Tuberous sclerosis complex (TSC) is characterized by the formation of hamartomas in multiple organs resulting from mutations in the TSC1 or TSC2 gene. Their protein products, hamartin and tuberin, respectively, form a functional complex that affects cell growth, differentiation, and proliferation. Several lines of evidence, including renal tumors derived from TSC2+/- animals, suggest that the loss or inhibition of tuberin is associated with up-regulation of cyclin D1. As cyclin D1 can be regulated through the canonical Wnt/β-catenin signaling pathway, we hypothesize that the cell proliferative effects of hamartin and tuberin are partly mediated through β-catenin. In this study, total β-catenin protein levels were found to be elevated in the TSC2-related renal tumors. Ectopic expression of hamartin and wild-type tuberin, but not mutant tuberin, reduced β-catenin steady-state levels and its half-life. The TSC1-TSC2 complex also inhibited Wnt-1 stimulated Tcf/LEF luciferase reporter activity. This inhibition was eliminated by constitutively active β-catenin but not by Dsh, suggesting that hamartin and tuberin function at the level of the β-catenin degradation complex. Indeed, hamartin and tuberin co-immunoprecipitated with glycogen synthase kinase 3 β and Axin, components of this complex in a Wnt-1-dependent manner. Our data suggest that hamartin and tuberin negatively regulate β-catenin stability and activity by participating in the β-catenin degradation complex.

The phenotype of patients with TSC encompasses the development of multiple focal lesions in the brain, heart, kidney, lung, and skin (1). While the underlying pathogenic mechanisms are unclear, the histology of these discrete, tumor-like lesions suggests defects in cell proliferation, differentiation, and cell size control. Studies in mammalian cells have shown that overexpression of TSC1 and TSC2 negatively regulates cell proliferation and induces G1/S arrest (2–4). In the case of tuberin, there appears to be an inverse correlation between tuberin level and p27(Kip1) expression and stability (5). Correspondingly, evidence supports a link between tuberin and cyclin D1 expression. Cortical tubers microdissected from TSC patients showed elevated cyclin D1 mRNA expression in the giant cells (6). Antisense inhibition of TSC2 in Rat1 fibroblasts resulted in up-regulation of cyclin D1 protein (3). Renal cortical tumors from the Eker rat model for TSC express elevated cyclin D1 compared with unaffected kidney tissue (7). As an in vivo target of the β-catenin pathway, cyclin D1 mRNA is responsive to the activity of the Tcf/LEF family of transcription factors (8, 9). This raises the possibility that TSC1 and TSC2 negatively regulate β-catenin signaling and, thereby, modulate the expression of cyclin D1.

β-Catenin is a highly conserved 95-kDa protein that participates in cell-cell adhesion through its association with members of the membrane-bound cadherin family, and in cell proliferation and differentiation as a key component of the Wnt/Wingless pathway (reviewed in Ref. 10). In its signaling role, β-catenin shuttles between the cytoplasm and the nucleus where it binds the Tcf/LEF family of transcription factors to activate downstream target genes (reviewed in Ref. 11). In the absence of the secreted factor, Wnt, β-catenin is phosphorylated by GSK3β and is targeted for ubiquitination and degradation. Upon Wnt stimulation, Disheveled (Dsh) is activated and blocks the ability of GSK3β to phosphorylate β-catenin. Other components of this degradation complex include Axin, serving as a scaffolding protein, and APC, a tumor suppressor protein. Disruption at multiple levels of this pathway has been shown to be oncogenic in humans and rodents. In this study, β-catenin protein levels were found to be elevated in renal tumors from Eker rats. Overexpression of tuberin and hamartin in cells down-regulated β-catenin levels, its half-life and its activity. Furthermore, we showed that TSC1 and TSC2 proteins co-immunoprecipitated with GSK3β and Axin, supporting a role of hamartin and tuberin in modulating the β-catenin pathway.
TSC1/TSC2 Inhibit β-Catenin Pathway

RESULTS

Expression of β-Catenin in Eker Rat Kidney Tumors—The Eker rat contains a germ-line mutation in the TSC2 gene (12) and spontaneously develops renal cortical epithelial tumors that have been shown to possess biallelic inactivation of TSC2 (L3-2). Commercially available antibodies were used to detect β-catenin, actin, and α-tubulin.
due to loss of heterozygosity, nonsense mutation, or null mutation (22). In a previous study, cyclin D1 levels were shown to be elevated in these kidney tumors compared with unaffected kidney tissue (7). Since cyclin D1 gene is a known target of the β-catenin signaling pathway and the accumulation of β-catenin has been shown to activate the transcription of the cyclin D1 gene (8, 9), β-catenin levels were examined in Eker rat kidney tumors. Tumors from three separate Eker rats were dissected as a value relative to \( \text{H9252} \). Tumors from three separate Eker rats were dissected as a value relative to \( \text{H9252} \). Time points were treated with ConA-Sepharose to remove cad-

