSK3β/Axin complex was also sensitive to Wnt-3a. Although GSK3β was detected in association with Axin following Wnt-3a treatment, GSK3β from Axin complexes of stimulated cells migrated more rapidly than did GSK3β from controls during SDS gel electrophoresis. Treatment of complexes with alkaline phosphatase had no effect on either mobility (data not shown). In other cells (see Figure 2A), GSK3β/Axin and GSK3β/Axin2 complexes are fully disrupted (as defined by the loss of coimmunoprecipitation) by Wnt treatment. To determine whether the GSK3β/Axin complexes were structurally distinct in naive or Wnt-treated L929 cells, we

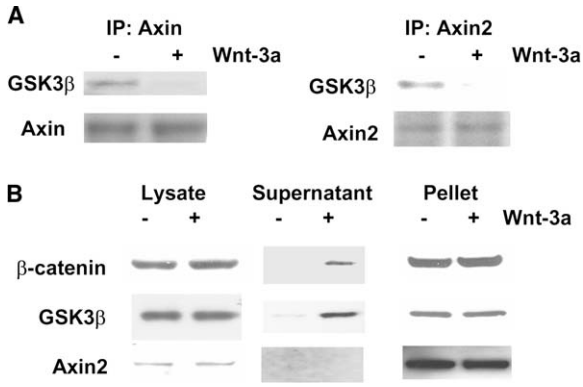

Figure 2. Wnt-3a Regulates the Cytosolic Release of GSK3β from Axin/GSK3β Complexes

(A) 3T3-L1 cells were cultured to confluence and treated with conditioned media with and without Wnt-3a for 2 hr. Cleared lysates were immunoprecipitated (IP) and blotted for GSK3β. Immunoblot controls were performed for each IP. Data are representative of four separate experiments.

(B) Stimulated and unstimulated cells were disrupted and lysates centrifuged at 500 × g for 5 min at 4ºC. Supernatant lysate fractions were then centrifuged at 390,000 × g for 90 min. Protein equivalent fractions of supernatants or of pellets from stimulated and unstimulated cells at each separation were analyzed by immunoblot with β-catenin, GSK3β (mouse), and Axin2 antibodies. Data are representative of three separate experiments.

investigated their sensitivities to very mild trypsin digestion. GSK3β/Axin complexes were immunoprecipitated, briefly exposed to trypsin (25 μg/ml for 2 min at 20ºC), and analyzed by immunoblot for Axin and GSK3β (Figure 1C). As seen, Axin is totally resistant to trypsin, irrespective of Wnt stimulation. Although GSK3β in complex with Axin in untreated cells is also trypsin resistant, treatment with Wnt structurally alters the GSK3β/Axin complexes in a manner that renders GSK3β completely sensitive to digestion by trypsin. The data suggest that Wnt induces an effective "unfolding" of GSK3β/Axin that may be functionally analogous to the dissociative changes observed with GSK3β/Axin2.

We suggest that Wnt-3a stabilizes β-catenin by rapidly targeting GSK3β/Axin and GSK3β/Axin2 complexes for functional repression; β-catenin becomes stabilized due to diminished phosphorylation by GSK3β. The observed GSK3β/Axin disruptions would precede Axin degradation (see [18]). Moreover, rapid alteration of GSK3β/Axin interaction would have the additional effect of eventually destabilizing Axin by reducing its phosphorylation by GSK3β [18–20]. While it has been proposed that the relative levels of Axin are a defining mode for destabilizing β-catenin [20, 21], our data (see Figures 1B and 2A) are more consistent with Axin degradation being secondary to the initial stabilization of β-catenin. Axin degradation may be imperative, but potentially as a more long-term, positive feedback mechanism to inhibit reformation of GSK3β/Axin degradation complexes rather than as the primary event that initiates β-catenin stabilization.

Wnt Stimulates the Release of GSK3 to the Cytosol

Data (Figure 1) suggest that Wnt-3a may promote the complete release of GSK3β from Axin complexes. Because the Axins appear to be membrane associated following Wnt stimulation, we were interested in determining whether GSK3β translocated to a separate subcellular compartment upon exposure to Wnt-3a. The cytosolic volume of L929 cells is relatively low, making it difficult to isolate uncontaminated subcellular fractions. We therefore investigated this hypothesis using mouse 3T3-L1 cells, a Wnt-responsive line with a larger cytosolic volume. As with L929 cells, Wnt-3a stimulation of 3T3-L1 cells promotes the release of GSK3β from Axin2 complexes (Figure 2A). GSK3β/Axin associations were also fully disrupted by Wnt-3a treatment of 3T3-L1 cells (Figure 2A), in contrast to that seen upon Wnt-treatment of L929 cells (see Figures 1A and 1C). The faster-mobility form of GSK3β found in association with Axin in L929 cells is not observed in 3T3-L1 cells (see Figures 1A, 1C, and 2A).

