Expression and Characterization of GSK-3 Mutants and Their Effect on β-Catenin Phosphorylation in Intact Cells*

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Glycogen synthase kinase-3 (GSK-3) is a serine-threonine kinase that is involved in multiple cellular signaling pathways, including the Wnt signaling cascade where it phosphorylates β-catenin, thus targeting it for proteasome-mediated degradation. Unlike phosphorylation of glycogen synthase, phosphorylation of β-catenin by GSK-3 does not require priming in vitro, i.e. it is not dependent on the presence of a phosphoserine, four residues C-terminal to the GSK-3 phosphorylation site. Recently, a means of dissecting GSK-3 activity toward primed and non-primed substrates has been made possible by identification of the R96A mutant of GSK-3β. This mutant is unable to phosphorylate primed but can still phosphorylate unprimed substrates (Frame, S., Cohen, P., and Biondi R. M. (2001) Mol. Cell 7, 1321–1327). Here we have investigated whether phosphorylation of Ser33, Ser37, and Thr41 in β-catenin requires priming through prior phosphorylation at Ser45 in intact cells. We have shown that the Arg96 mutant does not induce β-catenin degradation but instead stabilizes β-catenin, indicating that it is unable to phosphorylate β-catenin in intact cells. Furthermore, if Ser45 in β-catenin is mutated to Ala, β-catenin is markedly stabilized, and phosphorylation of Ser33, Ser37, and Thr41 in β-catenin by wild type GSK-3β is prevented in intact cells. In addition, we have shown that the L128A mutant, which is deficient in phosphorylating Axin in vitro, is still able to phosphorylate β-catenin in intact cells although it has reduced activity. Mutation of Tyr216 to Phe markedly reduces its activity in vitro, i.e. it is not dependent on the presence of a phosphotyrosine, four residues C-terminal to the GSK-3 phosphorylation site. In conclusion, we have found that the Arg96 mutant has a dominant-negative effect on GSK-3β-dependent phosphorylation of β-catenin and that targeting of β-catenin for degradation requires prior priming through phosphorylation of Ser45.

Glycogen synthase kinase-3 (GSK-3) is a serine-threonine kinase that is involved in both insulin and Wnt signaling. In both signaling pathways, GSK-3 is constitutively active and becomes inhibited upon binding of insulin or Wnt ligand to their respective receptors. As a result of insulin signaling, GSK-3 phosphorylates glycogen synthase, and inhibition of GSK-3 leads to accumulation of dephosphorylated, active glycogen synthase, thus leading to stimulation of glycogen synthesis. For Wnt signaling, the primary substrate of GSK-3 is β-catenin. Phosphorylation by GSK-3 targets β-catenin for ubiquitination and proteasome-mediated degradation. Inhibition of GSK-3 thus results in β-catenin stabilization. β-catenin then translocates into the nucleus, where it activates gene transcription in conjunction with transcription factors of the Lef/TCF family (1–3).

GSK-3 activity toward cellular substrates can be regulated at several levels, including serine and tyrosine phosphorylation of GSK-3 and interaction of GSK-3 with other proteins in multiprotein complexes, as well as priming of substrates through an independent phosphorylation event (4).

Upon binding of insulin to its receptor, Akt/protein kinase B is activated through a phosphatidylinositol 3-kinase-dependent cascade and phosphorylates GSK-3 at an N-terminal serine (Ser21 in GSK-3α and Ser27 in GSK-3β), resulting in inhibition of its activity (5). It has been shown recently that the phosphorylated N-terminus of GSK-3 acts as a competitive pseudosubstrate that occupies the substrate binding site of GSK-3 (6, 7). In addition to the N-terminal serine, the activity of GSK-3 is modulated through phosphorylation of a tyrosine residue (Tyr279 in GSK-3α and Tyr216 in GSK-3β). Tyrosine phosphorylation leads to increased GSK-3 activity, and mutation of the tyrosine to phenylalanine reduces its activity in vitro as well as in cells, as assessed by inhibition of c-Jun activity by GSK-3 (8). However, it is unclear whether tyrosine phosphorylation of GSK-3 plays a regulatory role under physiological conditions. For instance, tyrosine phosphorylation does not change in response to growth factors (9). The identity of the kinase responsible for the tyrosine phosphorylation event is not known, although it has been suggested that this may be an autophosphorylation event (4).

