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Wingless inactivates glycogen synthase kinase-3 via an intracellular signalling pathway which involves a protein kinase C

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The *Drosophila* gene product Wingless (Wg) is a secreted glycoprotein and a member of the *Wnt* gene family. Genetic analysis of *Drosophila* epidermal development has defined a putative paracrine Wg signalling pathway involving the zeste-white 3/shaggy (*zw3/sgg*) gene product. Although putative components of Wg- (and by inference Wnt-) mediated signalling pathways have been identified by genetic analysis, the biochemical significance of most factors remains unproven. Here we show that in mouse 10T1/2 fibroblasts the activity of glycogen synthase kinase-3 (GSK-3), the murine homologue of Zw3/Sgg, is inactivated by Wg. This occurs through a signalling pathway that is distinct from insulin-mediated regulation of GSK-3 in that Wg signalling to GSK-3 is insensitive to wortmannin. Additionally, Wg-induced inactivation of GSK-3 is sensitive to both the protein kinase C (PKC) inhibitor Ro31-8220 and prolonged pre-treatment of 10T1/2 fibroblasts with phorbol ester. These findings provide the first biochemical evidence in support of the genetically defined pathway from Wg to Zw3/Sgg, and suggest a previously uncharacterized role for a PKC upstream of GSK-3/Zw3 during Wnt/Wg signal transduction.

Keywords: glycogen synthase kinase-3/protein kinase C/ Ro31-8220/signal transduction/Wingless

Introduction

Wingless (Wg), the product of the *Drosophila melanogaster wg* gene, is a member of the Wnt family of developmental signalling molecules (reviewed in McMahon, 1992a,b; Nusse and Varmus, 1992). These proteins are secreted signalling molecules which play essential roles in developmental and cell fate processes. Genetic analysis of paracrine signalling by Wg during *Drosophila* epidermal development has defined a pathway from *wg* to zeste white-3/shaggy (*zw3/sgg*) via dishevelled (*dsh*) (Noordermeer *et al.*, 1994). The function of Dsh is currently unknown, although a mammalian counterpart has been cloned (Sussman *et al.*, 1994). Zw3/Sgg is a homologue of the mammalian protein-serine/threonine kinase glycogen synthase kinase-3 (GSK-3) (Plyte *et al.*, 1992). The loss of the *zw3/sgg* gene product results in the Wg-independent stabilization of engrailed expression (Siegfried *et al.*, 1992). These experiments have been used to argue that inactivation of Zw3/Sgg kinase forms part of a Wg signalling pathway (Siegfried *et al.*, 1992, 1994). However, direct biochemical evidence to support this genetic analysis and the identification of the signalling mechanism is currently lacking.

The mammalian GSK-3β isoform can partially compensate for the loss in *zw3/sgg* during *Drosophila* embryogenesis in *zw3/sgg* null mutants (Siegfried *et al.*, 1992). Recent data demonstrated that GSK-3 has an important function(s) during *Xenopus* development (Dominguez *et al.*, 1995; He *et al.*, 1995; Pierce and Kimelman, 1995), while a GSK-3 homologue in *Dictyostelium* plays a role in cell fate decisions (Harwood *et al.*, 1995). Mammalian GSK-3 has been implicated in signal transduction by growth factors including insulin (Welsh and Proud, 1993; Cross *et al.*, 1994, 1995; Welsh *et al.*, 1994), insulin-like growth factor-1 (IGF-1; Cross *et al.*, 1994), serum (Welsh *et al.*, 1994) and epidermal growth factor (EGF) (Saito *et al.*, 1994; Eldar-Finkelman *et al.*, 1995). These factors cause a rapid inactivation of GSK-3 kinase activity. GSK-3 has a broad spectrum of substrates, including the transcription factors c-Myc, c-Jun and c-Myb (reviewed in Plyte *et al.*, 1992). Phosphorylation of these substrates by GSK-3 appears to inhibit their function, consistent with the hypothesis that GSK-3 is an antagonist to processes essential to cell proliferation (Hughes *et al.*, 1993).

Phosphorylation of GSK-3β at Tyr216 is thought to be essential for protein function (Hughes *et al.*, 1993), although this tyrosine may not be essential for GSK-3 activity in all systems (Itoh *et al.*, 1995). This residue is analogous to the regulatory tyrosine of the mitogen-activated protein (MAP) kinase family, but GSK-3, unlike MAP kinases, is highly phosphorylated on tyrosine (and thus active) in resting cells. Although tyrosine dephosphorylation of GSK-3 correlates with the inactivation of GSK-3α by phorbol esters (Yang *et al.*, 1994), the majority of current studies show that GSK-3 is inactivated in response to growth factors via phosphorylation of serine residues, notably Ser9 in GSK-3β and Ser21 in GSK-3α (Sutherland *et al.*, 1993; Saito *et al.*, 1994; Stambolic and Woodgett, 1994). *In vitro*, several serine/threonine kinases have been demonstrated to phosphorylate and regulate GSK-3, including p70 S6 kinase, p90 S6 kinase ($p90^{rsk}$), Akt/protein kinase B (PKB) and some protein kinase C (PKC) isoforms (Goode *et al.*, 1992; Sutherland *et al.*, 1993; Stambolic and Woodgett, 1994; Sutherland and Cohen, 1994; Cross *et al.*, 1995). Of these kinases, only $p90^{rsk}$ and PKB have been demonstrated to phosphorylate and inactivate GSK-3 *in vivo* (Stambolic and Woodgett, 1994; Cross *et al.*, 1995). $p90^{rsk}$ itself is a target for regulation by MAP kinase (Sturgill *et al.*, 1988), and the MAP kinase cascade has been implicated in the signal

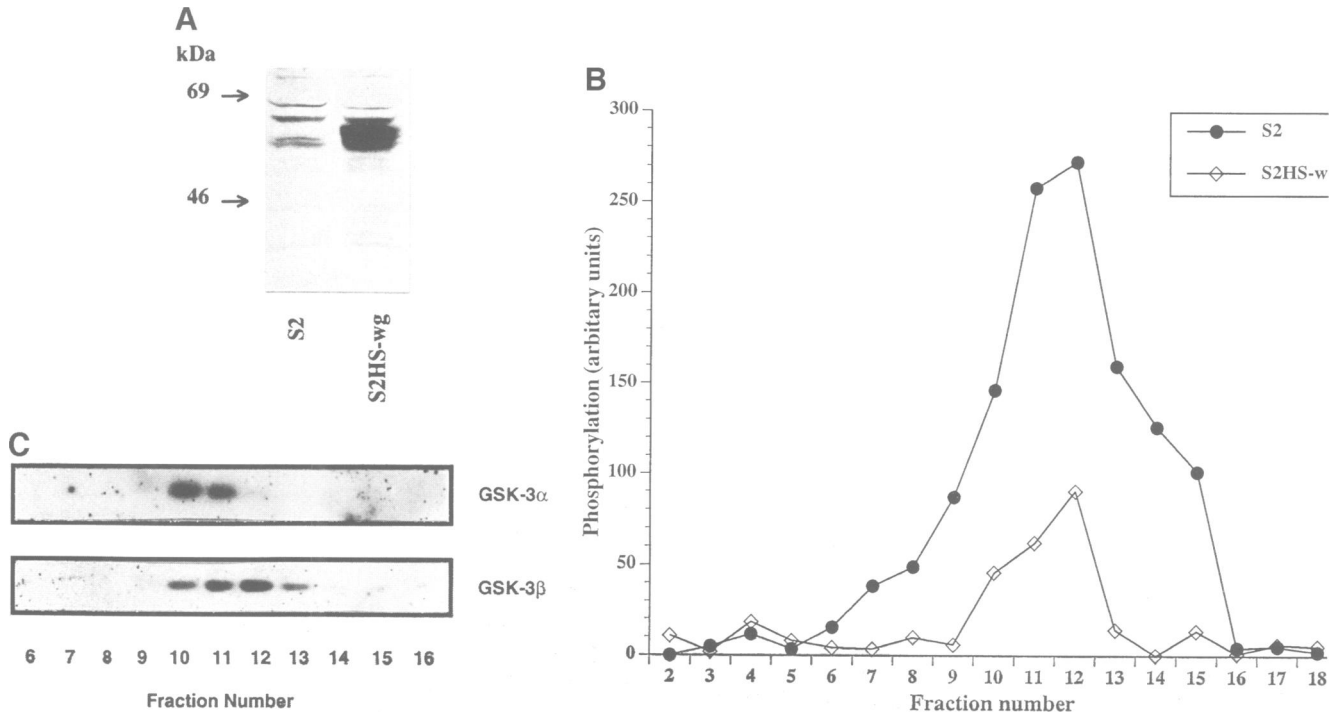

Fig. 1. GSK-3 activity in 10T1/2 mouse fibroblasts. **(A)** Schneider's *Drosophila* medium conditioned by heat-shocked S2 or S2HS-wg cells was Western blotted with the anti-Wg206 antiserum to demonstrate the presence of secreted Wg protein. **(B)** Cell extracts from confluent, serum-starved 10T1/2 fibroblasts were fractionated by FPLC on a Mono S column (Pharmacia) and analysed for GSK-3 activity. Confluent, serum-starved 10T1/2 cells were treated with 5× concentrated conditioned medium from heat-shocked S2 cells or S2HS-wg cells for 10 min prior to harvesting. Equal amounts of protein (as determined by Bio-Rad protein determination) were assayed. A representative experiment is shown. **(C)** FPLC Mono S column fractions were immunoblotted for the presence of GSK-3α and GSK-3β. Following addition of enhanced chemiluminescence reagents (Amersham), the blots were exposed to X-ray film for 16 h (GSK-3α) and 20 min (GSK-3β) respectively.

transduction of EGF to GSK-3 (Eldar-Finkelman *et al.*, 1995). PKB has been shown to be involved in insulin signalling to GSK-3 in a pathway which is independent of MAP kinase activation, but sensitive to the phosphoinositide (PI) 3-kinase inhibitor wortmannin (Cross *et al.*, 1995).

