RGS19 regulates Wnt–β-catenin signaling through inactivation of Gαo

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Summary
The Wnt–β-catenin pathway controls numerous cellular processes, including differentiation, cell fate decisions and dorsal-ventral polarity in the developing embryo. Heterotrimeric G-proteins are essential for Wnt signaling, and regulator of G-protein signaling (RGS) proteins are known to act at the level of G-proteins. The functional role of RGS proteins in the Wnt–β-catenin pathway was investigated in mouse F9 embryonic teratocarcinoma cells. RGS protein expression was investigated at the mRNA level, and each RGS protein identified was overexpressed and tested for the ability to regulate the canonical Wnt pathway. Expression of RGS19 specifically was found to attenuate Wnt-responsive gene transcription in a time- and dose-dependent manner, to block cytosolic β-catenin accumulation and Dishevelled3 (Dvl3) phosphorylation in response to Wnt3a and to inhibit Wnt-induced formation of primitive endoderm (PE). Overexpression of a constitutively active mutant of Gαo rescued the inhibition of Lef-Tcf-sensitive gene transcription caused by RGS19. By contrast, expression of RGS19 did not inhibit activation of Lef-Tcf gene transcription when induced in response to Dvl3 expression. However, knockdown of RGS19 by siRNA suppressed canonical Wnt signaling, suggesting a complex role for RGS19 in regulating the ability of Wnt3a to signal to the level of β-catenin and gene transcription.

Key words: RGS proteins, Wnt, β-catenin, Heterotrimeric G-proteins, RGS19, Frizzled, Gαo

Introduction
In unstimulated cells, cytosolic levels of the transcriptional co-activator β-catenin are tightly regulated by a multiprotein complex that includes glycogen synthase kinase 3β (GSK3β), axin and APC (product of the adenomatous polyposis coli gene) (Ikeda et al., 1998; Kishida et al., 1998; Zeng et al., 1997). Within the complex, β-catenin is phosphorylated and thereby targeted for degradation by the proteasome (Aberle et al., 1997). Upon binding of Wnt3a to its cognate cell-surface heptihelical receptor Frizzled (Fz1), the degradation complex is inhibited, allowing β-catenin to accumulate in the cytosol, translocate to the nucleus and activate target genes such as Myc and CyclinD1, by means of Lef-Tcf-sensitive transcription (Molenaar et al., 1996). The mechanism by which stimulation of Fz leads to the inhibition of the degradation complex for β-catenin is not fully understood, although it does involve activation of the phosphoprotein Dishevelled (Dvl) (Yanagawa et al., 1995).

Heterotrimeric G-proteins are essential to the Wnt–β-catenin pathway, in organisms ranging from Drosophila to vertebrates (Katanev et al., 2005; Liu et al., 2001; Liu et al., 2005; Malbon, 2004; Malbon, 2005; Slusarski et al., 1997). Gain- and loss-of-function studies in mouse F9 teratocarcinoma embryonic stem cells (Liu et al., 2001) as well as flies (Katanev et al., 2005) have demonstrated that Gαo, for example, is crucial for Wnt-stimulated target gene activation. Heterotrimeric G-proteins are a class of GTP-activated molecular switches comprising a Gα-subunit in complex with a Gβ-Gγ dimer (Cabrera-Vera et al., 2003). In the inactive state, GDP-bound Gα subunits are complexed with Gβ-Gγ, inhibiting activation of downstream signaling molecules. Upon activation of the G-protein by its cognate heptihelical receptor [i.e. a G-protein-coupled receptor (GPCR) such as Fz], GDP is exchanged for GTP by the Gα subunit, allowing dissociation of the Gβ-Gγ dimer. Both the GTP-bound Gα-subunits and the ‘free’ Gβ-Gγ dimers are available to activate downstream signaling components (Clapham and Neer, 1993), and both have been shown to function in Wnt signaling (Liu et al., 2001; Slusarski et al., 1997). The period of the activation cycle is determined by the hydrolysis of GTP, which terminates the activation.

Heterotrimeric Gα-subunits possess a slow, intrinsic GTPase activity that can be accelerated by a class of regulatory proteins termed RGS proteins (Hollinger and Hepler, 2002). Over 30 mammalian RGS proteins have been described, each having its own distinct repertoire of Gα-subunits that it regulates (Hollinger and Hepler, 2002). RGS proteins have been classified into subfamilies (RZ, R4, R7, R12, RA) based upon sequence conservation within the catalytic RGS domain (Ross and Wilkie, 2000). Members of each subfamily share functional characteristics, including regulation of similar subsets of Gα-subunits. RGS proteins also display modular domains important for protein interactions, for posttranslational modifications and for proper intracellular localization (Burchett, 2000). Several RGS proteins contain cystein-string regions necessary for palmitoylation and subsequent binding to the plasma membrane. Other RGS proteins possess PDZ, PTB and DEP domains necessary for formation of protein complexes (Burchett, 2000). RGS proteins have been shown to be essential in many G-protein-mediated
physiological processes, including visual (Chen et al., 2000) and dopaminergic signal transduction (Rahman et al., 2003), as well as orienting the mitotic spindle during cell division (Malbon, 2005; Martin-McCaffrey et al., 2004). These proteins can act as scaffolds, organizing receptors, G-proteins and their effectors, enhancing efficiency and specificity of signaling interactions (Abramow-Newerly et al., 2006).

