EGTA, 10 mM Hepes, and 1 μM tetrodotoxin (TTX). Pipettes were filled with 20 mM $BaCl_2$, 90 mM choline chloride, 10 mM tetraethylammonium (TEA), 10 mM Hepes, and 500 nM Bay K 8644. The pH was 7.3 for all solutions. If necessary, osmolarity was adjusted with sucrose to 300 mosM for the bath and 290 mosM for the pipette solution. We accomplished leak subtraction off-line by averaging 10 to 15 recorded leak currents of opposite polarity (same magnitude) and adding them to the records with channel openings.

18. Average current in a patch is given by $I = Np_O i$.
19. R. Horn, *Biophys. J.* **60**, 433 (1991); F. J. Sigworth and J. Zhou, in *Methods in Enzymology*, B. Rudy and L. E. Iverson, Eds. (Academic Press, New York, 1992), pp. 746–762. The method of maximum simultaneous openings was used to determine N by finding the maximal instantaneous current (I_{max}) in any of the 30 depolarizations to +10 mV (150 ms) and dividing by the single-channel amplitude i at +10 mV to give N ($N = I_{max}/i$). This method appears to be accurate when p_O is relatively high; in the present study, p_O for L-type channels was significantly elevated by the presence of Bay K 8644 and the use of maximally activating voltage (+10 mV). To ensure that p_O was sufficiently high at the instantaneous peak, we analyzed the fraction of repetitive trials on which the maximum value was observed (f_{max}). If f_{max} is above 0.1, the likelihood that the maximum peak overlap of current is an accurate estimate of N is quite high. In our patches, the mean ± SEM for f_{max} in each age group was as follows: young adult, 0.31 ± 0.03; mid-aged, 0.30 ± 0.06; and aged, 0.31 ± 0.03. Because a value of i at +10 mV was not available for each patch, we used the mean value of i at +10 mV for each group (Fig. 3A). The value of i did not differ at any voltage as a function of aging (Fig. 3A).
20. Patch area (a) is inversely correlated with pipette resistance (R) according to the relation $a = 12.6\,(1/R + 0.018)$ [B. Sakmann and E. Neher, in *Single Channel Recordings*, B. Sakmann and E. Neher, Eds. (Plenum, New York, 1983), pp. 37–51]. We carefully monitored patches for visible membrane in the pipette tip and for unusual suction durations. All patches for which extensive membrane (other than the small omega-shaped dome) was visible in the pipette tip or that required inordinately long suction for a seal were excluded from the study. There were relatively few of these, with a similar incidence in each group, and nearly all were excluded, in any case, by the criterion of a minimum seal resistance of 15 gigohm. In general, the suction time to seal was at least as rapid in the aged group as in the others, as indicated by the relatively high yield in this group (*12*).
21. To determine whether altered sensitivity to Bay K 8644 could account for aspects of these changes, we also measured the mean channel open time (*14*) during the step to −30 mV in patches used for the *I-V* analysis (Fig. 2B) and at −70 mV during the repolarization period (ROs) after steps to +10 mV in all patches in which ROs were present. In these multichannel patches, open time was most accurately measured at −30 mV and at −70 mV, when there were few simultaneous openings. The mean ± SEM values for open time at −30 mV were: young adult, 6.5 ± 1.4 ms; mid-aged, 6.5 ± 1.5 ms; and aged, 5.7 ± 0.8 ms; and at −70 mV (ROs) were: young adult, 5.5 ± 0.6 ms; mid-aged, 5.0 ± 0.5 ms; and aged, 5.7 ± 0.7 ms. These values do not differ statistically, indicating that Bay K 8644 affected the different age groups similarly. This conclusion is further supported by the lack of age difference in voltage dependence (Fig. 2B), because a shift in voltage dependence is also a hallmark of DHP agonists (*14*).
22. M. Gallagher and M. A. Pelleymounter, *Neurobiol. Aging* **9**, 363 (1988); K. M. Frick, M. G. Baxter, A. L. Markowska, D. S. Olton, D. L. Price, *ibid.*, **16**, 149 (1995). Briefly, 10 aged animals were trained in a Morris water maze to find a submerged platform, according to well-established procedures. Animals were given four trials a day, each from a different starting point, to find a consistently located platform. Training was for eight consecutive days. Acquisition latencies were averaged for each day and animals were ranked for their latency performance on the last 3 days of acquisition training. All behavioral studies were completed at least 3 weeks before recording began.
23. R. A. Deyo, K. T. Straube, J. F. Disterhoft, *Science* **243**, 809 (1989); A. Scriabine, T. Schuurman, J. Traber, *FASEB J.* **3**, 1799 (1989); K. McMonagle-Strucko and R. J. Fanelli, *Pharmacol. Biochem. Behav.* **44**, 827 (1993).
24. S. Govoni, R. A. Rius, F. Battaini, A. Bianchi, M. Trabucchi, *Brain Res.* **33**, 374 (1985); P. W. Landfield, D. G. Fleenor, J. C. Eldridge, B. S. McEwen, *Soc. Neurosci. Abstr.* **15**, 80 (1989).
25. L. M. Schwartz, E. W. McCleskey, W. Almers, *Nature* **314**, 747 (1985).
26. D. L. Armstrong and R. Eckert, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2518 (1987); D. L. Armstrong, M. F. Rossier, A. D. Shcherbatko, R. E. White, *Ann. N.Y. Acad. Sci.* **635**, 26 (1991); C. R. Artalejo, S. Rossie, R. L. Perlman, A. P. Fox, *Nature* **358**, 63 (1992); A. Sculptoreanu, T. Scheuer, W. A. Caterall, *ibid.*, **364**, 240 (1993).
27. K. D. Parfitt, B. J. Hoffer, M. D. Browning, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 2361 (1991); M. S. Magnoni, S. Govoni, F. Battaini, M. Trabuchi, *Rev. Neurosci.* **3**, 249 (1992).
28. K. C. Chen *et al.*, *Soc. Neurosci. Abstr.* **21**, 573 (1995).
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Binding of APC to the Human Homolog of the *Drosophila* Discs Large Tumor Suppressor Protein