Wg and Wnt-1 appear to be functionally equivalent in their ability to transform RAC311c mammary cells, indicating that elements of Wg/Wnt signalling (including the elusive receptor) have been highly conserved throughout evolution (Ramakrishna and Brown, 1993). As the study of mammalian Wnt signalling has been hampered by the lack of soluble mammalian Wnt factors, we have used a *Drosophila* S2 cell line (S2HS-wg) containing a *wg* cDNA under the control of the hsp70 promoter, which can condition medium with active soluble Wg protein (Cumberledge and Krasnow, 1993; van Leeuwen *et al.*, 1994). To study regulation of GSK-3 by Wg, we used 10T1/2 fibroblasts which have been shown previously to undergo morphological transformation in response to ectopic *Wnt-1* gene expression (Bradbury *et al.*, 1994). Using S2HS-wg-conditioned media, we demonstrate that murine GSK-3 is inactivated by Wg in these cells, providing direct biochemical evidence in support of genetically defined models. The signalling is shown to be distinct from both insulin- and EGF-mediated inactivation of GSK-3, suggesting that Wg utilizes a novel signal transduction pathway to GSK-3. Additionally, inhibitor experiments suggest that a phorbol 12-myristate13-acetate (TPA)-sensitive isoform(s) of PKC lies upstream of GSK-3 in Wg signal transduction.

Results

Wg inhibits GSK-3 activity

Growth-arrested 10T1/2 fibroblasts were treated with serum-free conditioned medium prepared from either heat-shocked S2HS-wg (van Leeuwen *et al.*, 1994) or control S2 cells. S2HS-wg conditioned media contained readily detectable amounts of secreted Wg protein (Figure 1A). GSK-3 was partially purified by FPLC on a Mono S column and its activity measured against a phosphopeptide representing the GSK-3 phosphorylation site in glycogen synthase (GS-1) (Welsh and Proud, 1993). After 10 min of treatment with S2HS-wg-conditioned medium, the total kinase activity against the GS-1 peptide was suppressed to <50% of the activity observed in control cultures or in cultures treated with control (S2) conditioned medium (Figure 1B). A Western blot of fractions across the Mono S gradient with antisera specific for either the α or β form of GSK-3 demonstrated the presence of both GSK-3 isoforms within the peak of kinase activity (Woodgett, 1990) (Figure 1C). However, as these antisera have similar sensitivities (Woodgett, 1990) and the exposure of the GSK-3α immunoblot in Figure 1C is ~20 times longer than that of the GSK-3β immunoblot, GSK-3α represented only a minor species in 10T1/2 fibroblasts (<5% total GSK-3 protein; D.C. and M.J.F., unpublished data). So, the observed peak of GSK-3 activity probably predominantly represents that of GSK-3β alone.

The use of 5× concentrated conditioned medium did not substantially alter the observed level of GSK-3 inhibition compared with 1× (non-concentrated) conditioned

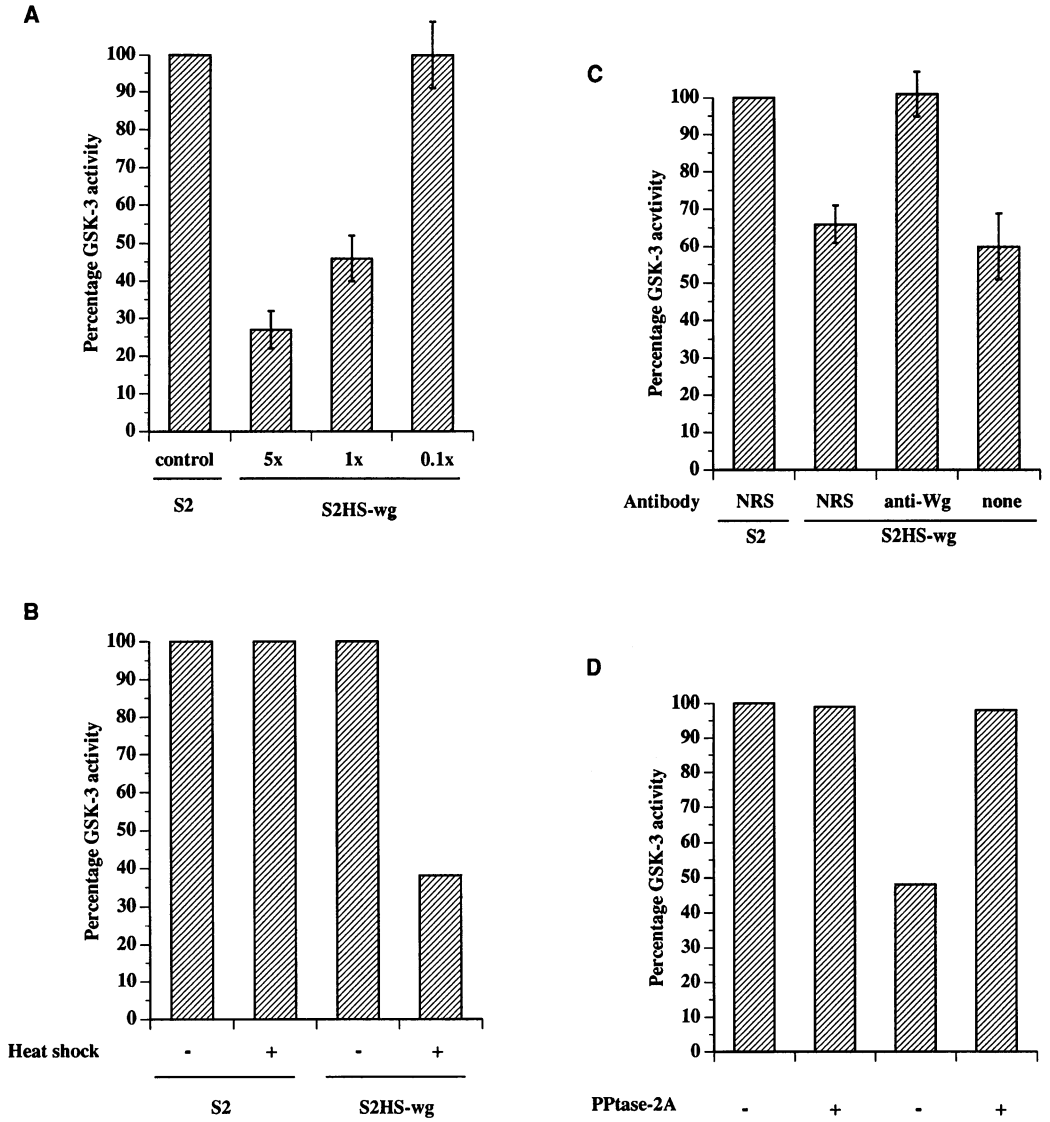

Fig. 2. Response of GSK-3 to conditioned medium. Cell extracts from confluent, serum-starved 10T1/2 cells were assayed for GSK-3 activity. (**A**) Titration of Wg-conditioned medium from heat-shocked S2HS-wg cells. Percentage activities are displayed relative to medium conditioned with heat-shocked S2 cells (either 5× or 1× concentrated). The 1× and 0.1× concentrated media (S2 and S2HS-wg) were diluted from 5× concentrated media with S2-conditioned media. Total GSK-3 activity was calculated as the area under the curve of quantified activity. The mean values from three independent experiments are shown. Bars indicate standard deviations. (**B**) Effect of heat-shocked and non-heat-shocked conditioned medium on GSK-3 activity; 5× concentrated medium was used. Percentage activities are shown relative to incubation of 10T1/2 cells with non-heat-shocked medium (either S2 or S2HS-wg). A representative experiment is shown. (**C**) Inhibition of Wg-induced GSK-3 antagonism by an anti-Wg neutralizing antiserum (van Leeuwen *et al.*, 1994). Percentage activities are displayed relative to S2-conditioned medium containing non-immunized rabbit serum (NRS). The mean values from three independent experiments are shown. (**D**) Restoration of GSK-3 activity by treatment with PPTase-2A. Experiments were performed either in the presence or absence of 0.5 U PPTase-2A. Percentage activities are displayed relative to 10T1/2 cells treated with S2-conditioned medium. The mean of two independent experiments is shown.

