Suppression of Glycogen Synthase Kinase Activity Is Not Sufficient for Leukemia Enhancer Factor-1 Activation*

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Glycogen synthase kinase-3 (GSK) can be regulated by different signaling pathways including those mediated by protein kinase Akt and Wnt proteins. Wnt proteins are believed to activate a transcription factor leukemia enhancer factor-1 (LEF-1) by inhibiting GSK, and Akt was shown to phosphorylate GSK and inhibit its kinase activity. We investigated the effect of an activated Akt on the accumulation of cytosolic β-catenin and LEF-1-dependent transcription. Although the activated Akt, mAkt, clearly inhibited the kinase activity of GSK, mAkt alone did not induce accumulation of cytosolic β-catenin or activate LEF-1-dependent transcription. On the contrary, coexpressed Wnt-1 and Frat activated LEF-1 but did not show significant inhibition of GSK-mediated phosphorylation of a peptide substrate. However, mAkt could act synergistically with Wnt-1 or Frat to activate LEF-1. In addition, the interaction of GSK for Axin appeared to decrease in the presence of mAkt, whereas the interaction for Frat remained unchanged. Consistently, a GSK mutant with substitution of a Phe residue for residue Tyr-216, which showed one-fifth of kinase activity of the wild-type GSK, exhibited a reduced association for Axin than the wild-type GSK. These results suggest that inhibition of GSK kinase activity is not sufficient for activation of LEF-1 but may facilitate the activation by reducing the interaction of GSK for Axin. The additional mechanism for LEF-1 activation may require dissociation of GSK from Axin as Frat facilitates the dissociation of GSK from Axin.

The Wnt family of secretory glycoproteins is one of the major families of developmentally important signaling molecules and plays important roles in embryonic induction, generation of cell polarity, and specification of cell fate. Wnt pathways are also closely linked to tumorigenesis. A large amount of knowledge about Wnt-mediated signal transduction comes from genetic studies in Drosophila. A genetic order of these signal transduction events has been established, in which Wg appears to negatively regulate Zeste-White 3 (Zw3) through Dishevelled (Dsh), thus relieving the suppression of Armadillo by Zw3 with a net result of up-regulation of Armadillo. Armadillo interacts with Pango- lin-regulating gene transcription. Recent evidence suggests that the Frizzled proteins may function as receptors for Wnt (for review, see Refs. 1–6).

Wnt-linked pathways are apparently conserved in higher organisms including mammals. More than 20 Wnt and 8 Frizzled homologues have been cloned and sequenced in mammals (5, 7, 8). Molecular cloning also revealed mammalian homologs to Dsh (9–11), Zw3, and Armadillo; they are Dvl, GSK-3β and β-catenin, respectively (5). Fly zw3 mutants and frog embryos expressing dominant negative mutants of GSK-3β showed phenotypes consistent with constitutive activation of the β-catenin by the Wnt pathway (5). This led to a model suggesting that Wnt inhibits GSK activity. Additionally, soluble Wg was shown to inhibit GSK activity in mouse fibroblasts (12).

Although the molecular mechanism by which Wnt inhibits GSK, presumably via Dsh/Dvl, remains to be elucidated, it appears to be better understood how GSK leads to regulation of gene transcription via β-catenin and transcription factors LEF-1/T cell factor (13–15). GSK-3β was initially found to form a complex with β-catenin and the product of adenomatous polyposis coli (APC) gene (16, 17). Recently, evidence indicated that Axin and its homologs are also part of the complex (18–20, 32). Axin binds directly to GSK-3β, β-catenin, and APC, whereas APC also directly binds to GSK-3β and β-catenin. GSK-3β is shown to phosphorylate β-catenin and APC. The phosphorylation is believed to play a role in promoting the degradation of β-catenin via the ubiquitination pathway (21). Axin and its homologs were also proposed to be involved in the degradation process (20). One of the models suggests that Axin functions as a scaffold protein to bring GSK and β-catenin together, thus facilitating the phosphorylation of β-catenin by GSK (18). APC was originally identified as a tumor suppressor gene. Mutations in APC that correlate to human colorectal cancers appear to lose the ability to destabilize β-catenin (16).