Stimulated and unstimulated cells were disrupted by filter membrane passage, and lysates were centrifuged at 500 × g for 5 min. Equivalent supernatant fractions from Wnt-stimulated and -unstimulated cells were then centrifuged further at 390,000 × g for 90 min. Protein equivalent fractions of supernatants from stimulated and unstimulated cells after each centrifugation were then analyzed for the relative levels of β-catenin, GSK3β, and Axin2. We also examined their relative distributions in the pelleted fractions of the same samples.

Although Wnt-3a promoted a dramatic rise in cytosolic levels of β-catenin in the 3T3-L1 cells (Figure 2B), only small whole-cell (i.e., lysate fraction) differences are seen between stimulated and unstimulated cells. These differences reflect the high basal levels of β-catenin found in association with cadherins in the cytoskeleton of 3T3-L1 cells [29]. There is also a marked increase in cytosolic GSK3β following Wnt stimulation, data consistent with the ability of Wnt-3a to promote dissociation of GSK3β/Axin complexes and release of GSK3β (Figure 2B). As expected, Axin2 was not detected at significant levels in the high-speed supernatants from either stimulated or unstimulated cells.

The release of GSK3β to the cytosol suggests the possibility that additional substrates may become available for phosphorylation by GSK3 upon Wnt-stimulation and there may be cross-talk between the Wnt pathway and other signaling networks.

Wnt Stimulation Does Not Rapidly Increase GSK3β/Dvl Interactions

Although we observe an increase in cytosolic GSK3β upon Wnt stimulation, potentially an increase in GSK3β interactions with Dvl complexes may promote disruption of GSK3β/Axin interactions. We tested this by using antibodies specific for the three mammalian Dvl isoforms. Lysates from cells treated with or without Wnt-3a were immunoprecipitated and associations probed with a series of antibodies to GSK3 (α-GSK3β [mouse]; nonphosphorylated, Y216/279 GSK3 [β and α; mouse]; pY216/279 GSK3 [β and α; mouse]; α-GSK3α [rabbit]; α-pS9 GSK3β [rabbit]; α-pY216 GSK3β [rabbit]). Little differences in GSK3β/DVl interaction were observed, regardless of the phosphorylation state of GSK3β (Figure 3, unpublished data). Under these conditions, the loss of GSK3β from Axin complexes is not accompanied by a detectable, reciprocal increase in GSK3β association

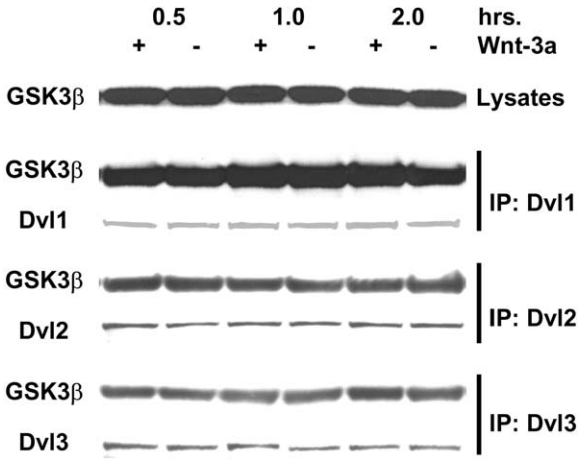

Figure 3. Wnt-3a Regulates Dvl/GSK3β Associations in L929 Cells

L929 lysates were prepared as in Figure 1A. Following immunoprecipitation by α-Dvl1, -2, or -3, GSK3β levels were measured by using α-GSK3β (mouse). Immunoblot controls were performed for each IP. Data are representative of two separate experiments and repeated with five different antibodies to GSK3.

with Dvl. These results indicate that Wnt-3a does not regulate the antagonistic functions of Axin and Dvl by enhancing the interactions of GSK3β with Dvl. We suggest that in response to Wnt-stimulation there is an initial release of GSK3β to the cytosol that is not related to an accompanying association with Dvl.

Depletion of $G\alpha_o$ and $G\alpha_q$ by siRNA Inhibits Wnt/β-Catenin Signaling

Molecular genetic data have suggested the involvement of heterotimeric G proteins in Wnt/β-catenin signaling [26, 27]. We therefore investigated the biochemical relationship of Gα protein function to GSK3β/Axin and β-catenin regulation using siRNA technology to specifically deplete $G\alpha_o$ and $G\alpha_q$ proteins (Figure 4A). Again we chose to use 3T3-L1 cells, because these have proven to be particularly responsive to oligonucleotide treatment [30]. Cells were treated with a control siRNA, $G\alpha_o$- or $G\alpha_q$- specific siRNAs, or simultaneously with siRNAs to both $G\alpha_o$ or of $G\alpha_q$. As seen (Figure 4A), the siRNA treatments were both effective and specific. Cells were obtained that were individually depleted to >80% of either $G\alpha_o$ or $G\alpha_q$ protein or of both $G\alpha_o$ and $G\alpha_q$.

siRNA-depleted and control cells were then treated with or without Wnt-3a. Levels of β-catenin were examined in the various cultures. To increase the precision of the assay and to avoid detection of β-catenin associated with the cell surface (see Figure 2B), we determined the effects of siRNA treatment on Wnt signaling using a C-terminal fragment of E-cadherin that specifically recognizes uncomplexed, cytosolic β-catenin. In addition, we examined the effect of Gα protein depletion on the associations of GSK3β with Axin and Axin2.