medium (Figure 2A). Inhibition of GSK-3 by S2HS-wg-conditioned media was comparable with that observed after the addition of insulin (5 μg/ml) or TPA (800 nM) to 10T1/2 cells (Figures 4, 5 and 7). By contrast, a 1 in 10 dilution of S2HS-wg-conditioned medium (0.1× conditioned medium) was sufficient to titrate out the GSK-3 response (Figure 2A). To exclude the possibility that constitutive differences between the medium conditioned by the S2HS-wg or S2 cells were responsible for GSK-3 inactivation, 10T1/2 cells were treated with medium conditioned by unshocked S2HS-wg cells (Figure 2B). No suppression of GSK-3 activity was detected relative to cells treated with S2-conditioned medium (either heat-shocked or unshocked), showing that heat shock treatment (and thus induction of Wg expression) was required for GSK-3 inactivation. To confirm that the observed inhibition of GSK-3 activity was due to soluble Wg, the conditioned medium was pre-incubated with either a neutralizing anti-Wg (van Leeuwen *et al.*, 1994) or control (non-immune) antiserum prior to assay on 10T1/2 fibroblasts. This antiserum has been shown previously to block the stabilization of armadillo by Wg in Cl-8 cells (van Leeuwen *et al.*, 1994). Treatment with anti-Wg antisera relieved the suppression of GSK-3 activity caused by S2HS-wg medium (Figure 2C). Two different non-immune control antisera, which were

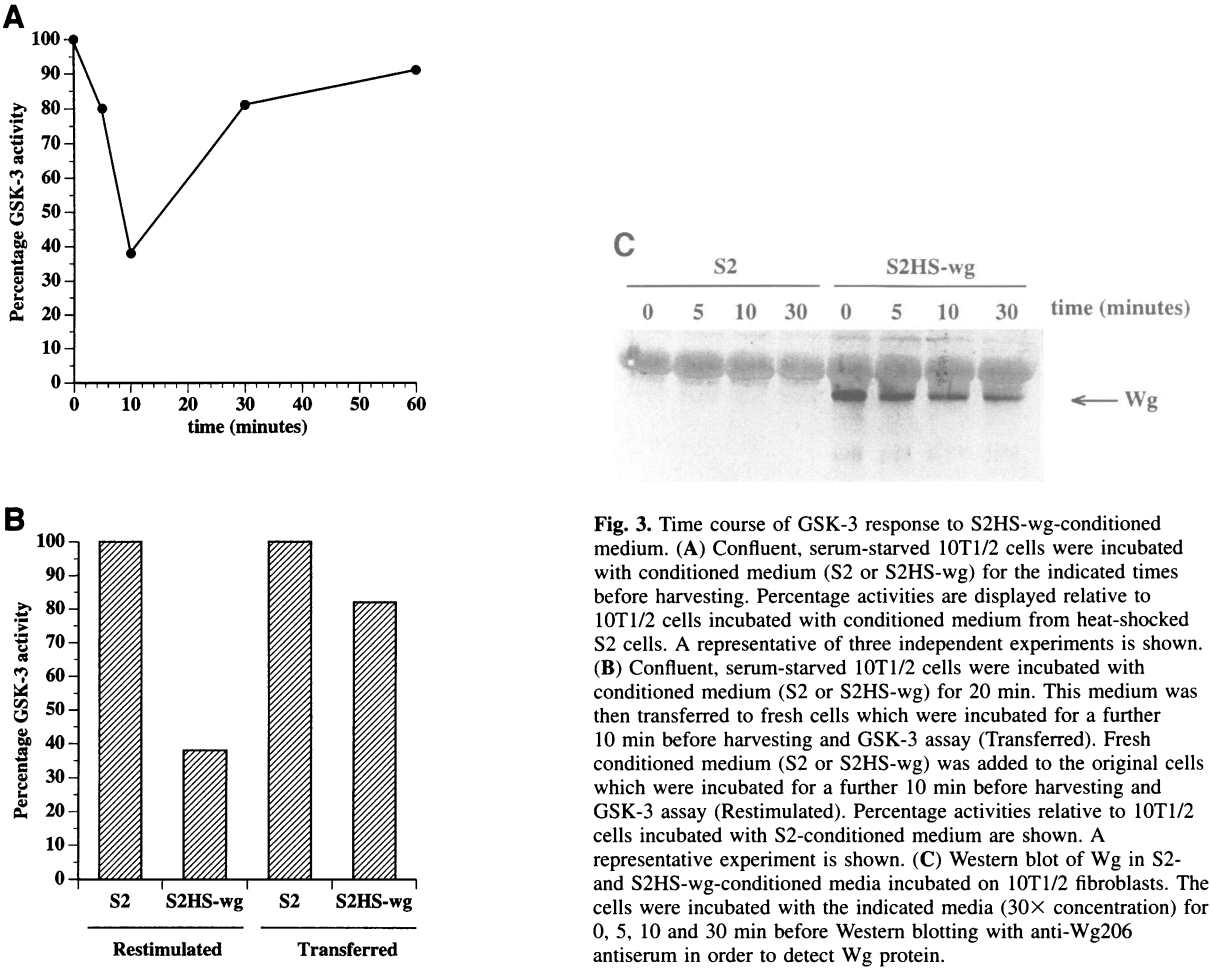

Fig. 3. Time course of GSK-3 response to S2HS-wg-conditioned medium. (**A**) Confluent, serum-starved 10T1/2 cells were incubated with conditioned medium (S2 or S2HS-wg) for the indicated times before harvesting. Percentage activities are displayed relative to 10T1/2 cells incubated with conditioned medium from heat-shocked S2 cells. A representative of three independent experiments is shown. (**B**) Confluent, serum-starved 10T1/2 cells were incubated with conditioned medium (S2 or S2HS-wg) for 20 min. This medium was then transferred to fresh cells which were incubated for a further 10 min before harvesting and GSK-3 assay (Transferred). Fresh conditioned medium (S2 or S2HS-wg) was added to the original cells which were incubated for a further 10 min before harvesting and GSK-3 assay (Restimulated). Percentage activities relative to 10T1/2 cells incubated with S2-conditioned medium are shown. A representative experiment is shown. (**C**) Western blot of Wg in S2- and S2HS-wg-conditioned media incubated on 10T1/2 fibroblasts. The cells were incubated with the indicated media (30× concentration) for 0, 5, 10 and 30 min before Western blotting with anti-Wg206 antiserum in order to detect Wg protein.

matched with the anti-Wg antisera for immunoglobulin content, had no effect upon GSK-3 activity (Figure 2C and D.Cook, unpublished data). Thus, secreted Wg protein was required for the observed antagonism of GSK-3 activity.

GSK-3 could potentially be inactivated in response to Wg by either serine phosphorylation or tyrosine dephosphorylation (Hughes *et al.*, 1993; Sutherland *et al.*, 1993; Saito *et al.*, 1994; Stambolic and Woodgett, 1994; Yang *et al.*, 1994). In order to address the mechanism by which S2HS-wg-conditioned media induced inactivation of GSK-3, we pre-incubated FPLC column fractions with the serine/threonine phosphatase protein phosphatase-2A (PPTase-2A) prior to kinase assay against GS-1 (Figure 2D). This treatment was sufficient to abolish the down-regulation in GSK-3 activity induced by S2HS-wg-conditioned media, restoring it to the levels of control cells. Addition of the serine/threonine phosphatase inhibitor okadaic acid (1 μM) to the PPTase-2A reaction mix prevented this reactivation of GSK-3 (D.Cook, unpublished data). Conversely, pre-treatment of 10T1/2 cells with the tyrosine phosphatase inhibitor sodium vanadate (500 μM) was unable to block inactivation of GSK-3 by S2HS-wg conditioned media (D.Cook, unpublished data). Thus, inactivation of GSK-3 by S2HS-wg conditioned media involved serine/threonine phosphorylation of the kinase.

Wg activity is lost after addition to 10T1/2 fibroblasts

To define further the GSK-3 response to S2HS-wg-conditioned medium, a time course was performed (Figure 3A). Inactivation of GSK-3 activity could be detected by 5 min and reached a maximal suppression of >50% at ~10 min. However, inhibition was transient and, after 30 min, the initial GSK-3 activity was almost fully restored. The response of GSK-3 to S2HS-wg-conditioned media is almost identical, in both level and duration, to that reported in response to EGF (Eldar-Finkelman *et al.*, 1995). We considered two explanations for the transient nature of the GSK-3 response to Wg. Firstly, that the Wg signalling pathway became desensitized to the presence of Wg within the S2HS-wg-conditioned media and, secondly, that Wg activity within the conditioned media was utilized, inactivated or sequestered during incubation with 10T1/2 cells.