Akt, also known as protein kinase B, is regulated by phosphotidylinositol-3 kinases (22). Many extracellular stimuli activate phosphotidylinositol-3 kinases via growth factor receptors and G protein-coupled receptors. Akt-mediated signaling has been subjected to intense study and found to be involved in a variety of biological processes, including anti-apoptosis, stimulation of protein synthesis and gene transcription, and regulation of metabolism. GSK is one of well characterized substrates and effectors of Akt. Akt can phosphorylate both GSK-3α and -3β and inhibit their kinase activity (22–25).

Recently, a novel GSK regulator, GSK-binding protein (GBP), was identified from Xenopus for its ability to bind to GSK and inhibit phosphorylation of tau proteins when GBP and tau were coexpressed in Xenopus embryos (26). The mammalian homolog of GBP, named Frat, has previously been cloned independently for its tumor-promoting activity in lymphocytes (27). Frat can also bind to GSK (26). The precise role
of the interaction between GSK and GBP/Frat is not clear. However, ectopic overexpression of GBP or its C-terminal GSK-binding domain could mimic the effects of Wnt in *Xenopus* (26), suggesting that GBP/Frat mediate either Wnt-1 signaling or a different pathway that can interact with Wnt-1.

We and others (28, 29) have previously shown that Wnt-1 activates LEF-1 in transfected mammalian cells using a reporter gene assay. In this report, we investigate the effect of Akt and Frat on regulation of LEF-1. Although Akt potently inhibited the kinases activity of GSK, Akt alone showed little effect on activation of LEF-1-dependent transcription. On the other hand, expression of Frat led to LEF-1 activation, but we could not observe any inhibition of GSK kinase activity by Frat. These results suggest that inhibition of GSK is not sufficient for activation of LEF-1 and that additional mechanism is required. Moreover, Akt can act synergistically with Wnt-1 or Frat in LEF-1 activation. This synergistic effect may be explained by the finding that phosphorylation of GSK by Akt attenuates the apparent affinity of GSK for Axin, although not affecting the affinity of GSK for Frat.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfection, and Luciferase Assay—**NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37 °C under 5% CO2. For the luciferase assays, cells (5 x 10^6 cells/well) were seeded into 24-well plates the day before transfection. Cells were transfected with 0.5 µg DNA/well using LipofectAMINE Plus (Life Technologies, Inc.), as suggested by the manufacturer. For kinase and immunoprecipitation assays, transfection was carried out in 12-well plates. The number of cells and amount of DNA were increased in proportions. Transfection was stopped by switching to normal growth medium after 3 h. Cell extracts were collected 24 h later for the luciferase assays, immunoprecipitation, and Western analysis. The constructs used in this study have previously been described (28).

Luciferase assays were performed using Roche Biochemical constant light luciferase assay kit. Cell lysates were first taken for determining fluorescence intensity emitted by coexpressed green fluorescence protein (GFP) proteins in a Wallac multilabel counter, which is capable of measuring fluorescence and luminescence. Then, luciferase substrate was added to the cell lysates, and luciferase activities were determined by measuring luminescence intensity using the same counter. Luminescence intensity was normalized against fluorescence intensity.