As seen (Figure 4B), control siRNA oligos had no effect on Wnt stabilization of β-catenin or the dissociation of GSK3β from Axin or Axin2 complexes. However, depletion by >80% of either $G\alpha_o$ or of $G\alpha_q$ (see Figure 4A) was able to significantly, but not fully, inhibit the stabilization of β-catenin by Wnt. The reduction in $G\alpha_o$

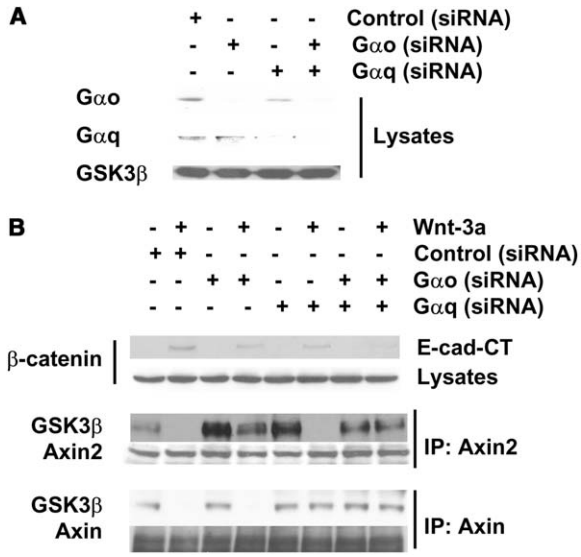

Figure 4. Effects of Depleting of $G\alpha_o$ and $G\alpha_q$ on Wnt Regulation of GSK3β Interactions and Stabilization of β-Catenin

(A) 3T3-L1 cells were cultured to 60%–70% confluence, media were removed, and control and specific siRNA oligonucleotides introduced. Lysates were examined for whole-cell levels of $G\alpha_q$, $G\alpha_o$, and GSK3β. Data are representative of three separate experiments. (B) siRNA-treated 3T3-L1 cells were cultured in media with and without recombinant Wnt-3a for 2 hr. β-catenin levels were measured in whole-cell lysates by direct immunoblot assay. Cytosolic, nonmembrane associated β-catenin levels were measured following affinity pull-down with a C-Terminal fragment of E-cadherin fused to GST (E-cad-CT). GSK3β levels were measured following immunoprecipitation by α-Axin2 or α-Axin. Immunoblot controls were performed for each IP. Data are representative of three separate experiments.

decreased the ability of Wnt to disrupt GSK3β/Axin2 complexes, but had no effect on GSK3β/Axin complexes. Conversely, depletion of $G\alpha_q$ inhibited Wnt destabilization of GSK3β/Axin complexes, but had only a minimal ability to affect GSK3β/Axin2 interactions (Figure 4B). Remarkably, cells simultaneously treated with siRNAs to both $G\alpha_o$ and $G\alpha_q$ were effectively resistant to Wnt-3a. β-catenin levels were unresponsive to Wnt-stimulation, and neither the GSK3β/Axin2 nor the GSK3β/Axin complexes were disrupted by treatment.

Stimulation of Permeabilized Cells with GTPγS Promotes β-Catenin Stabilization in the Absence of Wnt

We next sought to determine whether we could bypass the requirement for Wnt signaling to GSK3β/Axin2 and β-catenin by directly activating G proteins in vivo using GTPγS [5′-O-(3′-thiotriphosphate)], a nonhydrolyzable analog of GTP. L929 cells were permeabilized with *Staphylococcus aureus* α-toxin, with and without GTPγS [31] for 20 min. We reasoned that a permeablized cell system would be more reflective of the in vivo cellular organization than would a GTPγS-treated cell lysate. GSK3β/Axin2 were largely unaffected by α-toxin treatment alone, but simultaneous addition of GTPγS elicited a Wnt-like release of GSK3β from association with Axin2 (Figure 5A). We also observed a GTPγS-dependent release of GSK3β from Axin2 in 3T3-L1 cells, but not the release of GSK3β from complex with Axin (Figure 5B). $G\alpha_o$