To distinguish between these hypotheses, we stimulated 10T1/2 cells with S2HS-wg-conditioned media for 20 min. Media from these cells was then transferred to another dish of unstimulated 10T1/2 cells for an additional 10 min (Figure 3B—Transferred). Fresh S2HS-wg-conditioned media was added to the original cells and incubated for a further 10 min (Figure 3B—Restimulated). Restimulated cells demonstrated inactivation of GSK-3 activity which was comparable with the maximal inhibition of GSK-3 observed after a single addition of S2HS-wg-

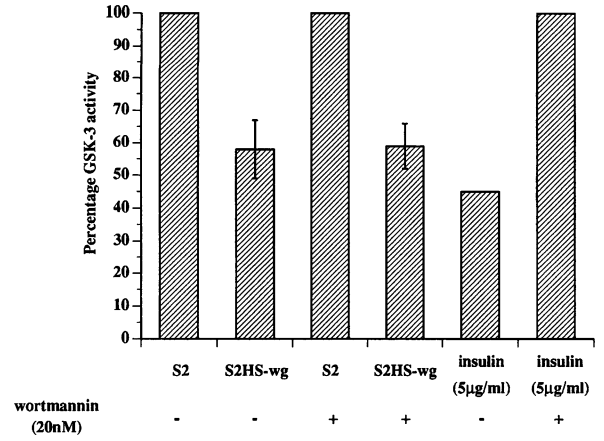

Fig. 4. Effect of wortmannin on GSK-3 inhibition by S2HS-wg-conditioned medium and insulin. Confluent, serum-starved 10T1/2 cells were incubated with conditioned medium (S2 or S2HS-wg) or conditioned S2 medium supplemented with insulin (5 μg/ml final concentration). Cells were pre-treated with either 20 nM wortmannin or carrier (DMSO 1:1000 v/v) for 10 min prior to incubation. Percentage activity is displayed relative to 10T1/2 cells treated with either S2-conditioned medium (plus or minus 20 nM wortmannin) or S2-conditioned medium containing insulin (5 μg/ml) plus 20 nM wortmannin. The insulin experiments shown are the average of duplicate experiments. All the other experiments shown are the mean of three independent experiments.

conditioned media to unstimulated 10T1/2 cells (Figures 2 and 3). However, transferred media produced only a marginal response (~20% suppression of activity) in fresh 10T1/2 cells. Despite the transient response of GSK-3 to Wg (Figure 3A), 10T1/2 cells still retained the capacity to respond to further stimulation by S2HS-wg-conditioned media. This would suggest that the signalling pathway was not becoming desensitized to the presence of Wg, but that Wg activity was being utilized/lost after addition to 10T1/2 fibroblasts. This was confirmed by a Western blot, which demonstrated a reduction in the detectable amount of Wg protein within the S2HS-wg-conditioned media following incubation with 10T1/2 cells (Figure 3C).

Inactivation of GSK-3 by Wg is insensitive to wortmannin

In order to identify elements upstream of GSK-3 in Wg signalling, we studied pathways that have been implicated in the regulation of GSK-3 by other antagonists. The insulin-dependent inactivation of GSK-3 is blocked by pre-treatment of cells with nanomolar concentrations of wortmannin (Cross *et al.*, 1994, 1995; Welsh *et al.*, 1994), suggesting that a PI 3-kinase lies upstream of GSK-3 in this signalling pathway (Arcaro and Wymann, 1993). Studies in L6 myoblasts further suggest that PKB lies downstream of PI 3-kinase and directly upstream of GSK-3 in this pathway (Cross *et al.*, 1995). Additionally, IGF-1- and serum-induced antagonism of GSK-3 are also sensitive to wortmannin (Cross *et al.*, 1994; Welsh *et al.*, 1994). We examined the effect of wortmannin on GSK-3 activity in response to S2HS-wg-conditioned medium. Addition of wortmannin (20 nM) inhibited PI 3-kinase activity (M.J.Fry, unpublished data) and was able to relieve insulin-induced suppression of GSK-3 activity completely (Figure 4). In contrast, this concentration of wortmannin failed to block suppression of GSK-3 activity by Wg (Figure 4). Thus, Wg signalling to GSK-3 does not involve a wortmannin-sensitive pathway and appears to be distinct from that of insulin (Cross *et al.*, 1994, 1995; Welsh *et al.*, 1994).

Inactivation of GSK-3 by Wg is insensitive to rapamycin

In vitro p70 S6 kinase is capable of inhibiting GSK-3 activity (Sutherland *et al.*, 1993; Sutherland and Cohen, 1994). The activation of this S6 kinase lies on a pathway which can be inhibited by the immunosuppressant rapamycin (Price *et al.*, 1992). It has been reported previously that insulin signalling to GSK-3 is insensitive to rapamycin (Cross *et al.*, 1994). Addition of rapamycin (100 nM) was also unable to inhibit suppression of GSK-3 by Wg (Figure 5A), making it unlikely that Wg signalling involves p70 S6 kinase. To confirm this, and to assess further the potency of rapamycin and wortmannin, we looked directly at the p70 S6 kinase protein by Western blot analysis (Figure 5B and C). It has been shown previously that activated p70 S6 kinase is phosphorylated at a number of sites, resulting in the appearance of protein species with retarded mobilities on SDS–PAGE gels. Some of the p70 S6 kinase phosphorylation sites have been shown to be sensitive to wortmannin and some to rapamycin, suggesting that at least two distinct pathways converge on p70 S6 kinase (Weng *et al.*, 1995). Over a 30 min time course, insulin stimulated an alteration in the electrophoretic mobility of p70 S6 kinase (Figure 5B). In contrast, neither S2 nor S2HS-wg-conditioned media had any apparent effect upon p70 S6 kinase, suggesting that this kinase is not involved in Wg signal transduction. Wortmannin and rapamycin both affected the mobility shift of p70 S6 kinase in response to insulin (Figure 5C). Wortmannin blocked the mobility shift to the level of unstimulated cells, whilst rapamycin completely blocked the shift down to a single fast migrating species. Thus, while neither inhibitor affected Wg signalling to GSK-3, both were active.

The role of the p42/p44 MAP kinase cascade during inhibition of GSK-3 by Wg

EGF signalling to GSK-3 can be partially blocked by two dominant-negative MAP kinase kinases (MEKs) suggesting that, in contrast to insulin, activation of the MAP kinase cascade to $p90^{rsk}$ represents a major route of GSK-3 regulation by EGF (Eldar-Finkelman *et al.*, 1995). To examine whether Wg signalling to GSK-3 also involved the MAP kinase pathway, we examined the kinase activity and phosphorylation status of p42/p44 MAP kinase in response to S2HS-wg-conditioned media (Figure 6). Previously described agonists of MAP kinase, EGF, platelet-derived growth factor (PDGF) and insulin, all showed increases (~9-, 6- and 5-fold respectively) in total MAP kinase activity (Figure 6A). These factors also caused increased Tyr204 phosphorylation of p42/p44 MAP kinase as measured using a specific antiserum raised against phosphorylated MAP kinase (Figure 6B). In contrast, no difference in either activity or phosphorylation of p42/p44 MAP kinase was seen between cells treated with either S2HS-wg- or S2-conditioned media (Figure 6A and B). However, MAP kinase was marginally activated (~2-fold) by both S2- and S2HS-wg-conditioned media in