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The adenomatous polyposis coli gene (*APC*) is mutated in familial adenomatous polyposis and in sporadic colorectal tumors, and its product binds to the adherens junction protein β-catenin. Overexpression of APC blocks cell cycle progression. The APC–β-catenin complex was shown to bind to DLG, the human homolog of the *Drosophila* discs large tumor suppressor protein. This interaction required the carboxyl-terminal region of APC and the DLG homology repeat region of DLG. APC colocalized with DLG at the lateral cytoplasm in rat colon epithelial cells and at the synapse in cultured hippocampal neurons. These results suggest that the APC-DLG complex may participate in regulation of both cell cycle progression and neuronal function.

The tumor suppressor gene *APC* is mutated in most cases of familial adenomatous polyposis (FAP), a dominantly inherited disease characterized by multiple adenomatous polyps in the colon (*1*, *2*). The *APC* gene is also somatically mutated in the majority of sporadic colorectal tumors (*2*). Mutation of *APC* is thought to be an early event in tumorigenesis (*3*).

The product of *APC* is a 300-kD homodimeric protein localized in the cytoplasm (*4*, *5*). The APC protein interacts with the adherens junction protein β-catenin, which suggests that APC may be involved in cell adhesion (*6*). APC also associates with microtubules and with a protein of unknown function, EB1, in cells overexpressing transfected *APC* (*7*). Overexpression of APC blocks progression from the G_0-G_1 to the S phase of the cell cycle (*8*).

To identify other proteins that associate with APC, we performed a two-hybrid screen of a human brain cDNA library using various regions of APC as "bait" (*9*). One clone that scored positive for interaction with the COOH-terminal region of APC contained a portion of the cDNA encoding DLG (amino acids 199 to 507), the human homolog of the *Drosophila* discs large tumor suppressor protein (*10*, *11*). To confirm that APC and DLG associate directly, we expressed each as a glutathione-S-transferase (GST) fusion protein and examined its ability to interact with the other protein, produced by in vitro translation (Fig. 1). In vitro–translated full-length DLG associated specifically with the COOH-terminal domain of APC (amino acids 2475 to 2843, APC-C369) fused to GST but not with GST alone. Likewise, in vitro–translated APC-C369 interacted with GST-DLG but not with GST alone.

