Differential Regulation of Glycogen Synthase Kinase 3β by Insulin and Wnt Signaling*

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Glycogen synthase kinase 3β (GSK3β) is a key component in many biological processes including insulin and Wnt signaling. Since the activation of each signaling pathway results in a decrease in GSK3β activity, we examined the specificity of their downstream effects in the same cell type. Insulin induces an increased activity of glycogen synthase but has no influence on the protein level of β-catenin. In contrast, Wnt increases the cytosolic pool of β-catenin but not glycogen synthase activity. We found that, unlike insulin, neither the phosphorylation status of the serine9 residue of GSK3β nor the activity of protein kinase B is regulated by Wnt. Although the decrease in GSK3β activity is required, GSK3β may not be the limiting component for Wnt signaling in the cells that we examined. Our results suggest that the axin-conductin complexed GSK3β may be dedicated to Wnt rather than insulin signaling. Insulin and Wnt pathways regulate GSK3β through different mechanisms, and therefore lead to distinct downstream events.

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1 The abbreviations used are: GSK3, glycogen synthase kinase 3; GS, glycogen synthase; PKB, protein kinase B; 4-HT, 4-hydroxytamoxifen; APC, adenomatous polyposis coli; DMEM, Dulbecco's modified Eagle's medium; LEF, lymphoid enhancer factor; TCF, T cell factor; HA, hemagglutinin; GBP, GSK3-binding protein; FRAT, frequently rearranged in advanced T-cell lymphomas; Dvl/Dsh, dishevelled.

Glycogen synthase kinase 3 (GSK3) was originally identified for its ability to phosphorylate and inhibit glycogen synthase (GS) (1, 2). It is a serine/threonine kinase that recognizes the target sequence SXXXS with the second serine phosphorylated (3). Many proteins other than GS also contain GSK3 as the substrate of GSK3 in vitro. These include ATP-citrate lyase, protein phosphatase 1, c-Jun, Myc, Myb, CREB, Tau, β-catenin, and IκB (4–6). GSK3 is also unusual in that its enzymatic activity remains high at resting state and decreases upon stimulation. GSK3 is conserved from yeast to mammals and has been implicated in strikingly versatile biological functions. However, how different signals regulate GSK3 is still unknown.

GSK3 plays an important role in the cellular response to insulin (7). The regulation of GSK3 by insulin has been shown to be mediated by protein kinase B (PKB). Upon insulin stimulation, threonine 308 (Thr-308) and serine 473 (Ser-473) residues of PKB are phosphorylated and PKB is activated (8). Subsequently, both GSK3 isotypes (GSK3α and GSK3β) in mammalian cells are phosphorylated on a serine residue at the N terminus (serine 21 of GSK3α and serine 9 of GSK3β) (9, 10), which leads to a decrease in GSK3 activity. Although this has usually been detected as a 50–70% drop, it is apparently sufficient to relieve the inhibition of GS and allow cells to complete glycogen synthesis.

Another in vitro substrate of GSK3β is β-catenin, a protein involved in cell adhesion, oncogenesis and development (11–13). Together with axin-conductin and APC, GSK3β is one of the components of a protein complex that regulates the stability of β-catenin (14–17). Phosphorylation of the GSK3β sites in the N terminus of β-catenin is believed to be a signal for degradation. When either APC or the GSK3β sites of β-catenin are mutated, as in 90% of colon cancer, levels of β-catenin are elevated (13). Excess β-catenin accumulates in the cytosol and nucleus, outside of cell adhesion complexes on cytoplasmic membrane where it normally resides. Nuclear β-catenin is capable of interacting with the LEF/TCF family DNA-binding proteins and activating transcription of genes containing LEF/TCF binding sites (18, 19). Increased β-catenin levels can also be achieved through the activation of Wnt/ Wingless signaling pathway (20). GSK3β has been placed between Dishevelled (Dvl) in mammalian cells, Dsh in other organisms) and β-catenin in the Wnt pathway based on a combination of genetic and biochemical evidence (21–23). It is not clear how the extracellular Wnt signal is transduced from the membrane receptor Fizzled to Dsh/Dvl, and then to GSK3β resulting in increased β-catenin levels. Decreases in the activity of GSK3β have been observed in mouse fibroblasts and Drosophila cells responding to wingless and Dsh (24, 25). Furthermore, inhibition of GSK3β activity by lithium salt or GSK3β-binding protein (GBP/FRAT) mimics Wnt signaling (26, 27). Recently, it is reported that Dvl and GBP/FRAT are able to associate with the axin-conductin-APC-β-catenin complex (23, 28). Moreover, this entire complex is believed to dissociate in response to Wnt signaling (20, 25, 28).