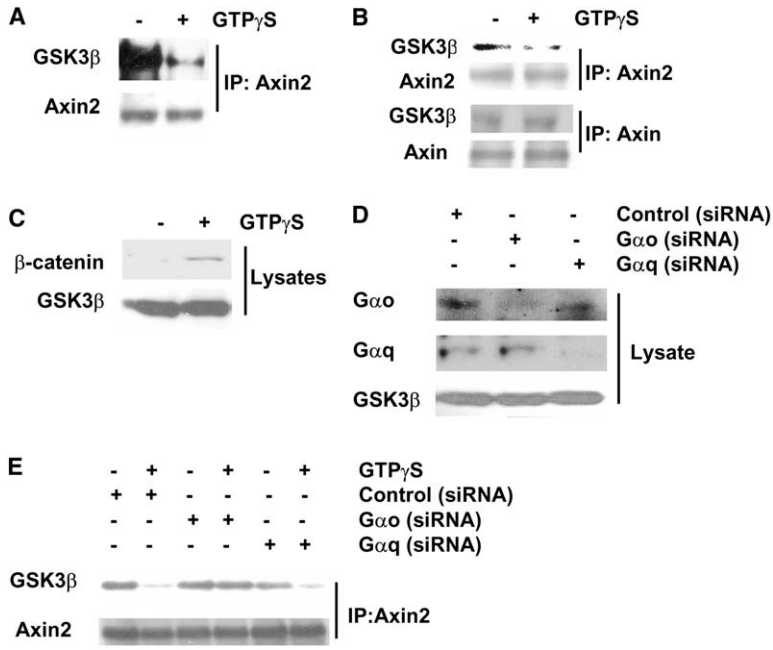

Figure 5. GSK3β/Axin2 Complexes and β-Catenin Levels Can Be Regulated by GTPγS

(A) L929 lysates were prepared from cells grown to confluence. Media were removed from cells and new media added containing α-toxin (5 μg/ml) with or without GTPγS (200 μM) for 20 min before lysate preparation. GSK3β and Axin2 levels were measured following immunoprecipitation by α-Axin2. Data are representative of three separate experiments.

(B) 3T3-L1 cells were cultured to confluence. Media were removed from cells, and new media added containing α-toxin (5 μg/ml) with or without GTPγS (200 μM) for 20 min. Lysates were prepared and GSK3β, Axin2, or Axin levels were measured following immunoprecipitation by α-Axin2 or α-Axin. Data are representative of three separate experiments.

(C) L929 lysates were prepared from cells grown to confluence. Media were removed from cells, and new media added containing α-toxin (5 μg/ml) with or without GTPγS (200 μM) for 3 hr before lysate preparation. β-catenin and GSK3β were measured by immunoblot assay of the whole-cell lysates. Data are representative of two separate experiments.

(D) 3T3-L1 cells were cultured to 60%–70% confluence, media were removed, and control and specific siRNA oligonucleotides introduced. Lysates were examined for whole-cell levels of $G\alpha_q$, $G\alpha_o$, and GSK3β. These cells were subsequently used for the GTPγS experiments described in (E).

(E) Media were removed from the siRNA-treated 3T3-L1 cells of (D), and new media added containing α-toxin (5 μg/ml) with or without GTPγS (200 μM) for 20 min. Lysates were prepared and GSK3β and Axin2 levels were measured following immunoprecipitation by α-Axin2.

is highly sensitive to activation by GTPγS, whereas other Gα proteins, notably $G\alpha_q$, have inherently low basal guanine nucleotide exchange properties [32, 33] and are less sensitive to activation by GTPγS treatment. Consistent with previous data (see Figure 4B), we suggest that, potentially, it is these latter Gα proteins that may predominate for Axin regulation.

We also examined whether direct treatment of permeablized cells with GTPγS would promote β-catenin stabilization in the absence of an exogenous Wnt signal. L929 cells were incubated for an extended (3 hr) time course, and β-catenin levels were assayed in control and GTPγS-treated cells (Figure 5C). Data indicate that the direct activation of G proteins with GTPγS mimics Wnt effects not only to disrupt GSK3β/Axin2 complexes, but also to stabilize β-catenin.

Finally, we examined the responsiveness of $G\alpha_o$-depleted cells to GTPγS. Cells were first treated with control, $G\alpha_o$-, or $G\alpha_q$- specific siRNAs (Figure 5D) and then subsequently permeabilized with *Staphylococcus aureus* α-toxin, with and without GTPγS for 20 min. Cells treated with control or $G\alpha_q$ siRNAs exhibited a GTPγS-dependent release of GSK3β from Axin2 (Figure 5E). However, cells depleted of $G\alpha_o$ (see Figure 5D) were unable to respond to GTPγS to disrupt GSK3β/Axin2 complexes (Figure 5E).

Although we cannot absolutely exclude a GTPγS effect on the ras-related family of small G proteins or even on a nonspecific path, collectively the data (see Figures 4 and 5 and below) indicate that $G\alpha_o$ participates in signaling via Wnt-activated Fz to destabilize GSK3β/Axin2 complexes leading to reduced phosphorylation and consequent stabilization of β-catenin.