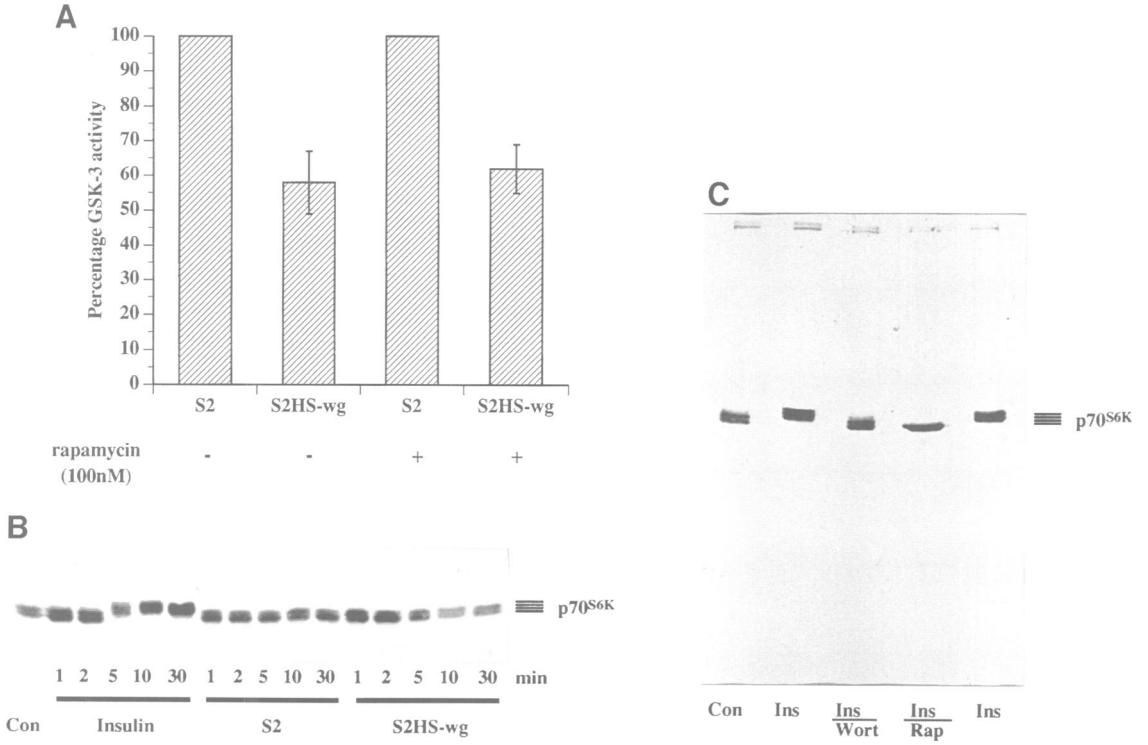

Fig. 5. Effect of rapamycin on GSK-3 inhibition by S2HS-wg-conditioned medium. (**A**) Confluent, serum-starved 10T1/2 fibroblasts were incubated with conditioned medium (S2 or S2HS-wg). Cells were pre-treated with 100 nM rapamycin or DMSO (1:1000 v/v) for 10 min prior to incubation. Percentage activity is displayed relative to 10T1/2 cells treated with S2-conditioned medium (plus or minus 100 nM rapamycin). The mean of three independent experiments is shown. (**B**) Confluent, serum-starved 10T1/2 cells were stimulated with insulin (5 μg/ml in S2-conditioned medium), S2- or S2HS-wg-conditioned medium for the indicated times. Con represents untreated cells. Cells were washed twice with ice-cold PBS and then lysed in boiling SDS–PAGE buffer. Lysates were used for immunoblot analysis and probed with an antibody against p70 S6 kinase. Detection was by enhanced chemiluminescence (ECL, Amersham). (**C**) Confluent, serum-starved 10T1/2 fibroblasts were pre-treated with either 10 nM wortmannin (Wort), 100 nM rapamycin (Rap) or an equivalent volume of vehicle (DMSO) alone (all other samples) for 30 min prior to stimulation with 5 μg/ml insulin (Ins) in DMEM for 10 min. Lysates were prepared and analysed as above.

comparison with Dulbecco's modified Eagle's medium (DMEM)-treated 10T1/2 fibroblasts (Figure 6A and B). Additional studies of MAP kinase activity following purification by FPLC anion exchange on a Mono Q column confirmed these observations (M.J.Fry, unpublished data). To determine whether the minor activation in p42/p44 MAP kinase induced by S2HS-wg-conditioned media was necessary for Wg signalling to GSK-3, 10T1/2 cells were treated with the MEK inhibitor, PD 98059 (Dudley *et al.*, 1995). PD 98059 (10 μM) was unable to prevent regulation of GSK-3 by S2HS-wg-conditioned media (Figure 6C). By contrast, this concentration of inhibitor blocked the small increase in levels of (activated) phosphotyrosine MAP kinase in response to both S2- and S2HS-wg-conditioned media (Figure 6D). These observations were confirmed by activity studies of p42/p44 MAP kinase purified by FPLC (M.J.Fry, unpublished data). Finally, we considered the regulation of $p90^{rsk}$ by S2- and S2HS-wg-conditioned media (Figure 6E). Like p70 S6 kinase, activated $p90^{rsk}$ is phosphorylated and exhibits retarded mobilities on SDS–PAGE gels. Western blot analysis of unstimulated 10T1/2 fibroblasts with an anti-$p90^{rsk}$ antiserum detected the presence of a protein doublet migrating at ~90 kDa (Figure 6C; DMEM). Following stimulation with either insulin or EGF, this doublet was replaced with a single detectable protein band of retarded mobility, presumably corresponding to phosphorylated (activated) $p90^{rsk}$. Reflecting their minor activations of p42/p44 MAP kinase, S2- and S2HS-wg-conditioned media had only relatively marginal effects on the mobility of $p90^{rsk}$ compared with unstimulated cells. Additionally, PD 98059 blocked the appearance of retarded mobility forms of $p90^{rsk}$ induced by S2, S2HS-wg and insulin (Figure 6C), although this concentration of PD 98059 was insufficient to prevent EGF regulation of $p90^{rsk}$. As PD 98059 had no effect on inactivation of GSK-3 by Wg, these data strongly suggest that MEK, p42/p44 MAP kinase and $p90^{rsk}$ are not involved in Wg signal transduction to GSK-3. Thus, Wg signalling to GSK-3 would also appear to be distinct from that reported for EGF, insulin, IGF-1 or serum (Welsh and Proud, 1993; Cross *et al.*, 1994, 1995; Saito *et al.*, 1994; Welsh *et al.*, 1994; Eldar-Finkelman *et al.*, 1995).

Signalling to GSK-3 by Wg involves a phorbol ester-sensitive PKC(s)

In vitro, several PKC isoforms are capable of phosphorylating and inhibiting GSK-3 (Goode *et al.*, 1992). Additionally, phorbol esters can inactivate GSK-3 via a pathway(s) which is insensitive to wortmannin (Welsh *et al.*, 1994). We considered the possibility that a PKC might be involved in Wg signalling by using the specific inhibitor Ro31-8220 (Davis *et al.*, 1989). This compound is a bis-indolylmaleimide based on the structure of the non-selective kinase inhibitor staurosporine, but having increased specificity for PKC isoforms. Ro31-8220 is