In this study, we investigated how different signals such as insulin and Wnt regulate GSK3β. Using mammalian cells that respond to both signals, we found that the downstream effect is specific to each pathway, despite the indistinguishable decrease in GSK3β activity. We also generated the first inducible system to conditionally activate Dishevelled in mammalian cells as an independent method to turn on the Wnt signaling pathway. Serine 9 of GSK3β is not regulated in cells that are activated by Wnt or Dishevelled. Furthermore, we have evidence that the axin-conductin complexed GSK3β is not significantly phosphorylated at serine 9 upon insulin stimulation and, therefore, may be protected from insulin signaling.
**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections—**Human embryonic kidney 293 cells were purchased from ATCC. C57MG, Rat2-MV7, and Rat2-Wnt1 cell lines were generously gifted from Dr. Anthony Brown. These cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Inc.). CHOIR cells were kindly given by Drs. Richard Roth (29) and Ira Goldfine, maintained in Ham’s F12 medium (Life Technologies, Inc.). All cell culture media were supplemented with 10% fetal calf serum (Life Technologies, Inc.) and 1% penicillin/streptomycin (Life Technologies, Inc.).

For conditioned media, Rat2-MV7 and Rat2-Wnt1 were grown to 95% confluency. Cells were washed with phosphate-buffered saline and maintained in serum-free DMEM overnight. The conditioned media were filtered through 0.22-\(\mu\)m filter units, aliquoted, and stored at -80°C until use. For insulin or Wnt stimulation, cells were grown to 70–80% confluence and serum-starved overnight, after which 5 \(\mu\)g/ml insulin or 0.2 mM conditioned medium was added.

Transfections of plasmids were performed by using LipofectAMINE Plus (Life Technologies, Inc.) or FuGENE6 (Roche Molecular Biochemicals) according to instructions from the manufacturer.

To generate 293-D-ER cells, 293 cells were transfected with the Dvl-ER plasmid. Selection with 1 \(\mu\)g/ml Geneticin (Life Technologies, Inc.) was used to select for 293-D-ERHis6 conductin cells.

**Plasmids—**Mammalian expression plasmid encoding human Dishevelled 2 was a generous gift from Dr. Misha Semenov (30). The coding region of Dishevelled 2 was also epitope-tagged with Glu-Glu (EE) tag and fused to the hormone binding domain of a modified version of the murine estrogen receptor (Dvl-ER). Conducint expression plasmid was from Dr. Walter Birchmeier (16), and was then epitope-tagged with the EE tag in pCDNA6. TOPTK reporter plasmid for TCF/LEF-dependent transcription was from Dr. Hans Clevers (31). GSK3\(\beta\) was amplified from this template by polymerase chain reaction and introdused into mammalian expression vector pBlueScriptSK+ from Dr. James Woodgett (33). GSK3\(\beta\) was amplified from this template by polymerase chain reaction and cloned into pcDNA3-based plasmid with an N-terminal HA tag. Mutants of GSK3\(\beta\) were created by using QuickChange site-directed mutagenesis kit (Stratagene). Three versions of kinase-dead GSK3\(\beta\) were made with amino acid substitutions at the ATP binding site: lysine 85 to alanine, lysine 85 and 86 to arginines, and lysine 85 to methionine plus lysine 86 to alanine.

**Antibodies—**Anti-GSK3\(\beta\) and anti-\(\beta\)-catenin antibodies were from Transduction Laboratory. Phosphotyrosine antibody 4G10 and anti-PKB antibody were from Upstate Biotechnology. Phosphospecific antibody against Ser-9 of GSK3\(\beta\) was from New England Biolabs. Phosphospecific antibodies against PKB were generously provided by Dr. David Stooke. Anti-E2 antibody was from Harlan Bioproducts. Anti-HA antibody was from Santa Cruz Biotechnology.