In contrast to insulin signaling, phosphorylation of the N-terminal serine is not involved in Wnt-mediated inhibition of GSK-3 (10). In the Wnt/β-catenin pathway, GSK-3 activity is regulated through interaction with binding partners in multiprotein complexes. These complexes include β-catenin, the scaffold protein Axin, and APC, the product of the adenomatous polyposis coli gene. Both Axin and APC are required for GSK-3-mediated phosphorylation of β-catenin and are also substrates for GSK-3 (11, 12). Phosphorylation of Axin by GSK-3 stabilizes the protein (13) and facilitates the binding between Axin and β-catenin (14, 15). Phosphorylation of APC by GSK-3 also facilitates its interaction with β-catenin (12, 16). Thus, phosphorylation of both Axin and APC by GSK-3 promotes the formation and stabilization of the β-catenin degradation complex. Wnt signaling is transmitted via Dishevelled, which is...
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MATERIALS AND METHODS

Plasmid Constructs and Transfection of HEK293T Cells—The V5-β-catenin-pcDNA3.1 expression vector was purchased from Invitrogen. To generate the Myc-GSK-3β expression construct, human GSK-3β was cloned from a cDNA library and incorporated into the pcDNA3.1 expression vector. The Myc epitope tag was added at the N terminus.

Wild Type and Mutant Myc-GSK-3β—10 μl of protein G-Sepharose was coupled to 5 μg of monoclonal anti-V5 antibody (Invitrogen), which was used to immunoprecipitate V5-β-catenin from 0.5 ml of precleared hypotonic cell lysate. The pellets were washed twice with 1 ml of Buffer 7.5 containing 0.5 M NaCl and then twice with 1 ml of Buffer A without 0.5 M NaCl. The pellets were then used for immunoblotting with the phospho-β-catenin antibody.

Immunoprecipitation of V5-β-Catenin—10 μl of protein G-Sepharose was coupled to 5 μg of monoclonal anti-V5 antibody (Invitrogen), which was used to immunoprecipitate V5-β-catenin from 0.5 ml of precleared hypotonic cell lysate. The pellets were washed twice with 1 ml of Buffer A (50 mM Tris/HCl, 0.1 mM EGTA, 0.1% (v/v) β-mercaptoethanol, pH 7.5) containing 0.5 mM NaCl and then twice with 1 ml of Buffer B without NaCl. The pellets were then used for immunoblotting with the phospho-

Immunoblotting—Cells were lysed as described above. Equal amounts of protein lysate or immunoprecipitated protein were subjected to SDS-PAGE, electrophoretically transferred to nitrocellulose membranes, and immunoblotted. The following antibodies were used: monoclonal anti-Myc (clone 9E10, Autogen Bioclear), monoclonal anti-v-My (Invitrogen), monoclonal anti-β-catenin (BD Transduction Laboratories), and polyclonal anti-phospho-β-catenin (Cell Signaling), which detects β-catenin when phosphorylated at Ser33, Ser37, and Thr41. Blots were developed using an enhanced chemiluminescence reagent (Amersham Biosciences).

β-Catenin/1Lef/TCF-regulated Gene Reporter Assay—Luciferase activity in cells transiently transfected with a Lef/TCF-regulated luciferase gene reporter construct was determined as previously described (32).