As Wnt signaling is under tight regulatory control that requires heterotrimeric G-proteins, we hypothesized that RGS proteins themselves play a role in modulating the output of Wnt signaling, and specifically our first analysis was of the Wnt–β-catenin pathway. We show that RGS19 specifically controls signaling of the Wnt–β-catenin pathway. Expression of RGS19 attenuates Dvl phosphorylation, β-catenin accumulation, Wnt-responsive gene transcription and blocks Wnt-induced differentiation of mouse 9 teratocarcinoma cells by inactivation of Goα. However, knockdown of RGS19 protein expression also suppresses Wnt-induced gene transcription and β-catenin accumulation, suggesting a complex role for RGS19 in the regulation of Wnt–β-catenin signal transduction.

Results

RGS protein expression in F9 cells: analysis at the mRNA level

The expression of RGS family members was investigated in mouse 9 teratocarcinoma (F9) cells. Mouse 9 cells have been used extensively to model early stages of mouse development (Lehtonen et al., 1989). These totipotent stem cells can be induced to differentiate into PE by several morphogens, including retinoic acid (Strickland and Mahdavi, 1978) and Wnt3a (Liu et al., 2001). F9 cells also have been used in studies of Wnt–β-catenin signal transduction owing to their expression of key mediators of the pathway, including Goα, Goβ, GSK3β, axin, β-catenin and Dvl (Liu et al., 2001). Reverse transcription PCR (RT-PCR) was employed to probe the presence of mRNA encoding RGS proteins in F9 cells. Specific primers were designed for each nucleotide sequence and validated for possible crossreactivity through homology searches on NCBI-BLAST. RNA was isolated from F9 cells and reverse transcribed to synthesize cDNA. Expression of 13 RGS proteins was detected (Fig. 1A,B), including members of each of the five RGS subfamilies: R4 (RGS3, RGS5, RGS16), R12 (RGS10, RGS12, RGS14), RZ (RGS20, RGS17, RGS19), RA (axin, conductin) and R7 (RGS9, RGS11).

RGS protein overexpression screen

The ability of overexpression of those RGS proteins identified in F9 cells to regulate Wnt–β-catenin signaling was examined. Wnt3a stimulation of Fz1 leads to β-catenin accumulation and activation of β-catenin-sensitive, Lef-Tcf-dependent transcription (Molenar et al., 1996). In order to probe the possible role of RGS proteins in Wnt–β-catenin–Lef-Tcf signaling, all RGS proteins found in the RT-PCR screen were transiently expressed in embryonic F9 cells [excluding the known Wnt signaling components axin (Zeng et al., 1997) and conductin (Behrens et al., 1998)]. The well-known promoter target of Wnt3a action, Lef-Tcf-sensitive transcription, was measured using a luciferase-based β-catenin-responsive promoter system. The reporter, termed M50, contains eight consensus Tcf-binding sites upstream of a minimal promoter driving luciferase expression (Veeman et al., 2003). Expression of RGS family members was investigated in F9 cells to regulate Wnt–β-catenin signal transduction owing to their expression of key mediators of the pathway, including Goα, Goβ, GSK3β, axin, β-catenin and Dvl (Liu et al., 2001). Reverse transcription PCR (RT-PCR) was employed to probe the presence of mRNA encoding RGS proteins in F9 cells. Specific primers were designed for each nucleotide sequence and validated for possible crossreactivity through homology searches on NCBI-BLAST. RNA was isolated from F9 cells and reverse transcribed to synthesize cDNA. Expression of 13 RGS proteins was detected (Fig. 1A,B), including members of each of the five RGS subfamilies: R4 (RGS3, RGS5, RGS16), R12 (RGS10, RGS12, RGS14), RZ (RGS20, RGS17, RGS19), RA (axin, conductin) and R7 (RGS9, RGS11).

Fig. 1. RGS protein expression in F9 cells: analysis at the mRNA level. (A) RT-PCR was performed on cDNA generated from RNA isolated from wild-type F9 cells. Primers specific for each RGS cDNA were utilized in the PCR reaction. PCR products were separated by electrophoresis on a 1% agarose gel and stained with ethidium bromide. RGS3, 5, 10, 12, 14, 20, 17, 9, 11, axin and conductin are expressed at the mRNA level in F9 cells. The lane labeled ‘No cDNA’ shows that the PCR reaction is not contaminated with exogenous DNA. bp, base pairs; MK, marker ladder. (B) Members of each RGS subfamily are expressed in F9 cells (R4, R12, RZ, R7, RA). All RGS proteins examined for mRNA expression are listed. Italic-bold type indicates expression in F9 cells.