**Cytosolic Fractionation, Immunoprecipitations, and Western Blots—**To prepare cytosolic fractions, cells were washed and collected in ice-cold phosphate-buffered saline. Cell pellets were resuspended in ice-cold hypotonic buffer (25 mM Tris, pH 7.5, 1 mM EDTA, 25 mM NaF, 1 mM dithiothreitol) with Complete protease inhibitor mixture (Roche Molecular Biochemicals). Cells were lysed after incubating on ice for 10 min (verified by microscopy). The lysates were subjected to ultracentrifugation at 100,000 \(\times g\) for 30 min at 4°C, and the supernatant was collected.

For immunoprecipitation, cells were washed twice in ice-cold phosphate-buffered saline, then lysed in IP buffer (125 mM NaCl, 25 mM NaF, 25 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10 mM β-glycerol phosphate, 5 mM sodium pyrophosphate, 1 mM NaVO\(_4\), 200 mM okadaic acid, 1 mM dithiothreitol) with Complete protease inhibitor mixture. Anti-GSK3\(\beta\), anti-HA, or anti-EE antibody was added to the lysates for 1 h at 4°C, and then Protein G beads (Sigma) were added for another 1 h. Immunoprecipitates were washed three times with IP buffer. To coimmunoprecipitate GSK3\(\beta\) with His\(_4\)-conductin, Ni-IP buffer was used. Ni-IP buffer was IP buffer without EDTA, EGTA, or dithiothreitol and supplemented with EDTA-free Complete protease inhibitor mixture (Roche Molecular Biochemicals). Nickel beads (ProBond resin, Invitrogen) were first blocked with 2 mg/ml bovine serum albumin in Ni-IP buffer for 2 h. After incubating with cleared lysates, nickel beads were washed three times with Ni-IP buffer supplemented with 200 mM imidazole and then once with Ni-IP buffer. Western blotting was carried out following standard procedures. 10% Tris-glycine polyacrylamide gels were used.

**Enzyme Assays—**For GSK3\(\beta\) kinase assays, GSK3\(\beta\) immunoprecipitates were washed once with kinase buffer (25 mM Tris, pH 7.5, 10 mM MgCl\(_2\)) first. Kinase reactions were performed in kinase buffer with 100 \(\mu\)M \([\gamma\text{P}]\)ATP and 100 \(\mu\)M 2BSP peptide as the substrate (synthesized by the Biomedical Resource Center, University of California, San Francisco, CA). 2BSP is based on the GSK3 target site in eIF2B (34). After 20 min at 30°C, the reactions were spotted on phosphocellulose P81 paper (Whatman), washed four times with 100 mM phosphoric acid, and counted in scintillation counter.

Luciferase assays were performed by using dual luciferase reporter assay system (Promega) in a Microplate Luminometer (EG&G Berthold). Transfection efficiency was normalized to the expression of Renilla luciferase from the cotransfected pRL-TK plasmid.

**RESULTS**

**GSK3\(\beta\) Is Involved in Wnt Signaling Pathway in Mammalian Cells—**To compare the regulation of GSK3\(\beta\) by insulin and Wnt, we needed to choose cell lines that respond to both signals. In this study, we used conditioned media from a stable Rat2 cell line expressing mouse Wnt-1 as a source of Wnt protein (36). We first tested the effect of the Wnt conditioned media on human embryonic 293 cells as this epithelial cell line does respond to insulin (37). We observed a maximal decrease in GSK3\(\beta\) activity at 10 min after the addition of Wnt media to cells (Fig. 1A). Wnt media also caused an accumulation of the cytosolic fraction of \(\beta\)-catenin, which peaked at about 3 h after stimulation (Fig. 1B). These results again place mammalian GSK3 upstream of \(\beta\)-catenin and downstream of Wnt. Similar results were also seen with C57MG (C57) cells, an immortalized mouse mammary gland epithelial cell line (data not shown). We then examined the relationship between Dsh/Dvl and GSK3\(\beta\) in 293 cells. Overexpression of Dsh/Dvl is known to activate the Wnt pathway and give rise to elevated levels of cytosolic \(\beta\)-catenin (23, 38). We utilized a luciferase reporter driven by TCP/LIF binding sites (TOPTK) to measure the activity of transient overexpression of human Dishevelled 2 (hDvl2) (Fig. 1C). Wild type GSK3\(\beta\) and a dominant negative form of the TCP4 transcription factor (DTCP4) blocked hDvl2 activity (Fig. 1C). GSK3\(\beta\) mutants that retain kinase activity, including serine 9 mutated to alanine or glutamic acid, and tyrosine 216 mutated to phenylalanine or glutamic acid, also retained the ability to block hDvl2 activity (see below and data not shown). In contrast, coexpression of kinase-dead mutants of GSK3\(\beta\) did not have any effect on the activity of hDvl2, nor did coexpression of active MERK, an irrelevant protein kinase (Fig. 1C). Similar results were also obtained from same experiments using CHOIR, a Chinese hamster ovary cell line stably expressing human insulin receptor (data not shown). Due to the high transfection efficiency of C57 cells, CHOIR and 293 cells were used as control experiments involving transient transfections. These observations confirmed that a decrease in GSK3\(\beta\) activity is necessary to convey signals from Dvl to \(\beta\)-catenin and GSK3\(\beta\) is downstream of Wnt in mammalian cells.