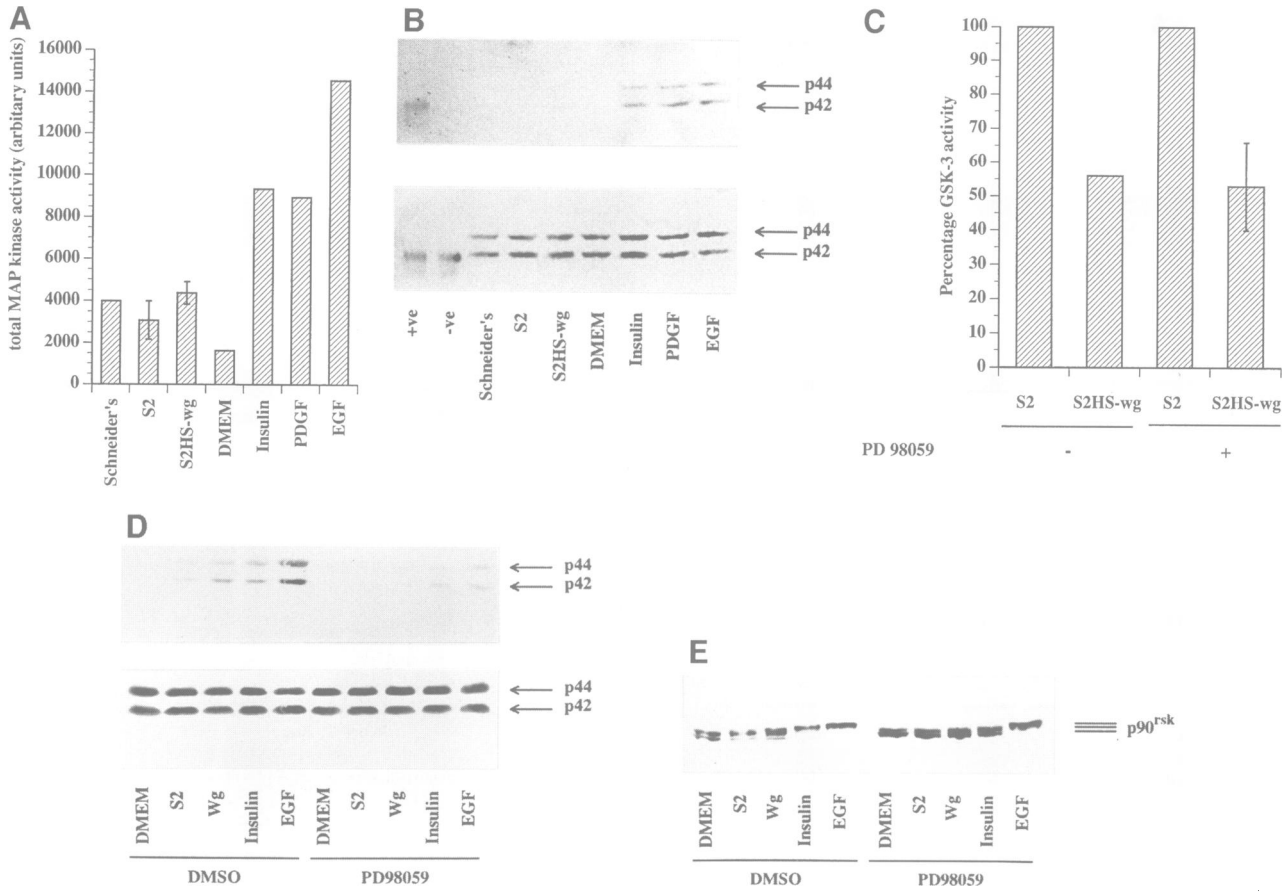

Fig. 6. Effect of S2HS-wg-conditioned media on p42/p44 MAP kinase and $p90^{rsk}$. (**A**) Confluent, serum-starved 10T1/2 cells were incubated with untreated Schneider's medium, conditioned medium (S2 or S2HS-wg), DMEM or DMEM supplemented with insulin (5 μg/ml), PDGF (200 ng/ml) or EGF (10 ng/ml). 10T1/2 cells were incubated for 10 min before harvesting. MAP kinase activity was calculated against a MAP kinase-specific peptide using the Biotrak MAP kinase (p42/p44MAP K) enzyme assay system (Amersham). Total MAP kinase activity is shown. The mean of three independent experiments is shown for S2- and S2HS-wg-treated cells. All other experiments represent the mean of two independent experiments. (**B**) Extracts used for MAP kinase assays were analysed by immunoblotting with an antiserum which only recognizes Tyr204 phosphorylated p42/p44 MAP kinase (New England Biolabs). The filter was stripped and re-blotted with an antiserum that recognizes all p42/p44 MAP kinase species (Santa Cruz Biotechnology) to demonstrate equal protein loading (lower panel). Positive (+ve) and negative (–ve) control samples represent Tyr204 phosphorylated and non-phosphorylated p42 MAP kinase (New England Biolabs) respectively. A representative experiment is shown. (**C**) 10T1/2 cells were pre-incubated for 30 min with either PD 98059 (10 μM) or an equivalent volume of carrier (DMSO). The indicated conditioned medium was added (±10 μM PD 98059) and the cells incubated for a further 10 min before harvesting. Percentage activities were calculated relative to cells treated with S2-conditioned media (±10 μM PD 98059). The mean of three experiments is shown for PD 98059-treated cells. The other bars represent the mean of two experiments. (**D**) 10T1/2 cells were pre-incubated for 30 min with either PD 98059 (10 μM) or an equivalent volume of carrier (DMSO). Cells were then incubated with DMEM, conditioned medium (S2 or S2HS-wg), or DMEM supplemented with insulin (5 μg/ml) or EGF (10 ng/ml) ±10 μM PD 98059. Cell extracts were analysed by immunoblotting with an antiserum which only recognizes Tyr204 phosphorylated p42/p44 MAP kinase (New England Biolabs). The filter was stripped and re-blotted with an antiserum that recognizes all p42/p44 MAP kinase species (Santa Cruz Biotechnology) to demonstrate equal protein loading (lower panel). A representative experiment is shown. (**E**) 10T1/2 cells were pre-incubated for 30 min with either PD 98059 (10 μM) or an equivalent volume of carrier (DMSO). Cells were then incubated with DMEM, conditioned medium (S2 or S2HS-wg), or DMEM supplemented with insulin (5 μg/ml) or EGF (10 ng/ml) ±10 μM PD 98059. Cell extracts were analysed by immunoblotting with an anti-$p90^{rsk}$ mouse monoclonal antiserum (Transduction Laboratories). A representative experiment is shown.

effective against both phorbol ester-sensitive and -insensitive PKC isoforms (Wilkinson *et al.*, 1994). Pre-treatment of 10T1/2 cells with 2 μM Ro31-8220 completely abolished inhibition of GSK-3 activity by Wg (Figure 7A). Concentrations of up to 20 μM Ro31-8220 had no detectable effect upon the regulation of GSK-3 by insulin (Figure 7A and D.Cook, unpublished data), showing that Ro31-8220 does not directly affect the activity of GSK-3 or cause a non-specific inhibition of cellular signalling. The effect of Ro31-8220 could be titrated out and, at a concentration of 20 nM, the inhibitor no longer had any detectable effect upon GSK-3 inactivation by Wg (Figure 7A).

To obtain further evidence for the involvement of PKC in Wg signal transduction to GSK-3, we considered the effects of a known PKC agonist, TPA, on GSK-3 regulation by Wg. Acute (10 min) treatment of 10T1/2 cells with TPA (800 nM) caused inactivation of GSK-3 (Figure 7B). This regulation was inhibited by 2 μM Ro31-8220 (Figure 7B), suggesting that 10T1/2 cells contain a pathway to GSK-3 in which PKC activation lies upstream of GSK-3. However, regulation of GSK-3 by TPA differed from that by Wg in that PD 98059 (10 μM) substantially blocked activation of MAP kinase by TPA but only partially blocked activation of $p90^{rsk}$ (Figure 7C). Thus, TPA could still potentially signal to GSK-3 via $p90^{rsk}$ in 10T1/2