only of RGS19 attenuated Wnt3a-induced gene transcription by more than 50% (Fig. 2A). Overexpression of other RGS proteins, however, including RGS3, 5, 9, 10, 11, 12, 14, 16, 17 and 20, had no effect on Wnt3a stimulation of Lef-Tcf-sensitive transcriptional activation. Western blot analysis of RGS proteins tagged with hemagglutinin (HA) showed similar levels of expression for most of the constructs tested (Fig. 2B). RGS5, RGS12 and RGS14 also were expressed in the F9 cells, albeit at levels less than those routinely observed for RGS19 and some other RGS proteins. Thus, we can state only that, when expressed at the cellular levels shown, RGS5, RGS12 and RGS14 fail to influence signaling of the Wnt–β-catenin canonical pathway. RGS19 expression had no effect on a control reporter plasmid containing eight mutated Tcf-binding sites (data not shown). In addition, RGS19 was a high-value target as the PDZ-domain-containing adaptor known as GAIp-interacting protein C-terminus (GIPC) interacts with both RGS19 (De Vries et al., 1998) and Fz (Tan et al., 2001). Both
overexpression and suppression of GIPC inhibits induction of neural crest by Fz3 in *Xenopus* (Tan et al., 2001). Based upon these observations, RGS19 was selected for a more detailed study of its role in Wnt–β-catenin signaling.

The results from the RT-PCR analysis of RGS19 mRNA were extended to the level of RGS19 protein expression. Crude cytosolic and cell membrane subcellular fractions were prepared from F9 cells, the proteins separated by SDS-PAGE and subjected to immunoblotting (IB). One band (molecular mass 29 kDa) in the cytosolic fraction and two bands (27 kDa and 29 kDa) in the membrane preparation were detected prominently by an RGS19-specific antibody (Fig. 2C). The doublet of RGS19 associated with the membrane is likely to reflect the known protein phosphorylation of this RGS protein, as evidenced by the reduction in the upper band after treatment with alkaline phosphatase, and as described in previous reports (Fischer et al., 2000).

**RGS19 overexpression attenuates Wnt3a-induced Lef-Tcf reporter activation**

A time-course for Wnt3a-stimulated gene transcription was performed in F9 cells. Wnt3a stimulated an approximately sixfold increase in gene transcription (as measured by luciferase activity, Fig. 3A). Wnt-stimulated gene transcription displayed a sharp increase at six to eight hours following stimulation by Wnt3a. Maximal Wnt-stimulated activity was observed at 10 hours, declining thereafter. Wnt–β-catenin signaling was probed in F9 cells transiently transfected with an expression vector harboring RGS19. Expression of RGS19 attenuated the maximal response of Wnt3a-stimulated gene transcription. Transient expression of RGS19 under these conditions suppressed the Wnt3a-induced response by at least 50%. Basal activity and the kinetics of the reporter gene response, by contrast, were largely unaffected by RGS19 expression.

In order to detail the relationship between RGS19 overexpression and Wnt3a-stimulated β-catenin–Lef-Tcf transcription, we sought to establish a dose-response relationship for increased expression of RGS19 protein and the Wnt-stimulated gene transcription response. Increasing levels of RGS19 expression were found to progressively inhibit Wnt3a-mediated gene transcription (Fig. 3B). In the absence of exogenous RGS19, Wnt3a promoted an approximately sixfold increase in reporter activation. At the lowest level of RGS19 expression tested (0.05 μg DNA/well), a small, but reproducible, decrease in Wnt3a-stimulated transcription also was observed. At higher levels of RGS19 expression (0.2 μg DNA/well), Wnt-stimulated gene transcription was inhibited by 33%. At the highest level of RGS19 expression tested (0.4 μg DNA/well), both Wnt3a-stimulated as well as basal transcriptional activity were found to be markedly suppressed. The expression level of HA-tagged RGS19 agrees well with the relative amount of DNA used in the transfections, as documented by immunoblotting of whole-cell extracts subjected to SDS-PAGE and immunoblotted with an antibody against HA (Fig. 3B). The expression of HA-RGS19 at the highest level tested represents less than 10% of endogenous RGS19 (data not shown).

To address the specificity of the effect of overexpression of RGS19 protein, a homologous member of the RZ subfamily (RGS17) was also expressed to analyze its influence on
Wnt3a-β-catenin signaling. RGS17 is 58% identical (76% similar) in amino acid sequence to RGS19 and in vitro accelerates the GTPase activity of several Gα subunits (Mao et al., 2004). Expression of RGS17 was found to have no effect on Wnt3a-induced activation of Lef-Tcf-sensitive transcription (Fig. 3C). Increased expression of RGS19, by contrast, progressively inhibited Wnt3a-mediated gene transcription (Fig. 3B,C). Expression of RGS17 did not alter Lef-Tcf transcription, even at the highest levels of transfection with the same expression vector harboring RGS19. Expression levels of HA-tagged RGS17 and HA-tagged RGS19 were similar, as documented by immunoblotting of whole-cell lysates subjected to SDS-PAGE and detected with an antibody against HA (Fig. 3D). Thus, expression of RGS19 protein attenuates the Wnt3a-β-catenin–Lef-Tcf pathway in a specific, dose-dependent and time-dependent manner, whereas expression of the highly homologous RGS17 protein does not.