**Immunoprecipitation Assay—**Cells were lysed with the lysis buffer containing 1% Nonidet P-40, 137 mM sodium chloride, 20 mM Tris, pH 7.4, 1 mM dithiothreitol, 10% glycerol, 10 mM sodium fluoride, 1 mM pyrophosphate, 2 mM sodium vanadate, and Complete® protease inhibitors (Roche Biochemical). The cell lysates were preclarified with 20 µl of protein A/G-Sepharose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 0.5 h at 4 °C and then incubated with 1 µl of anti-tag antibody (Berkeley Antibody Co., Richmond, CA) and 20 µl of protein A/G-Sepharose beads for 3.5 h on ice. The immunocomplexes were pelleted and washed 4 times with cold lysis buffer. For Western analysis, proteins were released from beads by boiling in SDS sample buffer. The samples were loaded on SDS-polyacrylamide gel electrophoresis gels, and proteins were electroblotted to nitrocellulose membranes. Results were visualized and quantified using a phosphoimager (Packard Instruments, Meriden, CT).

**RESULTS**

The effect of Akt on activation of LEF-1 was evaluated by determining whether the activated Akt can activate LEF-1-dependent transcription. The Akt mutant, mAkt, carrying a myristylation signal from Src, has been shown to be constitutively active (30). LEF-1 activity was determined using a reporter gene assay, in which multiple LEF-1 response elements were placed in front of a minimal promoter and a luciferase gene. We and others (19, 28, 29) have used this assay system to measure Wnt-1-induced LEF-1 activation in mammalian cells. As shown in Fig. 1, mAkt alone, unlike Wnt-1, did not activate LEF-1-dependent transcription, but it could act synergistically with Wnt-1 in activation of LEF-1. The effect of Akt on the accumulation of cytosolic β-catenin was also examined. Akt could not elevate the level of cytosolic β-catenin, whereas Wnt-1 could (Fig. 1C). To confirm that mAkt can indeed inhibit GSK activity in our assay system, we evaluated the GSK kinase activity by measuring the phosphorylation of a GSK substrate peptide derived from glycogen synthase by immunoprecipitated GSK. As previously reported (31), mAkt, when coexpressed with GSK, significantly inhibited the kinase activity of GSK. However, coexpression of Wnt-1 did not significantly affect the kinase activity of GSK (Fig. 1A).

We also determined the effect of Frat, the mammalian GBP homolog, on LEF-1 activation and GSK kinase activity. Consistent with the Wnt-like effect of GBP/Frat on *Xenopus* embryo development (26), expression of Frat-1 elevated the level of cytosolic β-catenin (Fig. 1C) and stimulated LEF-1-dependent transcriptional activity in 3T3 cells (Fig. 1B). In addition, Frat, like Wnt-1, could act synergistically with Akt in LEF-1-dependent transcriptional activity.
NIH 3T3 cells were cotransfected with cDNAs (0.25 μg) of Myc-GSK, Frat-HA and Axin-HA as indicated. LacZ plasmid was added to make the total amount of DNA equal (0.5 μg/transfection). Immunoprecipitation was carried out with anti-HA and anti-Myc antibodies as shown. One-half of immunocomplexes was analyzed by Western blotting using the anti-Myc antibody. The blot was developed using the chemiluminescence method and detected by an image analyzer (Raytest USA, Inc.) equipped with a cooled CCD camera (A, top panel). The levels of Myc-tagged GSK were quantified using an imaging analyzing software. The other half of immunocomplexes was used in a GSK kinase assay (A, lower panel). The kinase activity after subtraction of the activity of endogenous GSK was normalized against the levels of Myc-tagged GSK (B). For instance, the kinase activity of GSK pulled down via Frat was subtracted with that of endogenous GSK pulled down via Frat in cells expressing only Frat and then divided by the protein level. The kinase assay was carried out in triplicates. The experiment was repeated twice. C, 3T3 cells were transfected with LacZ and GSK-Myc. Part of cell extracts was analyzed by Western blot with an anti-GSK antibody (Santa Cruz Biotechnology, Inc.). The recombinant GSK-Myc is slightly larger than the endogenous GSK. The rest of cell extracts was subject to immunoprecipitation using antibodies as indicated. One-half of the immunocomplexes was detected with the anti-GSK antibody, whereas the other half was used in the kinase assay. The experiment was carried out in triplicate and repeated twice.