The $G\alpha_o$ Inhibitor PTX Antagonizes Wnt Signal Response

If $G\alpha_o$ were involved in Wnt regulation, we should observe an inhibition by pertussis toxin (PTX), a specific inhibitor of $G\alpha_{o/i}$, of Wnt signaling in native, untransfected cells. First, we examined the effects of PTX on β-catenin stabilization and specifically chose L929 cells for these experiments. It can be difficult to interpret regulatory effects on cells by simply examining total or even cytosolic levels of β-catenin. For example, cells that express an abundance of E-cadherin at the cell cortex may also exhibit inherently elevated levels of total cellular β-catenin. Yet, β-catenin molecules in complex with E-cadherin at the cortex are in effect sequestered from the Lef/Tcf signaling machinery; large differences in free, cytosolic β-catenin that result from Wnt or other stimuli may only appear as minimal increases (or decreases) when whole-cell lysates are examined (see Figure 2B). For similar reasons, one must be judicious when interpreting results obtained with PTX. There is the potential that long-term, PTX-sensitive, G protein-mediated signaling can alter the subcellular distribution of β-catenin, either directly or indirectly, by altering the effective affinity of E-cadherin for β-catenin.

L929 cells have extremely low levels of endogenous E-cadherin and membrane-associated β-catenin. In addition, L929 cells respond relatively rapidly to exogenous Wnt stimulation, increasing whole-cell and cytosolic levels of β-catenin by >20-fold (see Figure 1). Thus, the L929 cells present specific advantage for the study of PTX effects on Wnt signaling. In addition, we specifically assayed the effect of PTX on Wnt signaling making use of the C-terminal fragment of E-cadherin

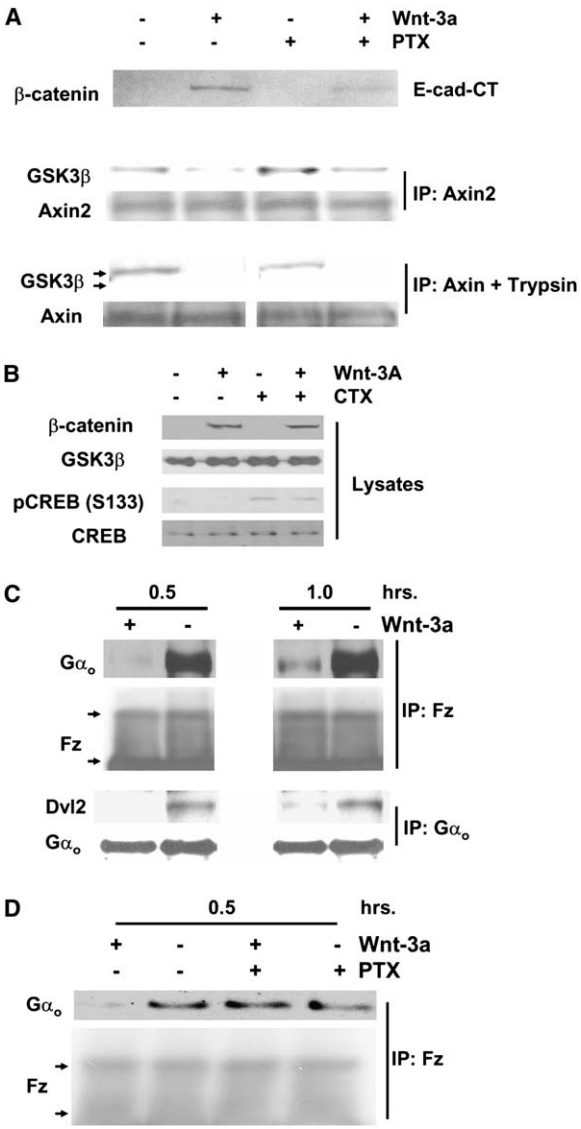

Figure 6. PTX Effects on Wnt-Regulated β-Catenin Stability and Protein Interactions

(A) L929 lysates were prepared from cells grown to confluence, treated with or without pertussis toxin (PTX, 1μg/ml) for 1.5 hr, and then cultured with conditioned media with or without Wnt-3a for 1 hr. Cytosolic, non-membrane-associated β-catenin levels (see Figure 4B) were measured following affinity pull-down with a C-Terminal fragment of E-cadherin fused to GST (E-cad-CT). GSK3β levels were measured directly or following immunoprecipitation with antisera to Axin2. Cleared lysates were also immunoprecipitated with α-Axin and immunoprecipitates analyzed by immunoblot following a very mild treatment with trypsin (see Figure 1C). Arrows indicate Wnt-regulated mobility differences for GSK3β in association with Axin. Immunoblot controls were performed for each IP. Data are representative of three separate experiments.