Constitutively active Gαo rescues inhibition of Wnt3a action by RGS19

RGS proteins regulate heterotrimeric G-protein signaling by accelerating GTP hydrolysis by the activated Gα subunit. If the hypothesis that RGS19 attenuates Wnt3a function by this mechanism is correct, then expression of a mutant G-protein that remains in the active state would be expected to rescue the RGS19 effect. We tested the ability of the expression of constitutively active Gα subunits to rescue the inhibitory effects of RGS19 on Wnt3a-mediated gene transcription (Fig. 4A). Transient transfection with an expression vector harboring the constitutively active Q205L mutant of Gαo was able to rescue Wnt3a-stimulated Lef-Tcf activation in cells expressing RGS19 protein (Fig. 4A, left-hand panel). Expression of the constitutively active Q209L mutant of Gα11, or the constitutively active Q209L mutant of Gαq, by contrast, failed to rescue the Wnt3a-stimulated transcription in RGS19-expressing cells (Fig. 4A, right-hand and lower panels).

**Fig. 3.** RGS19 overexpression attenuates Wnt3a-induced Lef-Tcf reporter activation. (A) F9 cells transiently transfected with vectors containing Fz1, M50 and RGS19 were treated with Wnt3a for the indicated lengths of time, then collected and subjected to luciferase assay. Overexpression of RGS19 (+ RGS19) blocked Wnt3a-induced reporter activation in a time-dependent manner. *P<0.05 for the difference between wild-type, Wnt3a-treated cells and those expressing RGS19 and Wnt3a-treated for the indicated time points. RLU, relative luciferase units. (B) F9 cells transiently transfected with vectors containing Fz1, M50 and the indicated amounts of RGS19 were treated with or without Wnt3a for 8 hours, then collected and subjected to the luciferase assay. The results show that increasing levels of RGS19 can block the Wnt3a-induced reporter activation. *P<0.05 for the difference between wild-type, Wnt3a-treated cells and those expressing RGS19 and Wnt3a-treated for the indicated time points. **P<0.05 for the difference between wild-type, untreated cells and those expressing RGS19 for the individual time points. Lower panel: immunoblotting (IB) western blot analysis was used to measure the expression of transiently transfected HA-tagged RGS19. Lysates were collected from cells transfected with the indicated amounts of HA-RGS19, then subjected to SDS-PAGE on a 12.5% acrylamide gel, transferred to nitrocellulose and probed with an antibody against HA. The expression of actin was used as a loading control. The results displayed are data from single experiments, representative of more than three independent tests. (C) F9 cells transiently transfected with vectors containing Fz1, M50 and the indicated amounts of RGS17 or RGS19 were treated with or without Wnt3a for 8 hours, then collected and subjected to luciferase assay. RGS19 suppressed Wnt3a signaling in a dose-dependent manner, whereas overexpression of RGS17 had little effect. Results are displayed as the difference in reporter activation upon Wnt3a stimulation (means ± s.e.) as compared with untransfected control and are representative of three independent experiments. *P<0.05 for the difference between wild-type cells and those expressing RGS19 at each concentration. (D) IB western blot analysis was used to measure the expression of transiently transfected HA-tagged RGS19 and RGS17. Lysates were collected from cells transfected with the indicated amounts of HA-RGS19 and HA-RGS17, then subjected to SDS-PAGE on a 12.5% acrylamide gel, transferred to nitrocellulose and probed with an antibody against HA.
Expression of either G-protein alone had no effect on Wnt3a-stimulated gene transcription. Expression of each G-protein was established by immunoblotting of whole-cell lysates subjected to SDS-PAGE and detected with antibodies specific for each protein (Fig. 4B). Note that the mutant form of G\(_{\alpha}\) displays a small, but reproducibly greater, mobility in these gels.

RGS19 expression attenuates the \(\beta\)-catenin stabilization that results from Wnt3a stimulation

A hallmark of canonical Wnt signaling is the intracellular accumulation of \(\beta\)-catenin (Peifer et al., 1994). If our hypothesis that RGS19 expression accelerates deactivation of G\(_{\alpha}\) and thereby suppresses Wnt3a-stimulated Lef-Tcf-sensitive transcription is correct, then one would predict that overexpression of RGS19 should counter the stabilizing effect of Wnt3a on intracellular \(\beta\)-catenin accumulation (Fig. 5). Basal levels of cytosolic \(\beta\)-catenin are low in the absence of stimulation of the canonical pathway by Wnt3a (Fig. 5A). Upon stimulation by Wnt3a, \(\beta\)-catenin accumulates in the cytosol, with a peak of intracellular accumulation of \(\beta\)-catenin occurring at 2-3 hours. This Wnt3a-stimulated accumulation of \(\beta\)-catenin was observed for more than 5 hours (Fig. 5A).

We tested our hypothesis by overexpressing RGS19 in F9 cells and then measuring \(\beta\)-catenin levels in the cytosol of cells not treated with Wnt3a (time=0). \(\beta\)-catenin levels were suppressed in F9 cells overexpressing RGS19 protein (time=0). The intracellular accumulation of \(\beta\)-catenin in response to stimulation by Wnt3a was obvious in control cells and clearly countered by overexpression of RGS19 protein. The maximal level of \(\beta\)-catenin still occurs at ~3 hours in cells expressing RGS19 protein but remains well below the \(\beta\)-catenin levels observed in the cytosol of cells transfected with an empty vector. Quantification of the data from multiple experiments confirms, at the level of \(\beta\)-catenin, our hypothesis that RGS19 regulates Wnt3a control of Lef-Tcf transcription (Fig. 5B).