RESULTS

Wild Type and Mutant Myc-GSK-3β Are Expressed at Similar Levels—Four mutant cDNA expression vectors, Myc-GSK-3β S9A, Y216F, L128A, and R96A, were generated by site-directed mutagenesis and expressed in HEK293T cells. All mutant proteins were expressed at similar levels compared with wild type Myc-GSK-3β (Fig. 1). The transfected proteins were much more abundant compared with endogenous GSK-3β protein, thus allowing for functional characterization of the mutant proteins. To assess the activity of the various mutants toward β-catenin, V5-β-catenin was cotransfected with wild type or mutant Myc-GSK-3β. GSK-3-dependent phosphoryla-

Another utilization of substrates by GSK-3 can be regulated by prior phosphorylation of the substrates. This occurs through phosphorylation at a serine or threonine residue four amino acids C-terminal to the GSK-3 phosphorylation site (4). For example, glycogen synthase can be primed by CK2 at Ser456 (21), which facilitates GSK-3-mediated phosphorylation at Ser452, followed by phosphorylation of Ser448, Ser444, and then Ser440. A similar motif in β-catenin (Ser29, Xγ3-γγ3, Yγ-Ser37, Xγ-Thr38-Xγ-Ser45) is known to be phosphorylated by GSK-3. However, no priming kinase has been identified, and it is at present unclear whether phosphorylation of β-catenin by GSK-3 requires priming (22). Binding of the F-box protein β-TrCP, a component of the E3 ubiquitin ligase, which results in inhibition of GSK-3-mediated phosphorylation and stabilization of β-catenin, eukaryotic initiation factor 2B (eIF2B), and the cAMP-response element-binding protein (CREB) peptide (18–20).

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tion of V5-β-catenin was measured directly using a phosphospecific antibody or indirectly by determining GSK-3-dependent stabilization of V5-β-catenin and transcriptional activation of a recombinant β-catenin/Lef/TCF-regulated luciferase reporter construct.

**Activity of the S9A, Y216F, and L128A Mutants**—Expression of S9A, Y216F, and L128A Myc-GSK-3β resulted in only small changes in total levels of cotransfected V5-β-catenin (Fig. 2A). Wild type Myc-GSK-3β and all three mutants significantly increased the phosphorylation of V5-β-catenin at Ser33, Ser37, and Thr41 (Fig. 2, A and B). However, the degree of phosphorylation of V5-β-catenin varied between the different mutants. Although both wild type and S9A Myc-GSK-3β increased the phosphorylation of β-catenin 4- to 5-fold, the increase with the L128A mutant was ~3-fold and with the Y216F mutant ~2-fold. However, only the difference between the effects of the Y216F mutant compared with wild type and S9A Myc-GSK-3β reached statistical significance (p < 0.05).

Wild type Myc-GSK-3β and the different mutants also led to a significant decrease in β-catenin/Lef/TCF-dependent reporter gene activity (Fig. 2C). The relative inhibitory effects of wild type Myc-GSK-3β and the different mutants were qualitatively similar to their effects on V5-β-catenin phosphorylation. However, as with Myc-GSK-3β-dependent phosphorylation of V5-β-catenin, only the difference between the effects of the Y216F mutant compared with wild type and S9A Myc-GSK-3β were statistically significant (p < 0.05).

**Expression of R96A Myc-GSK-3β Markedly Increases V5-β-Catenin Levels**—We next determined whether the R96A mutant of Myc-GSK-3β was still able to phosphorylate V5-β-catenin and target it for proteasome-dependent degradation. Surprisingly, we found that R96A Myc-GSK-3β markedly increased steady state levels of V5-β-catenin (Fig. 3A). This increase in the protein concentration was not due to a change in the mRNA level of transfected V5-β-catenin when the R96A mutant was cotransfected, as determined using a reverse transcriptase-PCR assay (not shown). Similarly, a marked increase in gene reporter activity was observed in the presence of R96A Myc-GSK-3β compared with the empty vector control (Fig. 3B), indicating that V5-β-catenin was stabilized and presumably would translocate to the nucleus to activate Lef/TCF-dependent transcription.