(B) L929 lysates were prepared from cells grown to confluence, treated with or without cholera toxin (CTX, 1 μg/ml) for 1.5 hr, and then cultured with conditioned media with or without Wnt-3a. Total β-catenin, GSK3β, CREB, and phospho-CREB levels were measured directly by immunoblot assay. Data are representative of two separate experiments.

(C) L929 cells were cultured to confluence and treated with conditioned media as in Figure 1. Cleared lysates were immunprecipitated with α-Fz or α-$G\alpha_o$ and blotted with α-$G\alpha_o$ and α-Fz or with α-Dvl2 and α-$G\alpha_o$, respectively. Arrows indicate mobility positions of the major Fz bands, although the lower band (~55 kDa) may partially represent detection of the heavy chain of IgG from the antibody. Data are representative of three separate experiments.

(D) L929 cells were cultured to confluence and treated with conditioned media with or without a PTX pretreatment. Cleared lysates were immunprecipitated with α-Fz and blotted with α-$G\alpha_o$ and α-Fz. Arrows indicate mobility positions of the major Fz bands. Data are representative of two separate experiments.

that only recognizes uncomplexed, cytosolic β-catenin (see Figure 4B).

In L929 cells, we see that the Wnt-stimulated increase in uncomplexed, cytosolic β-catenin levels is reproducibly attenuated (~50%) by pretreatment of cells with PTX (Figure 6A). PTX also significantly repressed the Wnt-induced release of GSK3β from Axin2 complexes but did not affect the sensitivity of GSK3β/Axin to trypsin (Figure 6A), indicating that the PTX effects were pathway specific. These data are consistent with the siRNA depletion experiments (see Figure 4B), which indicate that $G\alpha_o$ regulates the GSK3β/Axin2 complex, but not GSK3β/Axin. There was a modest PTX-dependent increase in GSK3β association with Axin2 in unstimulated cells (Figure 4A), but this probably results from an inhibitory effect of PTX on the low levels of endogenous Wnt produced by L929 cells. We suggest that signaling via the $G\alpha_{o/i}$ family primarily promotes regulation of GSK3β via Axin2, but not Axin; consequently, PTX would only partially inhibit β-catenin stabilization (see Figure 6A).

We also investigated the possibility that other Gα proteins could regulate Wnt signaling. Unlike PTX, which serves to inhibit the activation of targeted $G\alpha_{i/o}$ subunits, cholera toxin (CTX) generates constitutively active $G\alpha_s$. L929 cells were treated with and without Wnt-3a and CTX in various combinations. The effectiveness of CTX to activate a $G\alpha_s$ pathway was monitored by examining its influence on the PKA-dependent phosphorylation of the cAMP-responsive protein CREB. CTX was able to induce PKA-dependent phosphorylation of CREB on S133 (Figure 6B), regardless of the presence or absence of Wnt-3A. Although these results substantiate an ability of CTX to activate a $G\alpha_s$/cAMP pathway in this system, CTX did not stabilize β-catenin in the absence of Wnt-3A, inhibit β-catenin stabilization by Wnt, or synergize with Wnt-3a (Figure 6B). Consistent with other observations [26], these data indicate that $G\alpha_s$ does not appear to function during Wnt/β-catenin signaling and further substantiate the specific effects observed with PTX.

Given the results with PTX, we examined $G\alpha_o$ protein complexes in L929 cells by using antibodies that recognize most Fz isoforms and antibodies to Dvl (Figure 6C). We observed unambiguous coimmunoprecipitations of $G\alpha_o$ with Fz and with Dvl2 that were significantly and rapidly decreased by Wnt-3a treatment (Figure 6C). Interaction differences were not observed with reciprocal IP/IB blottings; Fz was similarly detected in $G\alpha_o$ immunoprecipitates regardless of Wnt treatment. $G\alpha_o$/Fz associations may not be fully disrupted upon Wnt treatment. Rather, Wnt-3a may stimulate a conformational change in $G\alpha_o$ protein complexes that make them poorly accessible to certain coimmunoprecipitations. These results do not demonstrate a direct interaction of Fz and $G\alpha_o$ or discern the complex or membrane compartment that they share. Nonetheless, the data demonstrate an interaction of $G\alpha_o$ with Wnt signaling