RGS19 attenuates Wnt–\(\beta\)-catenin signaling upstream of Dvl

Phosphorylation of Dvl3, an initial cellular response to stimulation of the canonical pathway by Wnt3a, is upstream of \(\beta\)-catenin degradation and downstream of activation of Fz1 (Gonzalez-Sancho et al., 2004; Yanagawa et al., 1995). Analysis by SDS-PAGE reveals nonphosphorylated and phosphorylated forms of Dvl3 in F9 cells (Fig. 6A). In the absence of Wnt stimulation (i.e. at 0 minutes), two Dvl3 isoforms (82 kDa, 85 kDa) are apparent, their presence presumed to reflect different degrees of protein phosphorylation. To establish whether these isoforms of Dvl3 are truly differentially phosphorylated forms of the protein, we treated the samples with purified calf intestinal alkaline phosphatase (Fig. 6B). Upon alkaline phosphatase treatment, only the band with highest mobility remained, providing evidence to support the tenet that the band of slower mobility is indeed the phosphorylated form of Dvl3.

Treating wild-type F9 cells with Wnt3a provokes a sharp increase in the relative amounts of slower migrating phosphoprotein.
Dv1 (Fig. 6A). In response to Wnt3a, the band of slowest mobility increases in intensity. If the hypothesis that RGS19 regulates Wnt3a signaling is correct, overexpression of RGS19 would be expected to suppress this proximal step in the pathway – that is, Wnt3a-stimulated phosphorylation of Dv1. Overexpression of RGS19 did not influence the pattern of Dv1 phosphorylation in those F9 cells not treated with Wnt3a (Fig. 6A). In the Wnt3a-treated cells, however, overexpression of RGS19 sharply reduced the amount of the slower migrating phosphorylated form of Dv1.

If the hypothesis that RGS19 exerts its inhibitory effects on Wnt–β-catenin signaling at the level of Gαq is correct, then activation of the pathway at a level downstream of the G-protein should be resistant to RGS19 action. Expression of Dv1 results in stimulation of Lef-Tcf-sensitive gene transcription (Fig. 6C). Expression of RGS19 is unable to block Dv1-mediated transcription, bolstering evidence that RGS19 inhibits Wnt action upstream of Dv1 phosphorylation.

Overexpression of RGS19 protein attenuates Wnt3a-induced formation of PE

Wnt3a and retinoic acid (RA) both stimulate F9 cells to form PE, an essential step in early stages of mouse development, through distinct signaling pathways. F9 cells promoted to PE no longer express embryonic markers but instead express increased PE-specific markers, including cytokertatin endo A,
recognized by the monoclonal antibody TROMA (Strickland and Mahdavi, 1978). These F9 cells form PE in response to Wnt3a or RA, demonstrated by an increase in the expression of the cytokeratin endo A marker (Fig. 7A). Cells transfected with an expression vector harboring RGS19 display a reduction in the PE-specific marker after Wnt3a stimulation (Fig. 7A). By contrast, RGS19 does not block the ability of RA to induce expression of cytokeratin endo A and formation of PE. Quantification of the data from several experiments indicates that overexpression of RGS19 attenuates Wnt3a-induced PE formation by 50% (Fig. 7B), similar in magnitude to its suppression of signaling from Gαo, to its suppression of the level of β-catenin and to the reduced level of induction of Lef-Tcf-sensitive gene transcription.

Indirect immunofluorescence was utilized to further characterize the expression of the cytokeratin endo A marker upon Wnt3a treatment (Fig. 7C). Untreated F9 cells do not stain with the TROMA antibody. However, cells treated with Wnt3a showed an increase in fluorescence, indicating expression of cytokeratin endo A and formation of PE. Overexpression of RGS17 had no effect on the ability of Wnt3a to induce formation of PE. However, cells expressing RGS19 were unable to form PE and showed decreased TROMA staining.

Knockdown of RGS19 attenuates Wnt3a-induced Lef-Tcf reporter activation and β-catenin accumulation

In order to further define the role of RGS19 in Wnt–β-catenin signaling, specific siRNA constructs were utilized to knockdown RGS19 protein expression in F9 cells. Cells treated with a ‘control’ siRNA sequence provided by the commercial supplier displayed a robust Lef-Tcf transcriptional response to Wnt3a stimulation (Fig. 8A). By contrast, cells treated with an siRNA directed against RGS19 showed a >50% decrease in Wnt3a-stimulated gene transcription. Two distinct siRNA sequences directed against RGS19 were tested, each giving similar responses to Wnt3a-mediated gene transcription (data not shown). Western blot analysis confirmed that the specific siRNA directed against RGS19 was effective, reducing RGS19 protein levels to less than 10% of those of control cells (Fig. 8B).