To further confirm the result that Frat does not inhibit the kinase activity of GSK, we compared the ability of GSK pulled down by Frat with that of GSK pulled down directly by antibody or via Axin to phosphorylate the GSK peptide substrate. In this way, it would be more likely to measure the activity of the GSK molecules bound to Frat. After normalization of the amounts of GSK proteins pulled down via different means, we found that Frat still showed no inhibitory effect on the GSK activity (Fig. 2, A and B). As a control, Axin did not show any effect on the GSK activity (Fig. 2, A and B), which is in agreement with a previous finding (18). The level and specific activity of endogenous GSK were compared with those of Myc-tagged recombinant GSK proteins (Fig. 2C). The expression levels and specific activities of endogenous and recombinant GSK proteins are similar.

To gain insights into the possible mechanism for the synergistic interaction between the Wnt and Akt pathways in regulation of LEF-1, we investigated the effect of Akt on interactions between Axin and GSK and between Frat and GSK. The interactions were studied using coimmunoprecipitation. Both Frat and Axin contain HA epitope tags, whereas GSK is Myc-tagged. When Akt was coexpressed with GSK and Axin, the presence of Akt decreased the amount of GSK immunoprecipitated by Axin (Fig. 3A). This suggests that inhibition of GSK activity may lead to reduction in the interaction of GSK for Axin. To corroborate this finding, we used a GSK mutant with a substitution of a Phe residue for residue Tyr-217. This GSK-YF mutant showed reduced kinase activity as shown in Fig. 3A. Moreover, GSK-YF pulled the same amount of Frat as the wild-type GSK. Thus, these results suggest that the activity of GSK may positively correlate with the affinity for Axin. In contrast, the presence of Akt appeared to have little effect on the amount of GSK coimmunoprecipitated with Frat (Fig. 3B). Moreover, GSK-YF pulled the same amount of Frat as the wild-type. Therefore, the kinase activity of GSK may have little to do with the interaction for Frat (Fig. 3B).

To further characterize the mechanism for Frat-mediated LEF-1 activation, we investigated the effect of Frat on the interaction between GSK and Axin. Cells were transfected with GSK and Axin in the presence and absence of Frat. GSK was immunoprecipitated, and the levels of Axin in the immunocom-
Fig. 4. Effect of Frat on the interaction between GSK and Axin. NIH 3T3 cells were cotransfected with cDNAs (0.1 µg) of Myc-GSK and Axin-HA in the presence and absence of Frat-Flag as indicated. LacZ plasmid was added to make the total amount of DNA equal (0.5 µg/ transfection). Immunoprecipitation was carried out with the anti-Myc antibody. The immunocomplexes were detected with anti-HA and anti-Flag antibodies. The total cell extracts were also subjected to Western analysis with the anti-HA antibody to make sure that the same amounts of Axin are present. The experiment was repeated three times.

The synergistic effect between Wnt and Akt suggests that growth factor-linked and G protein-linked pathways can potentially interact with the Wnt pathway, although the precise physiological relevance of this interaction is not clear. The fact that Akt also showed synergistic effect with Frat suggests that the interaction between Akt and the Wnt pathway may lie near or downstream of GSK if Frat activates LEF-1 by acting directly on GSK. Our current interpretation for the synergistic effect of Akt on Wnt-1-mediated LEF-1 activation is that Akt may weaken the interaction between GSK and Axin by suppression of GSK kinase activity. This idea is based on our observations that the wild-type GSK in the presence of Akt and the GSK-YF mutant with less intrinsic kinase activity showed lower apparent affinities for Axin but retained the same apparent affinity for Frat. Attenuation of the interaction of GSK-YF for Axin has been previously reported (18). It is, however, not clear how the kinase activity of GSK correlates with its apparent affinity for Axin. Residue Tyr-216, which is similar to those frequently referred to as activation loop sites found in many protein kinases, may be autophosphorylated (33) or phosphorylated by unknown tyrosine kinases. Phosphorylation of activation loop sites usually lead to activation of the kinases. The reduced activity of GSK-YF, which was also observed with GSK-YF produced from insect cells (34), agrees with this notion.