**Wnt and Insulin Signaling Lead to Distinct Downstream Events, although GSK3\(\beta\) Is Involved in Both Pathways—**Since GSK3\(\beta\) is a major player in both insulin and Wnt signaling, we...
compared changes in GSK3β activity upon insulin and Wnt stimulation. A similar decrease in GSK3β activity was observed in 3 cell lines, 293, CHOIR and C57 (Fig. 2A). We then examined the downstream events of activated insulin and Wnt pathways. The level of cytosolic β-catenin was increased in cells stimulated with Wnt but unchanged in insulin-treated cells (Fig. 2B). GS activity was analyzed in CHOIR and C57 cells. Insulin-stimulated cells yielded higher GS activity, while Wnt conditioned media had no effect (Fig. 2C). 293 cells had high basal GS activity, and no significant activity increase was detected with insulin treatment (data not shown). These data represent an example of specificity in signaling, yet raised the question how different downstream effects were achieved through a seemingly indistinguishable change in the activity of GSK3β, a common component of the two signaling pathways.

**Wnt Regulates GSK3 Activity through Mechanisms Other than Serine 9 Phosphorylation**—Serine 9 (Ser-9) is a key regulation site of GSK3β responding to insulin signaling (7). Using a phosphospecific antibody against phospho-Ser-9 in GSK3β, we were able to detect a clear increase of phosphorylation on this residue upon insulin stimulation in CHOIR and C57 cells (Fig. 3A). However, we did not observe any obvious difference of phospho-Ser-9 reactivity in samples treated with Wnt media or control media. The phosphotyrosine content remained constant before and after either insulin or Wnt stimulation. Similar results were observed from 293 cells (data not shown). Since PKB is known to be the upstream regulator of GSK3 in insulin signaling, we analyzed the phosphorylation status of two key residues in PKB, Thr-308 and Ser-473, using phosphospecific antibodies. There was a strong increase in phosphorylation of both residues responding to insulin but not to Wnt (Fig. 3B).
We also metabolically labeled cells in vivo with \[^{32}\text{P}]\text{orthophosphate before treatment with insulin or Wnt. We did not detect any significant changes in the total level of phosphorylation or phosphoamino acid analysis of GSK3\(\beta\), although it confirmed that the majority of phosphorylation was on serine residues (data not shown). Phosphopeptide mapping was also performed (Fig. 3C). The phosphopeptide pattern for GSK3\(\beta\) from C57 cells treated with control or Wnt media appeared to be essentially the same. Nevertheless, GSK3\(\beta\) from insulin-treated cells elicited a distinctive increase in phosphorylation at the positions corresponding to peptides containing Ser-9 (33). Similar patterns were obtained from 293 and CHOIR cells transiently expressing an HA-epitope-tagged wild type or S9A mutant of GSK3\(\beta\) were stimulated with insulin or Wnt conditioned media. The kinase activity of S9A-GSK3\(\beta\) no longer decreased in response to insulin (Fig. 4A), similar to what was reported previously (37). Upon Wnt stimulation, the S9A mutant exhibited an activity drop similar to that for the wild type kinase (Fig. 4A). We also tested the ability of S9A to block Wnt signal by hDvl2 (Fig. 4B). At higher expression level, both S9A mutant or wild type GSK3\(\beta\) efficiently blocked hDvl2-activated TCF/LEF-driven luciferase activity. Interestingly, at lower expression level, S9A was able to block hDvl2 activity roughly 2-fold better than the wild type GSK3\(\beta\). This is probably due to the higher intrinsic kinase activity of S9A mutant that we and others have observed (35). Similar effects were observed using CHOIR cells (data not shown).