In order to confirm that the attenuation of Lef-Tcf-mediated transcription by RGS19 knockdown was dependent upon the Wnt–β-catenin pathway, we examined β-catenin stabilization in response to Wnt3a in cells treated with or without the RGS19 siRNA (Fig. 8C). Cells transfected with the control siRNA displayed a marked accumulation of cytosolic β-catenin 2 hours after Wnt3a stimulation, and β-catenin levels remained stable past 4 hours. Knockdown of RGS19 countered the Wnt3a-mediated accumulation of β-catenin by 50%, at both 2-hour and 4-hour time-points while having no effect on basal β-catenin levels.

As both overexpression and knockdown of RGS19 attenuated Wnt–β-catenin signaling, we hypothesized that perhaps some optimal level of RGS19 was necessary for maximal signaling output. In order to address this issue, a ‘rescue’ experiment was performed by adding increasing amounts of RNAi-resistant RGS19 cDNA to cells lacking endogenous RGS19 (Fig. 8D). As shown above, transfection of F9 cells with siRNA directed against RGS19 attenuated Wnt3a-stimulated gene transcription by 50%. Addition of low levels of exogenous RGS19 (0.2 μg) was able to rescue the siRNA-mediated effect and restore wild-type levels of Wnt3a-induced gene transcription. However, increasing amounts of
exogenous RGS19 attenuated gene transcription in a dose-dependent manner, highlighting the crucial importance of the level of RGS19 expression in the regulation of Wnt–β-catenin signaling.

Discussion

The Wnt–β-catenin pathway plays a crucial role in development (Moon et al., 2004; Nusse, 2005), and dysregulation of this pathway can provoke diseases such as cancer (Reya and Clevers, 2005). Understanding the mechanisms by which cells respond to and modulate the Wnt downstream signals is therefore a focal point of investigation. Recent research has demonstrated that heterotrimeric G-proteins (including Go) are essential transducers of the Wnt–β-catenin–Lef-Tcf pathway, from fly to mouse (Katanaev et al., 2005; Liu et al., 2001; Liu et al., 2005; Malbon, 2004; Malbon, 2005; Slusarski et al., 1997). We investigated whether RGS proteins, which can regulate heterotrimeric G-proteins, might also regulate Wnt signaling. RGS proteins act as GTPase-activating proteins (GAPs) for heterotrimeric G-proteins, increasing the intrinsic rate of GTP hydrolysis and accelerating signal termination (Hollinger and Hepler, 2002). RGS family members have essential roles in many physiological processes, including cardiovascular function (Heximer et al., 2003) and visual signal transduction (Chen et al., 2000). Earlier work in Xenopus provided the first suggestion that RGS proteins impact development (Wu et al., 2000). These observations can now be understood more fully following demonstration of an obligate role for G-proteins in Wnt signaling (Malbon, 2005).
expression of mRNA for RGS proteins, focusing on the RZ subfamily member RGS19, in F9 mouse teratocarcinoma cells. RGS19, expressed in embryonic F9 cells, specifically attenuates Wnt3a-stimulated signaling, including Dvl phosphorylation, β-catenin accumulation, gene transcription and formation of PE. As G-proteins are known to be positive regulatory elements, our hypothesis was that RGS proteins, if involved, would counter the effects of Wnt3a on the Wnt–β-catenin–Lef-Tcf–PE pathway. We found that RGS19 overexpression, as well as its absence, suppressed the Wnt–β-catenin pathway.

Heterotrimeric G-proteins are molecular switches that cycle between active (GTP-bound) and inactive (GDP-bound) states through the action of specific GDP-GTP exchange factors (Cabrera-Vera et al., 2003). Both positive and negative regulatory elements are required for control over G-protein signaling output. Upon ligand binding, GPCRs act as positive regulatory elements by exchanging the inhibitory GDP for GTP on the G-protein α-subunit. RGS proteins inhibit G-protein activation by stabilizing the transition state of the Gα-subunit for GTP hydrolysis, resulting in GDP production (Hollinger and Hepler, 2002). If RGS19 acts as a GAP for Gαo in the Wnt–β-catenin pathway, a rescue experiment can be performed through the use of a constitutively activated, mutant form of a G-protein (which cannot be regulated by its cognate RGS proteins). Indeed, cells transfected with Q205L Gαo, but not Q209L Gαo1 or Q209L Gαo2, were able to restore wild-type levels of Wnt-responsiveness after RGS19 inhibition.

Phosphorylation of Dvl is required for inhibition of the β-catenin degradation complex upon activation of the Wnt–β-catenin pathway (Yanagawa et al., 1995). Although the kinase responsible for Wnt-stimulated phosphorylation remains elusive, epistasis experiments place Dvl downstream of the heterotrimeric G-protein Gαo (Katanaev et al., 2005) and, consequently, Dvl3 phosphorylation in response to Wnt3a. Expression of RGS19 suppressed Wnt3a-induced Dvl3 phosphorylation. In addition, RGS19 was unable to attenuate Dvl3-induced Lef-Tcf reporter activation, suggesting that RGS19 inhibition of Wnt–β-catenin signaling occurs upstream of Dvl.