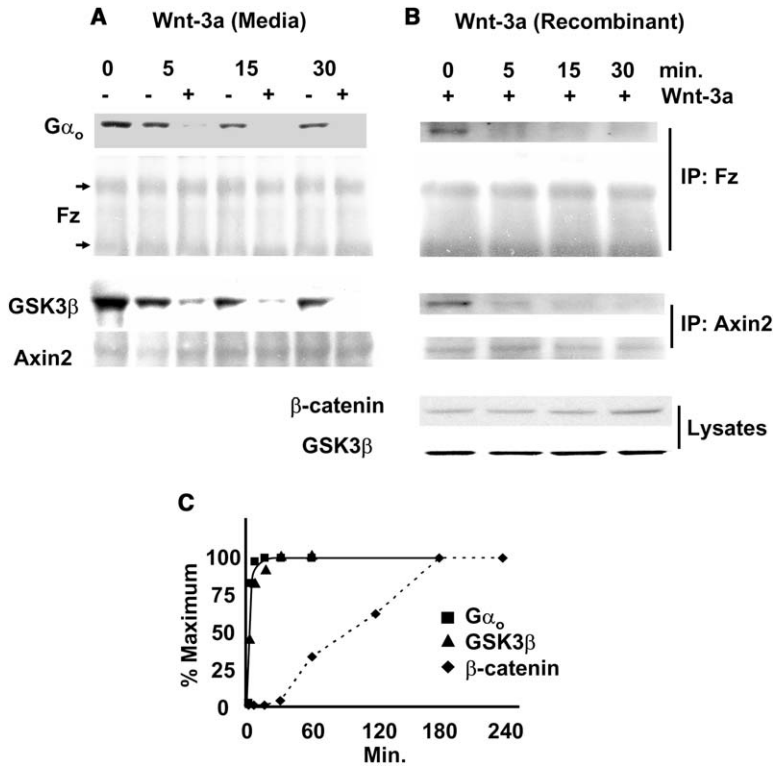

Figure 7. $G\alpha_o$ and Axin2 Protein Interactions Are Rapidly Altered by Wnt-3a

(A and B) L929 cells were cultured to confluence and treated with (A) conditioned media or (B) 600 ng/ml of recombinant Wnt-3a protein. Cleared lysates were immunprecipitated with α-Fz or α-Axin2 and blotted with α-$G\alpha_o$ and α-Fz or with α-GSK3β and α-Axin2, respectively. Arrows indicate mobility positions of the major Fz bands. In (B), Lysates were also examined for whole-cell levels of β-catenin and GSK3β.

(C) Graphical representations of Wnt-3a-induced, time-dependent changes in $G\alpha_o$/Fz and GSK3β/Axin2 interactions and in β-catenin levels. These data are compiled from four separate time courses.

components that is radically altered upon Wnt stimulation; further, PTX treatment inhibits these Wnt-dependent changes in $G\alpha_o$/Fz association (Figure 6D).

We next examined the kinetics of Wnt-induced changes in $G\alpha_o$/Fz, GSK3β/Axin2, and β-catenin stabilization. Within minutes of stimulation using Wnt-3a media, more than 80% of the $G\alpha_o$/Fz complexes were modified (Figure 7). GSK3β/Axin2 was also rapidly disrupted. We observed nearly identical temporal changes in $G\alpha_o$/Fz and GSK3β/Axin2 associations following stimulation with purified, recombinant Wnt-3a (Figure 7B). These modifications in protein-protein associations represent the most immediate cellular changes observed in response to Wnt signaling and significantly precede detectable β-catenin stabilization (Figure 7C). Data from others (see [18]) indicate that Axin degradation is similarly retarded.

Conclusions

We suggest that Wnt-signaling via Gα subunits regulates specific protein associations that disrupt GSK3β/Axin complexes and stabilize β-catenin. These biochemical data are consistent with an intermediary role for heterotrimeric G proteins in signal transduction from Fz/LRP to Axin/GSK3. Previous data from mammalian cells and *Drosophila* also provided genetic support for Gα function during Wnt regulation of GSK3/β-catenin signaling [26, 27]. Yet, it is not clear why G protein genes had not been identified previously in various genetic screens for Wnt modifiers or why mice that are nullizygous for an individual *Gα* gene do not exhibit phenotypes consistent with a *Wnt* null phenotype.

$G\alpha_o$ and $G\alpha_q$ exhibit effective functional redundancies but may preferentially regulate GSK3β/Axin2 and GSK3β/Axin, respectively. Given the complexity of the Fz family, the potential exists for regulation by additional Gα members. There may also be regulatory paths that potentiate β-catenin stabilization independently of Gα signaling. LRP5/6, in particular, may define a mechanism for direct signaling to the Axins [34–38] that requires Fz but functions separately from Gα activation. Although all Fz receptors contain an extracellular N-terminal, cysteine-rich domain (CRD) that serves as a requisite site for Wnt interaction, in vivo data in *Drosophila* indicate that CRDs may not be absolutely essential for Wnt signaling [38]. Perhaps direct binding of Wnt to Fz can be compensated by interaction with LRP5/6 or other components of the receptor complex. Still, loss of Wnt/Fz binding would suppress Gα activation. To this extent, disruption of Fz/Gα signaling may have a less severe effect on Wnt response that would be the loss of an individual Wnt, Fz, or LRP member. For example, while it is universally assumed that Dvl is absolutely required to transmit a Wnt signal, single and double *Dvl* null mice do not have simple Wnt-like phenotypes [39]. It should be emphasized that there are additional Wnt receptor systems (e.g., Ryk, Ror) that may function independently of Fz and, thus, also bypass G protein dependency [40–44], although definitive linkages of these signaling pathways to downstream components have not been established. It is also formally possible that Fz receptors are able to heterodimerize with a different, Gα-coupled receptor. In this context, interaction with such a hypothetical coreceptor may augment Fz/Wnt/β-catenin signaling in a Gα-dependent manner.