DLG contains three DLG homology re-

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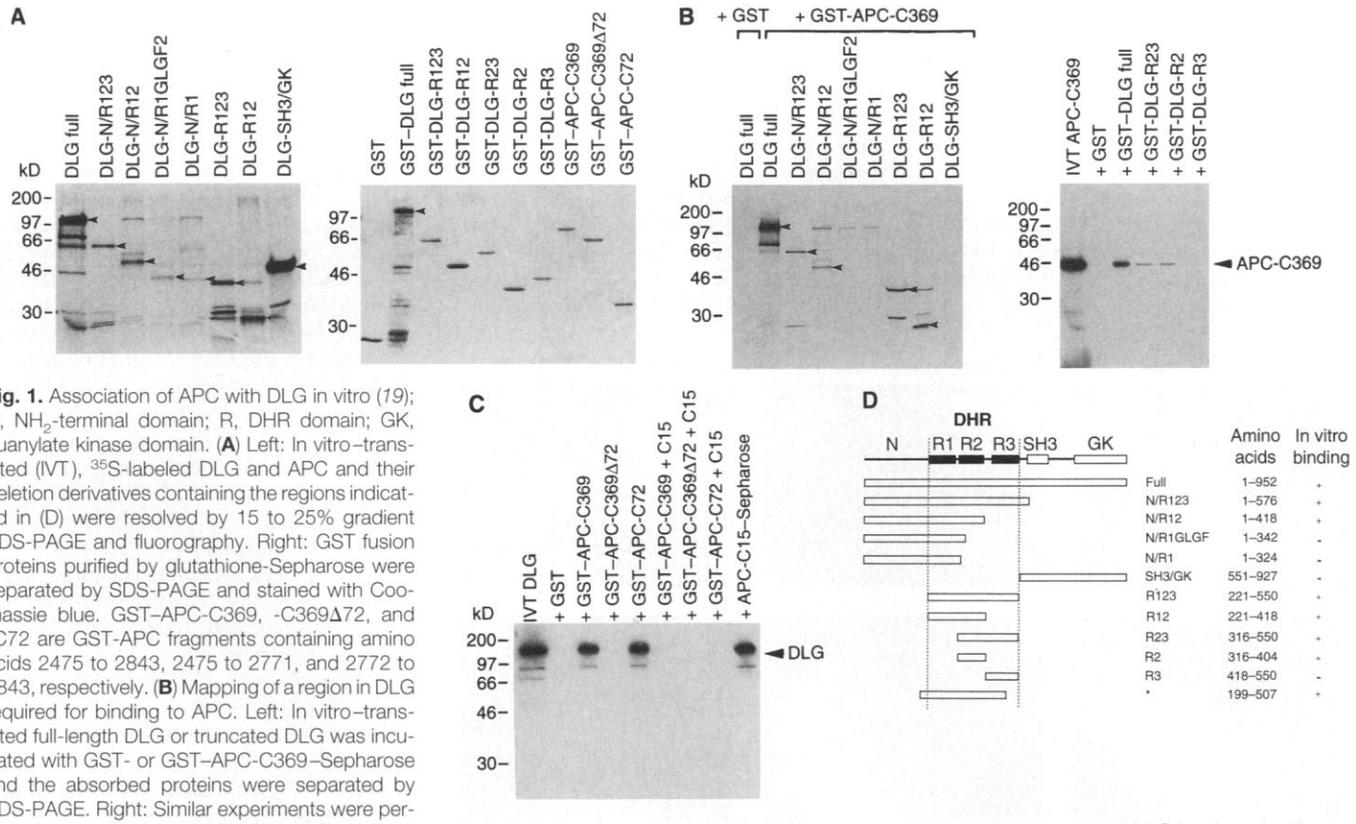