Intracellular accumulation of β-catenin in response to Wnt3a stimulation is a defining feature of canonical Wnt signaling (Peifer et al., 1994). Cytosolic β-catenin enters the nucleus, binds to members of the Lef-Tcf family of transcription factors and activates transcription of target genes (Molenaar et al., 1996). To confirm the specificity of the RGS19 inhibition of Lef-Tcf-mediated transcription, β-catenin accumulation in response to Wnt3a was measured in cells expressing RGS19. RGS19 expression diminished β-catenin accumulation in response to Wnt3a. This result agrees with the overarching hypothesis tested herein that RGS19 regulates Wnt–β-catenin signaling upstream of β-catenin accumulation. Likewise, expression of RGS19 suppressed Wnt3a-induced PE formation in F9 cells, as measured by expression of the marker cytokeratin endo A.

Wnt–β-catenin signaling is mediated through the Lef-Tcf family of transcription factors (Molenaar et al., 1996). Lef-Tcf-sensitive signaling was measured through the use of a luciferase-based reporter construct (Veeman et al., 2003). RGS19, but not other RGS proteins, attenuated gene reporter activation in a dose- and time-dependent manner. In order to probe the specificity of the RGS19-induced inhibition of Wnt3a signaling, a closely related RGS protein, RGS17, was also tested for its ability to alter Wnt3a-mediated gene transcription. Expression of RGS17 had no effect on Wnt3a-induced reporter activity. Therefore, although by in vitro reconstitution studies RGS17 has been shown to accelerate GTPase activity of several Gα proteins including Gαo, RGS19 is able (and RGS17 unable) to regulate Gαo in the context of Wnt3a–Fz1 signaling in vivo. This is not a surprising result, given our knowledge about RGS specificity. Early reports on RGS protein action by in vitro reconstitution revealed promiscuity of RGS proteins for G-protein α-subunits. RGS4 and RGS19, for example, were shown to act as GAPs in vitro for virtually all members of the Gα family (Berman et al., 1996). The results from in vivo approaches appear to be more complex (Xie et al., 2005). RGS4 and RGS19 can regulate signaling through Gα-coupled opioid receptors (Xie et al., 2005). However, each RGS protein has been observed to display a distinct preference for regulating signaling through specific receptors, even when coupled to the same G-protein (Xie et al., 2005). Therefore, RGS protein action is not only a function of the G-protein α-subunit but also of the GPCR whose action is being regulated and the cellular context that dictates the proper stoichiometry of signaling elements.

RGS proteins negatively regulate G-protein signaling by acting as GAPs for heterotrimeric G-proteins. Therefore, knockdown of RGS proteins would be expected to result in increased signaling output. However, recent studies in Caenorhabditis elegans (Ferkey et al., 2007) and humans (Nishiguchi et al., 2004) have shown that loss of specific RGS proteins can disrupt distinct G-protein-mediated physiological events. In C. elegans, chemosensory is controlled by G-protein-linked signaling cascades that are regulated by RGS-3 (homologous to mammalian RGS8) (Ferkey et al., 2007). Interestingly, animals lacking RGS-3 were unable to respond to strong sensory stimulation, despite the normal role of RGS-3 being to act as a negative regulator of this function. Similarly, light sensing in vertebrates is mediated by G-proteins and regulated by a specific RGS protein, RGS9 (Chen et al., 2000). Humans lacking functional RGS9 protein display slow photoreceptor deactivation, resulting in difficulty adjusting to rapid changes in light intensity (Nishiguchi et al., 2004). Therefore, despite the role of RGS9 as an inhibitor of G-protein signaling, loss of RGS9 also results in impaired visual signal transduction. In the present study, we show that both the extremes of overexpression and of knockdown of RGS19 protein attenuate Wnt3a–β-catenin signaling. Titration of RGS19 protein levels through either siRNA-mediated knockdown or overexpression of RNAi-resistant RGS19, however, highlights the crucial importance of proper levels of RGS19 for maximal Wnt–β-catenin signal transduction. In addition, overexpression or knockdown of the RGS19-binding partner GIPC inhibits Wnt signaling in Xenopus, suggesting a common necessity for proper physiological stoichiometry. That RGS proteins function as more than simple negative regulators of G-proteins reflects the need for more detailed probes into the complex roles for RGS19 in Wnt signal transduction.
Materials and Methods

Plasmids
Mammalian expression vectors pCDNA3.1 encoding RGS3, 5, 9, 10, 14, 16, 17, 19 and 20, Q290L, GnaA, Q290L, Gna11, and Q290L, Gna5, were obtained from UMR cDNA Resource Center (University of Missouri-Rolla, MO). Expression vectors containing Fz1 and M50 were provided by Randall Moon (Dept of Pharmacology, HHMI, University of Washington, Seattle, WA). Expression vectors encoding RGS11 and RGS12 were provided by David Siderovski (University of North Carolina, Chapel Hill, NC).

Antibodies
The rabbit antibody against RGS19 was provided by Susanne Mumbey (Dept of Pharmacology, University of Texas, Southwestern Medical Center, Dallas, TX). The monoclonal TROMA-1 antibody, recognizing the PE marker cytokeratin endo A, was generated by the University of Iowa Developmental Studies Hybridoma Bank (Iowa City, IA). The rabbit antibody against β-catenin and the mouse antibody against actin were obtained from Sigma (St Louis, MO). The mouse antibody recognizing Gna5 and Gna11 were obtained from Roche (Indianapolis, IN). The antibody recognizing Gna8 was obtained from BD Biosciences (San Jose, CA).