The current model for activation of LEF-1/T cell factor by Wnt proteins is that Wnt acts through Dvl to inhibit GSK. This model is mainly based on studies with Xenopus using dominant negative mutants of GSK in Xenopus (5). Our results clearly show that inhibition of GSK kinase activity may not be sufficient for LEF-1 activation, suggesting that there may be more to the current understanding of the pathway, at least in mammalian cells. The involvement of GSK in Wnt signaling appears to be different between Xenopus and mammalian cells. Although the kinase-deficient mutants of GSK are potent inducers of Wnt-like phenotypes in Xenopus embryos, they showed no effect on LEF-1 activation in mammalian cells (Ref. 35 and data not shown). Because Akt alone was also shown not to affect Wnt pathways in Xenopus (36), which is consistent with our finding, it is possible that the kinase-deficient mutants of GSK may exert additional effects in Xenopus embryos. Nevertheless, suppression of GSK kinase activity could have a significant effect on LEF-1 activation if we assume that Akt acts synergistically with Wnt by inhibiting GSK. The finding that GSK molecules with attenuated kinase activity have lower apparent affinities for Axin suggests that dissociation of GSK from Axin might be an important step leading to LEF-1 activation. This hypothesis is consistent with a current model that Axin facilitates the phosphorylation of β-catenin by bringing β-catenin and GSK together (18) and is supported by our findings that Frat activates LEF-1 and aids the dissociation of GSK from Axin. It is possible that Wnt-1 acts in a way similar to Frat or via Frat. In fact, we have found that Frat can interact with Dvl and that the Dvl-interacting domain of Frat can inhibit Wnt-1-induced LEF-1 activation (37). Thus, Dvl may recruit Frat to interact with GSK leading to dissociation of GSK from Axin. Reduction in the association of GSK for Axin led by either phosphorylation by Akt or attenuation in kinase activity by YF mutation would understandably facilitate the dissociation of GSK from Axin. The molecular mechanisms by which Wnt and Dvl regulate the stability of β-catenin and activation of LEF-1 via GSK, Frat, and Axin is now under investigation.