**Hamartin and Tuberin Regulate β-Catenin Levels**—To investigate a possible link between tuberin and the β-catenin signaling pathway, we analyzed the effects of tuberin expression on β-catenin steady-state levels. Endogenous β-catenin expression was assessed in HEK293T cells 48 h after transfection with increasing concentrations of TSC1 and TSC2 or control vectors in the presence or absence of Wnt-1 stimulation. In the absence of Wnt-1 stimulation, β-catenin levels were unchanged with or without overexpression of hamartin and tuberin (data not shown). Upon Wnt-1 stimulation, β-catenin levels were unchanged with or without overexpression of hamartin and tuberin (data not shown). Upon Wnt-1 stimulation, β-catenin accumulated over a 48-h period in vector control samples (Fig. 1B, compare lanes 1–4). However, β-catenin levels were significantly reduced with overexpression of wild-type tuberin and hamartin (Fig. 1B, compare lanes 1–7). In contrast, overexpression of a disease-causing TSC2 mutant (\( \Delta Y1571H \)) (14) resulted in a modest β-catenin accumulation (Fig. 1B, compare lanes 8–10). These results show an inverse relationship between β-catenin and hamartin/tuberin expression. With increasing expression of hamartin and wild-type tuberin, β-catenin levels diminished progressively (Fig. 1B, compare lanes 5–7). In contrast, steady-state β-catenin increased modestly with the expression of control vector (Fig. 1B, compare lanes 2–4) or with mutant tuberin (\( \Delta Y1571H \)) (Fig. 1B, compare lanes 8–10).

Next, we examined the effect of hamartin and tuberin expression on β-catenin half-life. HEK293T cells were transfected as described above and then subjected to pulse-chase following \( \text{[35S]} \)methionine incorporation. Lysates collected at specific time points were treated with ConA-Sepharose to remove cad-

**Hamartin and Tuberin Inhibit Wnt-1-Stimulated β-Catenin Transcriptional Activity**—To determine whether modulation of β-catenin levels by hamartin and tuberin affects its transcriptional activity, the ability of Wnt-1 to activate a Tcf/LEF-luciferase reporter construct (TOPFLASH) was examined in transient transfection assays using HEK293T cells. These cells were co-transfected with hamartin and tuberin constructs along with a vector for β-galactosidase to account for transfection efficiency. Parallel assays were performed using TOPFLASH, a mutant reporter, to monitor background activity. Upon stimulation with Wnt-1, cells with vector control revealed a 12-fold increase in luciferase activity relative to the non-stimulated cells (Fig. 2A, compare lanes 1 and 2). Co-expression of both wild-type tuberin and hamartin in Wnt-1 stimulated cells significantly reduced reporter activity (Fig. 2A, lane 3), while expression of hamartin or tuberin alone had only minor effects (Fig. 2A, lanes 5 and 6). Importantly, co-expression of the tuberin \( \Delta Y1571H \) mutant with hamartin did not suppress Wnt-1-stimulated TOPFLASH activity (Fig. 2A, lane 4) consistent with the effects on β-catenin protein levels described above (Fig. 1, B and C). Under the same conditions, hamartin and tuberin had no effects on TOPFLASH activity (Fig. 2A, lanes 7 and 8). Our data suggest that hamartin and tuberin, functioning as a complex, are capable of inhibiting Wnt-1 stimulated β-catenin-dependent transcriptional activity.
Hamartin and Tuberin Function within the Wnt/β-Catenin Signaling Pathway—To determine at what level in the Wnt/β-catenin signaling pathway hamartin and tuberin act, we examined the effects of TSC1 and TSC2 on TOPFLASH activity when stimulated by different components of the Wnt pathway. The CA-β-catenin mutant with its serine/threonine residues (Ser-33, Ser-37, Thr-41, Ser-45) replaced with alanine residues, thus preventing its phosphorylation and degradation (16), acts as a downstream stimulus and activates the TOPFLASH reporter by over 6-fold in HEK293T cells (Fig. 2B, compare lanes 1 and 2). Co-expression of hamartin and tuberin was ineffective in reducing CA-β-catenin stimulation of the Tcf/LEF reporter (Fig. 2B, compare lanes 2 and 3). Western blot of samples confirmed equal expression of CA-β-catenin (data not shown). As a control, a dominant negative mutant of Tcf-4 (ΔN-Tcf-4) that lacks the N-terminal β-catenin binding domain (18) was able to inhibit CA-β-catenin activity completely (Fig. 2B, lane 6). These results suggest that hamartin and tuberin act upstream of β-catenin.