Cell culture and transfection
Mouse F9 teratocarcinoma cells were obtained from the ATCC (Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal bovine serum plus penicillin (60 μg/ml) and streptomycin (100 μg/ml) in a humidified atmosphere chamber supplied with 5% CO₂ at 37°C. Cells were transfected by using Lipofectamine (Invitrogen, Carlsbad, CA), according to the manufacturer’s suggested protocol.

Reverse-transcription polymerase chain reaction
Specific primers for amplification of regions of genes encoding the various RGS proteins and cyclophilin A (CycA) were obtained from Operon (Huntsville, AL) (Table 1). RNA was isolated from wild-type F9 cells using the RNA STAT-60 protocol. Briefly, medium was removed from confluent cultures of F9 cells, treated first with concanavalin A (Con A) (Aghib and McCrea, 1995). Con A (covalently linked to sepharose) was obtained from Amersham Biosciences (Upsala, Sweden). Confluent F9 cells were washed once with PBS and lysed in RIPA buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS) and imaged with a Zeiss LSM510 inverted fluorescence microscope.

Membrane preparation
Confluent F9 cells were collected into a new tube and directly assayed as described below. A sample (20 μl) of lysate was added to 100 μl of luciferase assay buffer (20 mM Tricine, pH 7.8, 1.07 mM MgCl₂, 4 mM MgSO₄, 0.1 mM EDTA, 0.27 mM coenzyme A, 0.67 mM luciferin, 33.3 mM DTT, 0.6 μM ATP), and a luminometer (Berthold Lumat LB 9507) was used to measure luciferase activity.

Cytosolic β-catenin assay
In order to separate cytosolic from membrane-associated β-catenin, samples were treated first with concanavalin A (Con A) (Aghib and McCrea, 1995), Con A (covalently linked to sepharose) was obtained from Amersham Biosciences (Upsala, Sweden). Confluent F9 cells were washed once with PBS and lysed in RIPA buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) plus leupeptin, aprotinin and PMSE. After collection into microfuge tubes on ice, lysates were rotated at 4°C for 20 minutes and then centrifuged for 25 minutes at 20,000 g. The supernatant was transferred into a new tube and directly assayed as described below. A sample (20 μl) of lysate was added to 100 μl of luciferase assay buffer (20 mM Tricine, pH 7.8, 1.07 mM MgCl₂, 4 mM MgSO₄, 0.1 mM EDTA, 0.27 mM coenzyme A, 0.67 mM luciferin, 33.3 mM DTT, 0.6 μM ATP), and a luminometer (Berthold Lumat LB 9507) was used to measure luciferase activity.

Table 1. Primer sequences used to amplify RGS proteins and cyclophilin A (CycA)

| Protein targeted | Forward (5’-3’) | Reverse (5’-3’) |
|------------------|----------------|----------------|
| RGS1             | GAAGAACTGTCCATGCCAACAA | TCTTGGTTGCCAGAGTCAAGC |
| RGS2             | GAAGAGAGCCGAGAAGAAAT | GAGGAGACCTTGGTAGGTA |
| RGS3             | CTTGAGAAAGGTGTGCTC | TAGAGCCTGACAGAGGAGG |
| RGS4             | AGCTGCAGAAATGCTCA | AGAACCGAGAGAGAAGTC |
| RGS5             | AGAAGGAGCGGCCCATTCA | GCTGCTGTTGCTGATTTG |
| RGS13            | TGAGAAGACCTCGTCTTGG | TGGAGAATGTCTCTCCCTTG |
| RGS16            | CTCCTGCAGAAATGCTCA | TGTGCTGTTGCTGAGTTC |
| RGS18            | CATGAGGTCAGAAAGAGA | CTCTGGTCAGGCTCAGTAT |
| RGS10            | GATGAGAAGCCGAGAAGAAT | GATGAGAAGCCGAGAAGAAT |
| RGS14            | TGGCTGCTGCTGCTGCT | TGGCTGCTGCTGCTGCT |
| RGS15            | GCCTGCAGAAATGCTCA | AGAACCGAGAGAGAAGTC |
| RGS33            | GCCTGCAGAAATGCTCA | AGAACCGAGAGAGAAGTC |
| Axin              | CACCTGCTGCTGCTGCT | CTCCTGCTGCTGCTGCT |
| Conduit          | CTCGGAGCTGTGGACGA | AGAGGTGCTGCTGCTGCT |
| RGS6              | AAAGAGAAAGAGAAGAAGA | AGAGGTGCTGCTGCTGCT |
| RGS7              | TCTTGGTTGCCAGAGTCAAG | TCTTGGTTGCCAGAGTCAAG |
| RGS9              | GAGGAGGAGAGGAGGAGGAGG | GAGGAGGAGAGGAGGAGGAGG |
| RGS11             | CACCTGCTGCTGCTGCT | CTCCTGCTGCTGCTGCT |
| CycA             | AGAAGGAGCCGAGAAGAAT | AGAAGGAGCCGAGAAGAAT |
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