The R96A Mutant of GSK-3β, but Not Kinase-inactive GSK-3β, Acts as Dominant-negative—Our results suggested that the R96A mutant is unable to phosphorylate β-catenin and initiate its degradation and that it increases β-catenin by competing with endogenous GSK-3β. To test this further, we measured luciferase gene reporter activity in the presence of the GSK-3 inhibitor SB-415286 (32). As reported previously (32), inhibition of endogenous GSK-3 in untransfected cells markedly increased gene reporter activity (Fig. 4). When cells were transfected with only V5-β-catenin, basal gene reporter activity was highly elevated; addition of SB-415286 increased transcriptional activity of the β-catenin/Lef/TCF-dependent reporter construct further. Recombinant overexpression of Myc-GSK-3β decreased basal gene reporter activity, which was reversed in the presence of the GSK-3 inhibitor. In contrast, R96A Myc-GSK-3β markedly increased basal gene reporter activity. Addition of SB-415286 led to only a small further increase in transcriptional activity, suggesting that GSK-3-dependent phosphorylation and degradation of β-catenin was already inhibited because R96A Myc-GSK-3β acted as dominant-negative.

We next compared the effect of the R96A mutant with that of kinase-inactive Myc-GSK-3β. We found that although the R96A mutant markedly increased the total level of cotransfected V5-β-catenin, expression of kinase-inactive K85M/K86I Myc-GSK-3β did not result in any increase in V5-β-catenin compared with cells transfected with empty vector and V5-β-catenin (Fig. 5).
Mutation of Ser 45 in /H9252-Catenin to Ala Prevents Phosphorylation of Ser33, Ser37, and Thr41 by GSK-3

The R96A mutant of GSK-3/H9252 has been shown to be unable to phosphorylate primed substrates, while retaining the ability to phosphorylate unprimed substrates in vitro (7). Thus, our finding that it inhibits the GSK-3-mediated degradation of /H9252-catenin suggests that phosphorylation of the two serine residues (Ser 33 and Ser37) in the /H9252-TrCP recognition motif of /H9252-catenin requires priming by means of prior phosphorylation of Ser45 and subsequently of Thr41. To determine whether this is the case, Ser45 in V5-β-catenin was mutated to Ala. This resulted in a marked stabilization of V5-β-catenin (Fig. 6A), as had been previously observed when the residue was deleted (27). Coexpression of wild type Myc-GSK-3β decreased levels of wild type V5-β-catenin but did not affect S45A V5-β-catenin levels (Fig. 6A).

DISCUSSION

The aim of this study was to gain insight into the regulation of the GSK-3β-mediated phosphorylation of β-catenin in cells.
In particular, conflicting data have been reported with respect to the requirement of priming phosphorylation on β-catenin to facilitate its utilization as a GSK-3 substrate. In glycosynthase, GSK-3 phosphorylates the four N-terminal serines in the motif Ser640-3-Ser648 (12, 14). Mutation of these serines by GSK-3 is sequential from the C to the N terminus and is dependent on prior phosphorylation of Ser645 by CK2. GSK-3β is known to phosphorylate a similar motif in β-catenin: Ser33-3-Ser37-3-Thr41-3-Ser45. Although only Ser33 and Ser37 are part of the recognition motif for binding of β-TrCP to β-catenin, mutations of all of these serine and threonine residues, including Thr41 and Ser45, are known to stabilize β-catenin and to be associated with various cancers (27–29). This suggests that, like glycosynthase, β-catenin is phosphorylated in a sequential manner beginning at the C terminus. However, it has been shown in vitro that phosphorylation of β-catenin by GSK-3 does not require priming. Consistent with this, the R96A mutant of GSK-3β was shown in vitro to phosphorylate β-catenin as efficiently as the wild type enzyme (7).