Next, TOPFLASH activity was measured in the presence of ectopically expressed Dsh, an effector that is stimulated by the Wnt-Frizzled receptor complex upstream of β-catenin (see Ref. 10). Transient overexpression of Dsh stimulates TOPFLASH activity by ~3-fold in control vector transfected cells (Fig. 2C, compare lanes 1 and 2). This activity was inhibited to near baseline levels upon overexpression of hamartin and tuberin (Fig. 2C, lane 3). Hamartin alone did not reduce the activity, while tuberin alone slightly decreased activity (Fig. 2C, lanes 4 and 5). Again, ΔN-Tcf-4 reduced activity toward unstimulated levels (Fig. 2C, lane 6). Together, these results are consistent with hamartin and tuberin exerting an effect on the Wnt signaling pathway at a level between Dsh and β-catenin (i.e. the β-catenin degradation complex).

Hamartin and Tuberin Interact with Components of the β-Catenin Degradation Complex—The β-catenin degradation complex is comprised of several proteins, including APC, Axin, and GSK3β, and is responsible for the regulation of cytoplasmic β-catenin (see Ref. 10). To determine whether hamartin and tuberin physically interact with components of the β-catenin degradation complex, co-immunoprecipitation assays were performed in HEK293T cells ectopically expressing hamartin and tuberin along with GSK3β. Anti-tuberin antibodies brought down GSK3β only in samples where both were overexpressed (Fig. 3A, panel i, lane 4). This band was not observed in samples where the GSK3β construct was co-expressed with vector control (Fig. 3A, panel i, lane 2) or in samples without the GSK3β construct (Fig. 3A, panel i, lanes 1 and 3). As expected, hamartin co-immunoprecipitated with tuberin in sample where both were overexpressed (Fig. 3A, panel ii, lanes 3 and 4). Conversely, immunoprecipitation of GSK3β brought down both tuberin and hamartin only in samples where all three were overexpressed (Fig. 3A, panels iv and v, lane 4). The expression of ectopic proteins was verified in cell lysates (Fig. 3A, panels vii, viii, and ix). Compared with the level of overexpression, the amount of interacting protein was relatively small, suggesting that only a fraction of tuberin/hamartin and GSK3β can associate with one another.

If hamartin and tuberin interact with the GSK3β that function in the β-catenin degradation complex, one would predict that other components of the complex such as Axin would co-immunoprecipitate with hamartin and tuberin. To test this hypothesis, hamartin and tuberin were ectopically expressed in the presence of c-Myc-tagged Axin and then subjected to immunoprecipitation analyses. Using anti-tubulin and anti-c-Myc antibodies, tuberin and tagged Axin co-precipitated in samples where they were both overexpressed (Fig. 3B, panels i and v).
lanes 5 and 6). Hamartin also co-immunoprecipitated with Axin (Fig. 3B, panel vi, lanes 5 and 6). Tuberin, hamartin, and c-Myc-tagged Axin were not detected in vector control samples (Fig. 3B, panels i, iii, v, and vi, lanes 1–3) or in samples without the tagged construct (Fig. 3B, panels i, iii, v, and vi, lane 4). We conclude that the tuberin-hamartin complex can associate with GSK3β and Axin possibly as part of the β-catenin degradation complex.

Finally, to examine whether tuberin interacts with the endogenous GSK3β complex, co-immunoprecipitation assays were performed in HEK293T cells with and without Wnt stimulation. A band corresponding to GSK3β was found to co-immunoprecipitate with tuberin but not in preimmune serum control samples (Fig. 3C, compare lanes 1 and 3). Furthermore, the amount of co-immunoprecipitated GSK3β was reduced upon Wnt-stimulation (Fig. 3C, compare lanes 1 and 2), suggesting that this interaction can be modulated by Wnt-1.

DISCUSSION

The TSC1 and TSC2 tumor suppressor genes have recently been implicated to play a role in negatively regulating mTOR in the PI3K signaling cascade (23, 24). As a result, tumors secondary to the inactivation of these genes have elevated levels of p70 S6 kinase activity that is reversible by rapamycin, a specific mTOR inhibitor (7). While this pathway may explain some of the complex phenotype exhibited by TSC pathology (25). While this pathway may explain some of the complex phenotype exhibited by TSC pathology (25), it is conceivable that upon stimulation, activated Akt phosphorylates tuberin and disables it from the complex. Accordingly, tuberin acting downstream of Akt may play a role in coordinating signals transduced through the Wnt and PI3K pathways, thus providing a mechanism for the pleiotropic effects of the TSC1 and TSC2 genes in tuberous sclerosis. The molecular components and their regulation by which TSC1 and TSC2 interact with the GSK3β complex remain to be identified.