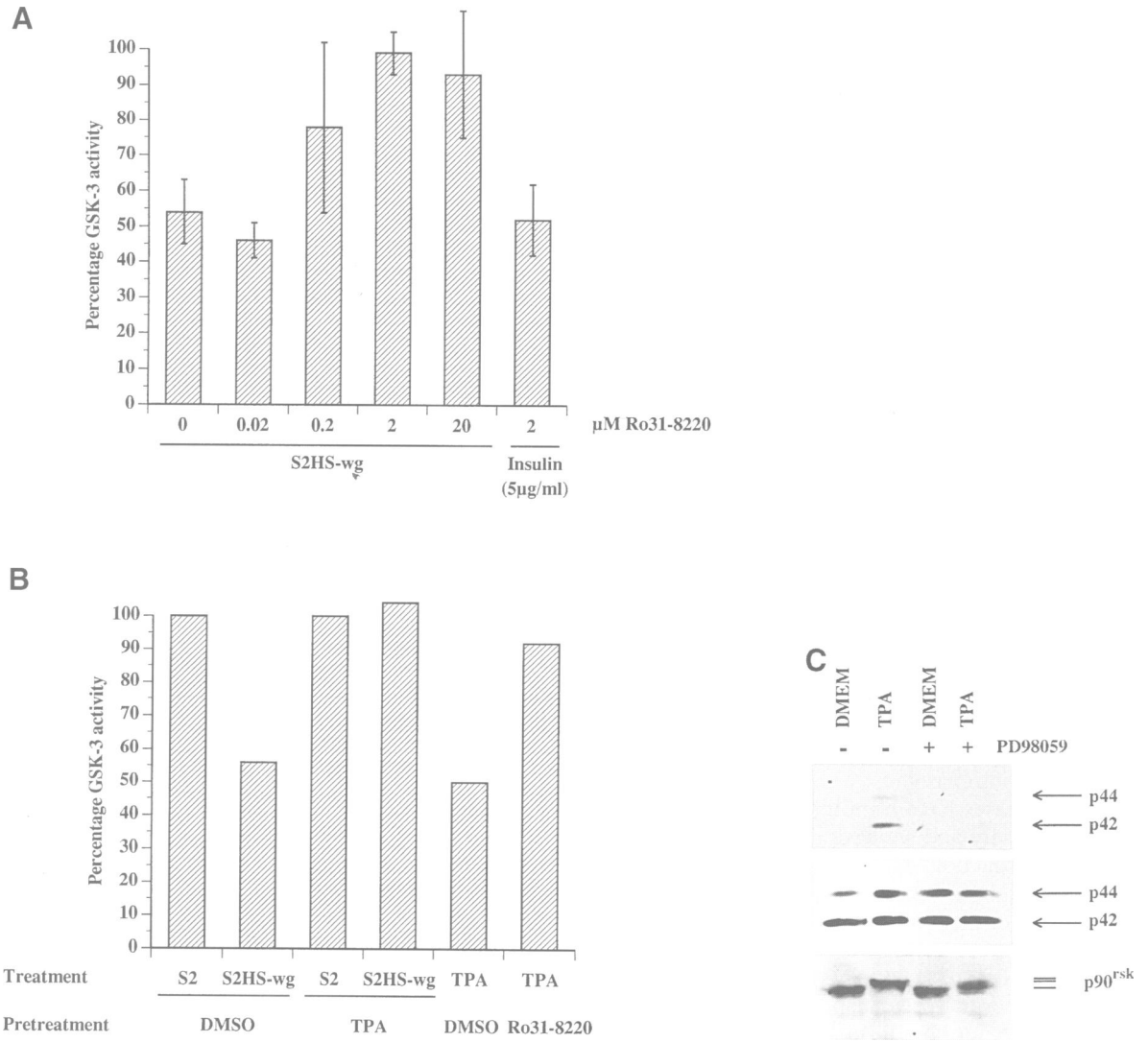

Fig. 7. Prevention of GSK-3 inhibition in response to Wg by Ro31-8220 and pre-treatment with TPA. (**A**) Confluent, serum-starved 10T1/2 cells were incubated with conditioned medium (S2 or S2HS-wg) or conditioned S2 medium supplemented with insulin (5 μg/ml final concentration). Cells were pre-treated with either the indicated concentration of Ro31-8220 or DMSO (1:1000 v/v) for 10 min prior to incubation. Percentage activity is displayed relative to 10T1/2 cells treated with S2-conditioned medium containing the equivalent concentration of Ro31-8220. The mean of three experiments is shown. (**B**) Following pre-treatment, confluent, serum-starved 10T1/2 cells were incubated with conditioned media (S2 or S2HS-wg) or S2-conditioned medium supplemented with TPA (800 nM). Cells were pre-treated with TPA (800 nM) or carrier (DMSO 1:1000 v/v) for 48 h prior to incubation with S2- or S2HS-wg-conditioned medium. Cells were pre-treated with Ro31-8220 (2 μM) or carrier (DMSO 1:1000 v/v) for 10 min prior to 10 min incubation with S2-conditioned medium supplemented with TPA (800 nM). Percentage activity relative to 10T1/2 cells treated with S2-conditioned medium is displayed. The mean of two experiments is shown. (**C**) Effect of PD 98059 on regulation of p42/p44 MAP kinase and $p90^{rsk}$ by TPA. 10T1/2 cells were pre-incubated for 30 min with either PD 98059 (10 μM) or an equivalent volume of carrier (DMSO). Cells were then incubated with DMEM or DMEM supplemented with TPA (800 nM) ±10 μM PD 98059. Cell extracts were analysed by immunoblotting with an antiserum which only recognizes Tyr204 phosphorylated p42/p44 MAP kinase (New England Biolabs; top panel). The filter was stripped and re-blotted with an antiserum that recognizes all p42/p44 MAP kinase species (Santa Cruz Biotechnology) to demonstrate equal protein loading (middle panel). Cell extracts were also immunoblotted with an anti-$p90^{rsk}$ mouse monoclonal antiserum (Transduction Laboratories; bottom panel). Representative experiments are shown.

fibroblasts. Prolonged treatment with TPA has been shown to down-regulate some PKC isoforms (for example, see Adams and Gullick, 1989; Olivier and Parker, 1992). Pre-treatment of 10T1/2 cells with TPA (800 nM) for 48 h had no substantial effect upon GSK-3 regulation by insulin (D.Cook, unpublished data), but was sufficient to abolish Wg-induced antagonism of GSK-3 activity (Figure 7B). This result supports the observations with Ro31-8220 and provides further evidence supporting the hypothesis that Wg signalling to GSK-3 involves a TPA-sensitive isoform(s) of PKC.

Discussion

We have demonstrated regulation of GSK-3 kinase activity by Wg in murine 10T1/2 fibroblasts. These studies provide the first direct biochemical evidence in support of the proposed genetic model of paracrine Wg signalling to Zw3/Sgg (Hooper, 1994; Siegfried *et al.*, 1994) and demonstrate the potential of this system as a tool to study aspects of Wg/Wnt signal transduction in more detail. The inactivation of GSK-3 by Wg may be a characteristic response to several members of the Wnt family, as recent

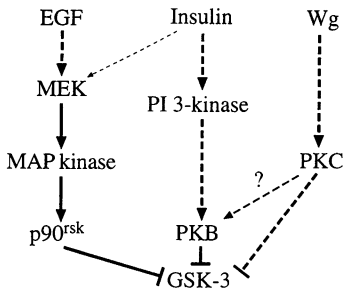

Fig. 8. Models of possible signal transduction routes to GSK-3 utilized by EGF, insulin and Wg.

data has shown that soluble *Xenopus* Xwnt-8 protein is also capable of inhibiting GSK-3 activity (D.Cook and T.C.Dale, unpublished data in collaboration with Dr Min Ku, Boston). This response does not appear to be a phenomenon unique to 10T1/2 fibroblasts, as GSK-3 is also inactivated in L6 myoblasts following treatment with S2HS-wg-conditioned media (D.Cook, unpublished data). Wg presumably binds to endogenous murine Wnt receptors and activates a conserved Wnt signal transduction pathway in 10T1/2 fibroblasts. To date, genetic analysis had provided the only description of factors involved in signal transduction by Wg/Wnt. However, using this biochemical model, we have also identified a TPA-sensitive PKC(s) as a putative component of Wg/Wnt signal transduction to GSK-3 in 10T1/2 fibroblasts.

A number of kinases have been implicated in suppression of GSK-3 activity and we have considered their role in Wg signal transduction. Insulin signalling to GSK-3, was originally thought to occur via the MAP kinase/p90^{rsk} cascade because wortmannin inhibited both activation of MAP kinase and p90^{rsk} as well as inhibition of GSK-3 kinase (Welsh and Proud, 1993; Cross *et al.*, 1994; Welsh *et al.*, 1994). However, recent data using PD 98059 to inhibit MEK activation by insulin has indicated that, although activation of MAP kinase and p90^{rsk} lie downstream of the wortmannin block, their activity is not required for GSK-3 regulation. Instead the major route of insulin signalling to GSK-3 involves PKB (Figure 8; Cross *et al.*, 1995). In contrast, the activation of p90^{rsk} and inactivation of GSK-3 by EGF can be partially blocked by using dominant-negative MEK, suggesting that this pathway does play a role in signal transduction to GSK-3 by EGF (Figure 8; Eldar-Finkelman *et al.*, 1995). Unlike insulin and EGF, Wg does not activate either MAP kinase or p90^{rsk} in comparison with S2 controls (Figure 6). Additionally, the marginal activations of MAP kinase and p90^{rsk} induced by S2- and S2HS-wg-conditioned media can be inhibited by PD 98059 without affecting GSK-3 regulation by Wg (Figure 6C). Thus, signal transduction through MAP kinase to p90^{rsk} is not required for regulation of GSK-3 by Wg.

In 10T1/2 cells, the pathways used by insulin and Wg to regulate GSK-3 are quite distinct. As has been observed in other cell types, insulin signalling to GSK-3 in 10T1/2 fibroblasts is sensitive to wortmannin, suggesting that it may occur via a PI 3-kinase-regulated step (Cross *et al.*, 1994; Welsh *et al.*, 1994). In contrast, inactivation of GSK-3 by Wg is unaffected by wortmannin and would thus be predicted not to involve a PI 3-kinase (Figure 4). Conversely, signalling by Wg, but not insulin, is sensitive to Ro31-8220 and TPA pre-treatment (Figure 7). While Ro31-8220 inhibits PKC activity (Davis *et al.*, 1989; Wilkinson *et al.*, 1994), long-term pre-treatment with TPA results in down-regulation (proteolysis) of TPA-sensitive PKC isoforms (Adams and Gullick, 1989; Olivier and Parker, 1992). Thus, the mechanisms by which Ro31-8220 and TPA inhibit PKC are fundamentally different and, taken together, strongly suggest a previously undescribed role for a TPA-sensitive PKC(s) in Wg/Wnt signalling to GSK-3.

Several PKC isoforms have been shown to be capable of inactivating GSK-3β (but not GSK-3α) *in vitro* (Goode *et al.*, 1992). Thus, the simplest model that integrates a PKC into Wg/Wnt signal transduction is one in which PKC directly phosphorylates and inactivates GSK-3. This hypothesis is consistent with our observations, as GSK-3β represents >95% of the total GSK-3 protein (and activity) in 10T1/2 fibroblasts (D.Cook and M.J.Fry, unpublished data) and inactivation of GSK-3 is reversible by treatment with PPTase-2A (Figure 2D). The genetic description of Wg signal transduction places dishevelled (*dsh*) upstream of *zw3/sgg* and so, in this model, Dsh (or its mammalian Dvl homologues) would have to lie either upstream of or on a parallel signalling pathway to PKC. However, PKC regulation of GSK-3 may be indirect and PKC could therefore lie upstream of, as yet, unidentified factors. In this model, the relationship of Dsh/Dvl to PKC is unclear. Given the previously highlighted differences between insulin, EGF and Wg signalling, Wg/Wnt signal transduction to GSK-3 may represent a novel route to GSK-3 regulation involving a PKC (Figure 8). We are currently addressing potential Wg-mediated mechanisms of PKC activation as well as identifying the PKC isoform(s) involved in signal transduction to GSK-3.

Materials and methods

Cell lines

10T1/2 fibroblasts (Reznikoff *et al.*, 1973), a gift from Dr J.M.Bradbury (University of Cambridge, UK), were maintained at 37°C in DMEM (Imperial Laboratories, UK) supplemented with 5% (v/v) fetal calf serum (FCS, Imperial Laboratories, UK). S2 and S2HS-wg cells were cultured at 25°C in Schneider's *Drosophila* medium supplemented with 10% FCS. S2 and S2HS-wg cells were a gift from Professor R.Nusse (Stanford University, CA).

Antisera

The rabbit antiserum anti-Wg206 was raised against a glutathione-*S*-transferase–Wg fusion. A 5′ *Eco*RV fragment of the Wg coding region (van den Heuvel *et al.*, 1989) was cloned into the *Sma*I site of pGEX5X-Z (Pharmacia) and transformed into *Escherichia coli* strain BL-21 (Stratagene). Production of the fusion protein and subsequent rabbit immunization were performed as described previously (van den Heuvel *et al.*, 1989). The neutralizing anti-Wg antiserum was a gift from Professor R.Nusse (Stanford University, CA) and previously has been demonstrated to block Wg function (van Leeuwen *et al.*, 1994). Antisera to GSK-3α and GSK-3β have been described previously (Woodgett, 1990). The anti-phosphorylated p42/p44 MAP kinase rabbit antiserum was obtained from New England Biolabs. The anti-Erk-1 (sc-94) rabbit and anti-p70 S6 kinase (sc-230) rabbit antisera were obtained from Santa Cruz Biotechnology. The anti-p90^{rsk} [ANTI-RSK (p90^{rsk})] mouse monoclonal antiserum was obtained from Transduction Laboratories.