Here we have used this mutant to investigate the mechanism of GSK-3-mediated phosphorylation of β-catenin in intact cells. In contrast to in vitro studies, we have found that the R96A mutant prevents GSK-3-mediated phosphorylation and degradation of β-catenin. Furthermore, mutation of Ser45 in β-catenin to Ala prevented phosphorylation of the Thr41, Ser37, and Ser33 residues in β-catenin. These results indicate that like glycosynthase β-catenin is phosphorylated in a sequential manner, beginning at the C terminus, in cellular systems. If Ser45 is phosphorylated, R96A GSK-3β is unable to catalyze the phosphorylation of Thr41 because the substrate is primed. Consequently the mutant enzyme acts as a dominant-negative. It is presently unclear whether priming of β-catenin through phosphorylation of Ser45 is mediated by GSK-3 itself or by a different kinase.

We have also found that the L128A mutant of GSK-3β was able to phosphorylate β-catenin in intact cells, although its activity was reduced compared with wild type enzyme. The L128A mutant has been reported to be deficient in phosphorylating, but not in binding, Axin in vitro while retaining the ability to phosphorylate primed substrates (7). The ability of the L128A mutant to phosphorylate β-catenin in intact cells would thus be in agreement with the mechanism of β-catenin phosphorylation by GSK-3. The reduced activity of L128A mutant GSK-3β may be due to impaired GSK-3-dependent phosphorylation of Axin and/or APC, which has been shown to promote the formation and stabilization of the β-catenin degradation complex (12, 14–16). Thus, reduced phosphorylation of β-catenin by the L128A mutant may result from destabilization of the β-catenin signaling complex.

In contrast to the R96A mutant, we have found that kinase-inactive GSK-3β was unable to act as a dominant-negative in signaling to β-catenin. Although there are several reports showing dominant-negative effects of kinase-inactive GSK-3 in Wnt signaling in Xenopus (26, 33–35), activation of the Wnt signaling pathway by kinase-inactive GSK-3 in a mammalian cell system has been reported only in a study by Staal et al. (36). That study found that kinase-inactive GSK-3β and lithium activate β-catenin-dependent Tcf-1-mediated transcription in C57MG but not in Jurkat T cells. GSK-3 does not bind β-catenin directly but requires interaction with Axin and APC to phosphorylate β-catenin. It is possible that the dominant-negative activity is dependent on the interaction with Axin, because kinase-inactive GSK-3β has been shown to be unable to interact with Axin (11, 37). This implies that the interaction of GSK-3 with Axin is dependent on its kinase activity. Because Axin may be an unprimed substrate for GSK-3 (7), it is possible that the R96A mutant retains the ability to phosphorylate and bind Axin. This would allow access for the R96A mutant, but not kinase-inactive, GSK-3 into the β-catenin degradation complex. Thus, only R96A mutant, but not kinase-inactive, GSK-3, would be able to compete with endogenous GSK-3.

Finally, as expected, we have shown that mutation of Ser33 to Ala did not affect GSK-3β-mediated phosphorylation of β-catenin, whereas mutation of Tyr216 to Phe markedly reduced the phosphorylation of β-catenin in intact cells. These findings are consistent with other studies, in which tyrosine phosphorylation has been shown to increase the activity of GSK-3β toward various substrates in vitro and in intact cells, including c-Jun (8) and Tau (38). Our results suggest that tyrosine phosphorylation is also required for GSK-3β-dependent phosphorylation of β-catenin, although it is not clear whether it may serve a regulatory role in the Wnt/β-catenin pathway.

In conclusion, this study shows that phosphorylation of β-catenin at Ser33, Ser37, and Thr41 in intact cells requires priming through phosphorylation of Ser45. The Arg306 mutant, which is unable to phosphorylate primed substrates, but not kinase-inactive GSKβ3, has a dominant-negative effect on the phosphorylation of β-catenin.

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FIG. 7. S45A mutant V5-β-catenin cannot be phosphorylated by Myc-GSK-3β at Ser33, Ser37, and Thr41. Cells were cotransfected with wild type or S45A mutant V5-β-catenin and empty vector or wild type Myc-GSK-3β as indicated. Cell lysates were immunoprecipitated using V5 antibody. Immunoprecipitates were subjected to SDS-PAGE and analyzed by immunoblotting with phospho-β-catenin-specific or V5 antibody.
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