Acknowledgments—We thank David Kimelman for providing the GSK3β construct and members of the Yeung laboratory for critical reading and assistance in the preparation of the manuscript.

REFERENCES

1. Gomez, M. R. (1995) Brain Res. 777, 55–57
2. Jin, F., Wienceke, R., Xiao, G. H., Mante, J. C., Jr., DeClue, J. E., and Yeung, R. S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9154–9159
3. Soucek, T., Fisch, O., Wienceke, R., DeClue, J. E., and Hengstschläger, M. (1997) J. Biol. Chem. 272, 25031–25038
4. Milone, A., Rosner, M., Nellist, M., Halley, S., Bernaschek, G., and Hengstschläger, M. (2000) Hum. Mol. Genet. 9, 1721–1727
5. Soucek, T., Yeung, R. S., and Hengstschläger, M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15653–15658
6. Crino, P. B., Trojanowski, J. Q., Dichter, M. A., and Eberwine, J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14152–14157
7. Kenerson, H. L., Aicher, L. D., True, L. D., and Yeung, R. S. (2002) Cancer Res. 62, 5645–5650
8. Shitutman, M., Zhirinsky, S., Simcha, I., Albanese, C., D’Amico, M., Pestell, R., and Ben-Ze’ev, A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5522–5527
9. Tetsu, O., and McCormick, F. (1999) Nature 398, 422–426
10. Daniels, D. L., Ekolf Spink, K., and Weis, W. I. (2001) Trends Biochem. Sci. 26, 672–678
11. Miller, J. R., Hocking, A. M., Brown, J. D., and Moon, R. T. (1999) Oncogene 18, 7860–7872
12. Yeung, R. S., Xiao, G. H., Jin, F., Lee, W. C., Testa, J. R., and Knudson, A. G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11413–11416
13. Xiao, G. H., Shariatnejad, P., Jin, F., Golemis, E. A., and Yeung, R. S. (1997) J. Biol. Chem. 272, 6997–6100
14. Aicher, L. D., Campbell, J. S., and Yeung, R. S. (2001) J. Biol. Chem. 276, 21017–21021
15. Plank, T. L., Yeung, R. S., and Henske, E. P. (1998) Cancer Res. 58, 4766–4770
16. Yost, C., Torres, M., Miller, J. R., Huang, E., Kimelman, D., and Moon, R. T. (1998) Genes Dev. 12, 1445–1454
17. Sokol, S. Y., Klingsensmith, J., Perrimon, N., and Itoh, K. (1995) Development (Camb.) 121, 1637–1647
18. 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
19. Turner, D. L., and Weintraub, H. (1994) Genes Dev. 8, 1344–1347
20. Pierce, S. B., and Kimelman, D. (1996) Dev. Biol. 175, 256–264
21. Williams B. O., Barish, G. D., Klymkowsky, M. W., and Varmus, H. E. (2000) Oncogene 19, 5720–5728
22. Rajino, K., and Hino, O. (1999) Contrib. Nephrol. 128, 45–50
23. Guscharov, E. A., Guscharov, D. A., Esters, A., Hunter, D. S., Glassberg, M. K., Yeung, R. S., Walker, C. L., Nonnau, D., Kwiatkowski, D. J., Chou, M. M., Panettieri, R. A., Jr., and Krymskaya, V. P. (2002) J. Biol. Chem. 277, 30658–30667
24. Manning, B. D., Ten, A. E., Logsdon, M. N., Blenis, J., and Cantley, L. C. (2002) Mol. Cell 10, 151–162
25. Kugoh, H., Kleymenova, E., and Walker, C. L. (2002) Mol. Carcinog. 33, 131–136
26. Fukumoto, S., Hsieh, C. M., Maemura, K., Layne, M. D., Yet, S. F., Lee, K. H., Matsui, T., Rosenzweig, A., Taylor, W. G., Ruini, J. S., Ferrarella, M. A., and Lee, M. E. (2001) J. Biol. Chem. 276, 17479–17483
27. Bernabeu, G., Li, S., Brown, S. J., Braverman, R., Vass, W. C., Cheadle, J. P., Halley, D. J., Sampson, J. R., Wienceke, R., and DeClue, J. E. (2000) Oncogene 19, 6306–6316
The Tuberin-Hamartin Complex Negatively Regulates β-Catenin Signaling Activity
Baldwin C. Mak, Ken-Ichi Takemaru, Heidi L. Kenerson, Randall T. Moon and Raymond S. Yeung

J. Biol. Chem. 2003, 278:5947-5951.
doi: 10.1074/jbc.C200473200 originally published online January 2, 2003

Access the most updated version of this article at doi: 10.1074/jbc.C200473200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 27 references, 16 of which can be accessed free at http://www.jbc.org/content/278/8/5947.full.html#ref-list-1