Production of conditioned medium

Schneider's medium conditioned by S2 or S2HS-wg cells was produced essentially as described previously (van Leeuwen *et al.*, 1994). Equal cell numbers (~1–2×10^6 cells/ml) of S2 and S2HS-wg cells were heat-shocked at 37°C for 30 min. The cultures were allowed to recover at

25°C for 90 min before being transferred to serum-free Schneider's medium and incubated at 25°C for 3 h. Cells were removed by centrifugation at 2000 *g* for 5 min. Any cellular debris was removed by further centrifugation at 100 000 *g* for 30 min. Medium was concentrated using a Centriprep 10 column (Amicon).

Growth factors

Insulin was obtained from Sigma and acid activated by resuspension in 6 mM HCl. PDGF BB and EGF were obtained from R & D systems.

Inhibitors

Ro31-8220 was a gift from Dr E.J.Murray and Dr D.Bradshaw (Roche Products, Welwyn Garden City, UK). Rapamycin was a gift from Professor P.Cohen (Dundee, UK) and wortmannin was obtained from Sigma. PD 98059 was a gift from Dr D.T.Dudley (Parke–Davis Pharmaceutical Research, Ann Arbor, MI). The 1000× stocks (Ro31-8220 20 mM to 20 μM, wortmannin 20 μM, rapamycin 100 μM and PD 98059 10 mM) were made up in dimethyl sulfoxide (DMSO). The 1000× Ro31-8220 stocks were made fresh prior to use. Wortmannin, rapamycin and PD 98059 stocks were stored at –70°C. Inhibitors were diluted (1:1000) in DMEM containing 0.5% (v/v) FCS, added to 10T1/2 cells and then incubated for 10 min at 37°C prior to addition of conditioned medium. Where indicated, inhibitors were also diluted (1:1000) in S2- or S2HS-wg-conditioned medium before addition to fibroblasts. Control incubations contained carrier (DMSO 1:1000 v/v).

TPA

TPA was obtained from Sigma. A 1000× stock solution (800 μM) was made in DMSO. For short-term (10 min) treatment of 10T1/2 fibroblasts, TPA was diluted (1:1000) in Schneider's *Drosophila* medium before addition to the cells. For long-term treatment, TPA was diluted (1:1000) and added to the cells for 32 h in DMEM/5% FCS. Sixteen hours prior to assay, the medium was changed from 5 to 0.5% FCS/DMEM containing TPA (800 nM). The TPA concentration was maintained during incubation of cells with conditioned medium. Control incubations contained DMSO (1:1000 v/v).

GSK-3 assay

GSK-3 was partially purified and assayed essentially as described previously (Welsh and Proud, 1993). Briefly, $1–2\times10^7$ 10T1/2 cells were growth arrested and serum starved. Fibroblasts were grown to confluence and then maintained for 72 h in 5% FCS/DMEM. Sixteen hours prior to assay, the medium was changed from 5 to 0.5% FCS/DMEM. The cells were washed twice in serum-free DMEM before the addition of S2- or S2HS-wg-conditioned medium. Then, 10 ml of conditioned medium (sufficient to cover the monolayer) was added to the cells which were then incubated for 10 min at 37°C. After stimulation, the cells were washed twice in phosphate-buffered saline (PBS) then harvested by scraping into 1 ml of buffer A [25 mM Tris–HCl (pH 7.5), 1.5 mM EDTA, 50 mM sodium fluoride, 1 mM benzamidine, 0.5 mM dithiothreitol (DTT), 0.5 mM sodium vanadate, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μg/ml pepstatin, 1 μg/ml antipain, 1 μg/ml leupeptin and 100 nM okadaic acid]. Cell lysates were flash-frozen in liquid nitrogen and stored at –70°C. Lysates were pre-cleared by centifugation (30 min at 10 000 *g* in a microfuge) and filtration through 0.2 μm Anotop filters (Fisons) before being fractionated on a Mono S HR 5/5 column (Pharmacia). GSK-3 was eluted from the column with a linear 0–500 mM NaCl gradient in buffer B [25 mM Tris–HCl (pH 7.5), 1 mM EDTA and 0.5 mM DTT) at 0.75 ml/min. Fractions of 0.5 ml were collected and 35 μl samples of column fractions were assayed for GSK-3 activity against 25 μM of pre-phosphorylated GSK-3 peptide (YRRAAVPPSPSLSRHSSPHQSEDEEE; S indicates the pre-phosphorylated serine residue) as described previously (Welsh and Proud, 1993). Reactions were incubated at 30°C for 30 min. Phosphorylated species were separated by Tricine–SDS–PAGE (Schagger and Von Jagow, 1987). Phosphorylation of the target peptide was visualized by exposing the gel to a phosphor imager screen and quantified by analysis on a Molecular Dynamics PhosphorImager using ImageQuant software. GSK-3 activities were normalized to the total protein loaded onto the column (as determined by Bio-Rad protein determination).

PPTase-2A treatment of FPLC fractions

FPLC column fractions were incubated with magnesium chloride (10 mM final concentration) in the presence or absence of 0.5 U of PPTase-2A. Fractions were incubated at 30°C for 20 min and the reaction terminated by the addition of okadaic acid (1 μM final concentration). GSK-3 kinase assays were then performed on the fractions as described above.

MAP kinase assay

A total of $1–2\times10^6$ growth-arrested and serum-starved 10T1/2 fibroblasts were washed twice in serum-free DMEM. Conditioned medium (S2 or S2HS-wg), untreated Schneider's *Drosophila* medium or DMEM [containing insulin (5 μg/ml), PDGF (200 ng/ml), EGF (20 ng/ml)] were added to the cells and incubated at 37°C for 10 min. Cells were washed twice in ice-cold PBS and then lysed on ice for 10 min in 100 μl of buffer S [20 mM Tris–HCl (pH 8.0), 40 mM sodium tetrapyrophosphate, 50 mM sodium fluoride, 5 mM magnesium chloride, 100 μM sodium vanadate, 10 mM EGTA, 1% (v/v) Triton X-100, 0.5% sodium deoxycholate, 20 μg/ml leupeptin, 20 μg/ml aprotinin and 3 mM PMSF]. Cells were scraped into chilled microfuge tubes and cellular debris pelleted by centrifugation at 10 000 *g* in a microfuge for 10 min at 4°C. The supernatants were transferred to a fresh microfuge tube. Duplicate 15 μl aliquots of supernatant were assayed for total MAP kinase activity against a specific MAP kinase substrate peptide using the Biotrak MAP kinase (p42/p44MAPK) enzyme assay system (Amersham) according to the manufacturer's instructions.

Immunoblotting procedures

Soluble Wg was detected in 200 μl of 15× concentrated S2- or S2HS-wg-conditioned media resolved on 10% SDS–polyacrylamide gels. GSK-3α and GSK-3β were detected in 50 μl aliquots of Mono S FPLC fractions resolved on 10% SDS–polyacrylamide gels. $p90^{rsk}$ and p42/p44 MAP kinases were detected in 50 μl samples of lysates made for MAP kinase assay resolved on 8 and 12.5% SDS–polyacrylamide gels respectively. Cell extracts which were used to analyse p70 S6 kinase were harvested into boiling SDS–PAGE buffer [50 mM Tris–HCl (pH 6.8), 100 mM DTT, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue and 10% (v/v) glycerol] and resolved on 10% SDS–polyacrylamide gels. After electrophoresis, the proteins were transferred to nitrocellulose. Respective blots were blocked in PBS containing 5% low fat milk (PBS/5%) and then incubated with the relevant antisera, in PBS/5%, at the following concentrations: anti-Wg206 antiserum (1:2000 v/v), anti-GSK-3α (1:750 v/v), anti-GSK-3β (1:750 v/v), anti-phosphorylated p42/p44 MAP kinase (1:1000 v/v), anti-Erk-1 (1:5000 v/v), anti-$p90^{rsk}$ (1:1000 v/v) or anti-p70 S6 kinase (1:1000 v/v). Bound immunoglobulins were detected using either horseradish peroxidase-conjugated goat anti-rabbit secondary antibody or horseradish peroxidase-conjugated sheep anti-mouse secondary antibody and enhanced chemiluminescence (Amersham). p42/p44 MAP kinase filters were stripped for reprobing by incubation for 30 min in 62.5 mM Tris–HCl (pH 6.7), 100 mM 2-mercaptoethanol, 2% (w/v) SDS at 50°C, followed by two large volume washes in PBS containing 0.05% Tween-20.

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