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REFERENCES
1. Dickinson, M. E., and McMahon, A. P. (1992) Curr. Opin. Genet. Dev. 2, 562–566
2. Nusse, R., and Varmus, H. E. (1992) Cell 69, 1073–1087
3. Perrimon, N. (1994) Cell 76, 781–784
4. Klingensmith, J., and Nusse, R. (1994) Dev. Biol. 166, 396–414
5. Cadigan, K. M., and Nusse, R. (1997) Genes Dev. 11, 3286–3305
6. Dale, T. C. (1998) Biochem. J. 329, 209–223
7. Chan, S. D., Karpe, D. B., Fowlkes, M. E., Hooks, M., Bradley, M. S., Vuong, V., Ilambo, T., Liu, M. Y., Arnaud, C. D., Strewler, G. J., and Nissenson, R. A. (1992) J. Biol. Chem. 267, 25202–25207
8. Wang, Y., Macke, J. P., Abella, B. S., Andreassen, K., Worley, P., Gilbert, D. J., Copeland, N. G., Jenkins, N. A., and Nathans, J. (1996) J. Biol. Chem. 271, 4468–4476
9. Sussman, D. J., Klingensmith, J., Salinas, P., Adams, P. S., Nusse, R., and...
Perrimon, N. (1994) *Dev. Biol.* **166**, 73–86
10. Klingensmith, J., Yang, Y., Axelrod, J. D., Beier, D. R., Perrimon, N., and Sussman, D. J. (1996) *Mech. Dev.* **58**, 15–28
11. Tsang, M., Lijam, N., Yang, Y., Beier, D. R., Wynshaw-Boris, A., and Sussman, D. J. (1996) *Dev. Dyn.* **207**, 253–282
12. Cook, D., Fry, M. J., Hughes, K., Woodgett, J. R., and Dale, T. (1996) *EMBO J.* **15**, 4520–4536
13. Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O., and Clevers, H. (1996) *Cell* **86**, 391–399
14. Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B., and Clevers, H. (1997) *Science* **275**, 1784–1787
15. Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K. W. (1997) *Science* **275**, 1787–1790
16. Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S., and Polakis, P. (1996) *Science* **272**, 1023–1026
17. Rubinfeld, B., Souza, B., Albert, I., Muller, O., Chamberlain, S. H., Masiarz, F. R., Munemitsu, S., and Polakis, P. (1993) *Science* **262**, 1731–1734
18. Ikeda, S., Kishida, S., Yamamoto, H., Murai, H., Koyama, S., and Kikuchi, A. (1996) *EMBO J.* **15**, 1371–1384
19. Sakanaoka, C., Weiss, J. B., and Williams, L. T. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 3020–3023
20. Behrens, J., Jerchow, B., Wurtele, M., Grimm, J., Ashrand, C., Wirtz, R., Kuhl, M., Wedlich, D., and Birchmeier, W. (1998) *Science* **280**, 596–598
21. Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997) *EMBO J.* **16**, 3797–3804
22. Alessi, D. R., and Cohen, P. (1998) *Curr. Opin. Genet. Dev.* **8**, 55–62
23. Hawkins, P. T., Welch, H., McGreger, A., Gebert, S., Krugmann, S., Anderson, K., Stokoe, D., and Stephens, L. (1997) *Biochem. Soc. Trans.* **25**, 710–714
24. Downward, J. (1998) *Curr. Opin. Cell Biol.* **10**, 262–267
25. Coffer, P. J., Jin, J., and Woodgett, J. R. (1998) *Biochem. J.* **335**, 1–13
26. Yost, C., Farr, G. H., Pierce, S. B., Ferkey, D. M., Chen, M. M., and Kimelman, D. (1998) *Cell* **93**, 1031–1041
27. Jonkers, J., Korswagen, H. C., Acton, D., Breuer, M., and Berns, A. (1997) *EMBO J.* **16**, 441–450
28. Li, L., Yuan, H., Xie, W., Mao, J., Carriso, A. M., McMahon, E., Sussman, D., and Wu, D. (1999) *J. Biol. Chem.* **274**, 129–134
29. Hsu, S. C., Galceran, J., and Grosschedl, R. (1998) *Mol. Cell Biol.* **18**, 4807–4818
30. Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. (1999) *Cell* **81**, 727–736
31. Cross, D. A., Alesi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995) *Nature* **378**, 785–789
32. Yamamoto, H., Kishida, S., Kishida, M., Ikeda, S., Takaoka, S., and Kikuchi, A. (1999) *J. Biol. Chem.* **274**, 10681–10684
33. Wang, Q. M., Fiol, C. J., DePaoli-Roach, A. A., and Roach, P. J. (1994) *J Biol. Chem.* **269**, 14566–14574
34. Hughes, K., Nikolakaki, E., Frye, S. E., Totty, N. P., and Woodgett, J. R. (1993) *EMBO J.* **12**, 803–808
35. Smalley, M. J., Sara, E., Paterson, H., Naylor, S., Cook, D., Jayatilake, H., Fryer, L. G., Hutchinson, L., Fry, M. J., and Dale, T. C. (1999) *EMBO J.* **18**, 2823–2835
36. Torres, M. A., Eldar-Finkelman, H., Krebs, E. G., and Moon, R. T. (1999) *Mol. Cell Biol.* **19**, 1427–1437
37. Li, R., Yuan, H., Weaver, C. D., Mao, J., Farr, G. H., III, Sussman, D. J., Jonkers, J., Kimelman, D., and Wu, D. (1999) *EMBO J.* **18**, 4233–4240