Response of GSK3\(\beta\) to Insulin and Wnt in Axin-Conductin Complex—GSK3\(\beta\) has been found in the axin-conductin-APC-\(\beta\)-catenin complex (16, 17). Because of the multitude of biological processes that GSK3\(\beta\) is involved in, it is reasonable to hypothesize that only a fraction of the total cellular GSK3\(\beta\) is
in the axin-conductin complex. We investigated the response of GSK3β in the axin-conductin complex to insulin or Wnt. From CHOIR cells with insulin or Wnt treatment, endogenous GSK3β was coimmunoprecipitated with ectopically expressed EE-tagged conductin (EE-conductin). Only a small fraction of that of the untreated sample.

Activities were expressed as the percentage of the untreated sample. Error bars represent standard deviations from at least three independent experiments with duplicates in each experiment.

Response of GSK3β to Inducible Dishevelled—As the most upstream intracellular component of the Wnt pathway, Dvl is capable of activating downstream molecules independent of the extracellular ligand Wnt (23, 25, 38). To further substantiate our findings described in the earlier sections, we also investigated the response of GSK3β to activated Dvl. We chose to generate a fusion protein between Dvl and a modified version of the hormone binding domain of the murine estrogen receptor (Dvl-ER) and use this as our inducible system to achieve rapid activation of the Wnt pathway (39). Expression of Dvl-ER activates TOPTK reporter activity in a hormone (4-hydroxytormoxifen (4-HT))-dependent manner (data not shown). A 293 cell line was then made to stably express Dvl-ER (293-D-ER). As shown in Fig. 6A, cytosolic β-catenin accumulated upon 4-HT treatment in 293-D-ER cells (293-D-ER-His6-conductin) so that the conductin-bound pool of GSK3β seemed to be less prominent than the total pool of GSK3β.

DISCUSSION

GSK3β has been implicated in mediating many diverse signals in various cell types. It is believed that many signals can down-regulate the kinase activity of GSK3β. How different
signaling pathways achieve specificity through GSK3β remains unclear. In this study, we investigated the differential regulation of GSK3β by two extracellular signals, insulin and Wnt. Although both signals decrease GSK3β activity to a similar extent, we found that insulin and Wnt lead to very distinct downstream events. Furthermore, unlike in insulin signaling, Ser-9 of GSK3β is not phosphorylated by the Wnt signaling pathway.

In any given organism, many cells will receive and respond to multiple extracellular signals. The cell lines that we chose for this study, for example, are able to respond to both insulin and Wnt. Insulin stimulation leads to increased glyco-gen synthase activity and up-regulate β-catenin level (41). However, we found that insulin did not cause β-catenin accumulation and Wnt did not increase glycogen synthase activity. Therefore, the effect of each signaling pathway is highly specific. A similar finding was reported by Staal et al. (42) that T cell activation causes decrease in GSK3β activity but no change in β-catenin accumulation.

A large body of evidence suggests that the regulation of GSK3β by insulin is a phosphorylation event at Ser-9 via activated PKB (7, 10). One way to achieve specificity via a common intermediate in different pathways could be through different posttranslational modifications. GSK3β activity can be down-regulated independent of Ser-9 phosphorylation or PKB in exercised muscle (43). We demonstrated that Wnt signaling did not cause Thr-308 or Ser-473 phosphorylation on PKB, nor was Ser-9 of GSK3β modified. Supporting our data, Yuan et al. (44) reported that activation of PKB alone is not sufficient to mimic Wnt signaling. However, in their report, exogenous PKB had a synergistic effect on β-catenin with exogenously expressed Wnt1 or Frat1. We did not find any synergistic effect if we stimulated cells with insulin and Wnt simultaneously (data not shown). It is possible that, although activating Wnt signaling does not rely on the activity of PKB, nor was Ser-9 of GSK3β modified. Supporting our data, it is not certain whether the levels of overexpression of GSK3β and axin-conductin are comparable. Nevertheless, one explanation is that GSK3β is not the limiting factor, thus supporting the idea that a subpopulation of GSK3β is dedicated to form complexes with axin-conductin and only this pool of GSK3β participates in Wnt signaling. Furthermore, we observed that the GSK3β complexed to transiently or stably expressed conductin was significantly protected from Ser-9 phosphorylation by insulin. Further analysis through the investigation of this complex will shed light on our understanding of how GSK3 is regulated in the Wnt signaling pathway.

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