Experimental Procedures

Wnt-3a Media and Recombinant Mouse Wnt-3a

Conditioned media (CM) were harvested from confluent monolayers of control L929 cells and a clonal L929 cells stably transfected to

secrete Wnt-3a. CM stored at −80ºC and thawed retained Wnt-3a activity for several weeks when stored at 4ºC. Wnt-3a and control condition medium were prepared essentially as described [28], except that serum-free medium was used for conditioning. Recombinant mouse Wnt-3a was from R&D Systems. The Wnt-3a was reconstituted with PBS containing 0.2% BSA and added into plates with L929 cells.

Antibodies

Axin (rabbit, H-98), Axin2 (goat, M-20), Dvl1 (mouse, 3F12), Dvl2 (mouse, 10B5), Dvl3 (mouse, 4D3), Frizzled (rabbit, H-300), Gαo (mouse, A2), Gαq (rabbit, E-17), GSK3α (rabbit, H-75), GSK3β (rabbit, H-76), p-GSK3β (Ser 9, rabbit), and p-GSK3β (Tyr216, rabbit) are from Santa Cruz Labs. β-catenin (mouse) and GSK3β (mouse) are from BD Transduction Laboratories. Non-Tyr-phosphorylation-GSK3 (4G-1E, mouse) and Tyr-phosphorylation-GSK3 (5G-2F, mouse) are from Upstate Labs. Antibodies to CREB and pCREB (S133) are from Santa Cruz Labs.

siRNA Treatment

Control siRNA, Gαo siRNA, and Gαq siRNA were purchased from Santa Cruz, CA. Transfection of siRNA into 3T3-L1 cells was performed according to the protocol of the manufacturer; incubation time was 60–70 hr. In some experiments, these cells were then treated with α-toxin and GTPγS, as described below.

Immunoprecipitations and Western Blotting

L929 or 3T3-L1 cells were cultured to confluence and lysed in 0.5% Triton X-100, 0.5% NP40, 0.25% Gelatin, 1 × TBS (pH 7.4), 1 mM EDTA, 1 mM DTT, 1 mM Na_3VO_4, 5 mM NaF, and protease inhibitor cocktail (Roche) for 20 min on ice. Lysates were centrifuged for 5 min at 2.5 K at 4ºC. The lysates were normalized by protein concentration assays, rotated with proper antibodies and Protein A or Protein G beads (Sigma) overnight at 4ºC, and precipitated at 2.5 K rpm. Precipitates were washed three times in 0.1% NP40, 0.25% Gelatin, 1 × TBS (pH 7.4), 1 mM EDTA, 1 mM Na_3VO_4, and 5 mM NaFl. Sample buffer was added to the final precipitates, and the samples were heated for 5 min at 95ºC. The samples were loaded onto NuPAGE (Invitrogen), and the running buffer and transfer buffer always included phosphatase inhibitors.

GST-E-cad-CT fusion protein was prepared and used to select unbound β-catenin in whole-cell lysates as described [45]. The incubation time of cell lysates with the fusion protein/Glutathione-agarose (Santa Cruz) complex was 30 min.

Limited Tryptic Digestion

L929 cells were treated with or without recombinant Wnt-3a (200 ng/ml) for 1 hr Two aliquots of the lysates of each condition were incubated with Axin antibody and Protein A beads. The pellets were washed three times with the washing buffer. Pellets from one of the aliquots were resuspended with 1 ml of TBS with 25 μg of trypsin (TPCK treated, Sigma) for 2 min at 20ºC. The pellets were spun down again, and the following treatments were the same as those of the other aliquot pellets.

Sequential Centrifugation for Preparation of Cytosolic Fractions

Stimulated and unstimulated 3T3-L1 cells were disrupted by membrane filter passage and lysates centrifuged at 500 × g for 5 min at 4ºC. Supernatant fractions were then centrifuged again at 390,000 × g for 90 min. Protein equivalent fractions of supernatants from stimulated and unstimulated cells at each stage of fractionation were analyzed by immunoblot assay.

α-Toxin and GTPγS Treatment

L929 or 3T3-L1 cells were grown to confluence in 6-well plates. Media were removed and new media added containing α-toxin (5 μg/ml; List Biological Labs) with or without GTPγS (200 μM; Sigma). For Axin/GSK3 binding assays, the incubation time was 20 min, while for β-catenin stabilization assays; the incubation time was 3 hr.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.current-biology.com/cgi/content/full/15/22/1989/DC1/.

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