Fig. 1. Association of APC with DLG in vitro (*19*); N, NH_2-terminal domain; R, DHR domain; GK, guanylate kinase domain. (**A**) Left: In vitro–translated (IVT), ^{35}S-labeled DLG and APC and their deletion derivatives containing the regions indicated in (D) were resolved by 15 to 25% gradient SDS-PAGE and fluorography. Right: GST fusion proteins purified by glutathione-Sepharose were separated by SDS-PAGE and stained with Coomassie blue. GST–APC-C369, -C369Δ72, and -C72 are GST-APC fragments containing amino acids 2475 to 2843, 2475 to 2771, and 2772 to 2843, respectively. (**B**) Mapping of a region in DLG required for binding to APC. Left: In vitro–translated full-length DLG or truncated DLG was incubated with GST- or GST–APC-C369–Sepharose and the absorbed proteins were separated by SDS-PAGE. Right: Similar experiments were performed with in vitro–translated APC-C369, GST, and GST-DLG derivatives. (**C**) Mapping of a region in APC required for binding to DLG. In vitro–translated full-length DLG was incubated with GST-APC derivatives. Addition of the peptide corresponding to the COOH-terminal 15 amino acids of APC (C15) abrogated coprecipitation of DLG with GST-APC derivatives. The lane labeled APC-C15–Sepharose contains in vitro–translated DLG incubated with peptide C15 that had been immobilized to Sepharose. (**D**) Schematic representation of DLG deletion mutants and corresponding APC binding activities. +, detectable activity; −, no detectable activity; ∗, the portion of DLG found in the initial two-hybrid screen.

peat (DHR) domains [characterized by the conserved sequence Gly-Leu-Gly-Phe (GLGF)], a Src homology 3 (SH3) domain, and a domain homologous to guanylate kinases (GK) (Fig. 1D) (*10*, *11*). The clone isolated by the two-hybrid system encoded a region containing DHR-1, DHR-2, and a part of DHR-3. We next used various GST fusion and in vitro–translated proteins to delineate the regions of DLG and APC that interact. The NH_2-terminal domain, the SH3 domain, and the COOH-terminal GK domain did not interact with APC, whereas a fragment containing DHR-1, -2, and -3 associated with GST–APC-C369 (Fig. 1, B and D). A fragment containing DHR-2 alone and fragments containing DHR-1 and -2 or DHR-2 and -3 interacted weakly with APC. However, neither DHR-1 nor DHR-3 alone showed substantial APC binding activity.

An APC derivative lacking the COOH-terminal 72 amino acids (GST–APC-C369Δ72) did not associate with DLG, but a derivative containing these amino acids (GST–APC-C72) did associate. This interaction was inhibited by a synthetic peptide corresponding to the COOH-terminal 15 amino acids of APC (APC-C15) (Fig. 1C). Furthermore, this synthetic peptide itself exhibited DLG binding activity, suggesting that the COOH-terminal portion of APC is sufficient for DLG binding.

We next examined whether APC binds to DLG in vivo. We subjected a lysate from embryonic mouse brain to immunoprecipitation with antibodies against the NH_2-terminal region of APC (anti–APC-NH_2) and then immunoblotted the immunoprecipitate with antibodies raised against the NH_2-terminal region of DLG (anti–DLG-NH_2). A 140-kD DLG doublet was detected (Fig. 2). Coprecipitation of APC and DLG was inhibited by preincubation with the APC fragment used for immunization. Immunoprecipitation of the lysate with anti–DLG-NH_2 or anti-DLG antibodies against the DHR domains (anti–DLG-DHR), with subsequent immunoblotting with anti–APC-NH_2, also revealed an association between APC and DLG. In these experiments, preincubation of the antibodies to DLG with the antigenic DLG fragments prevented coprecipitation of DLG and APC. In contrast, antibodies against the COOH-terminal region of APC coprecipitated negligible amounts of DLG, presumably because the region recognized by the antibodies overlaps with that interacting with DLG.

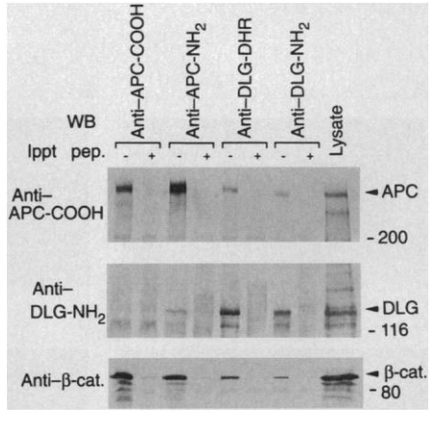

Fig. 2. Association of APC with DLG and β-catenin in vivo. Lysates prepared from embryonic mouse brain were subjected to immunoprecipitation with the indicated antibodies, fractionated by 5% SDS-PAGE, and immunoblotted with the indicated antibodies (*20*). pep. + indicates that antibodies were preincubated with antigen before use in immunoprecipitation. Ippt, immunoprecipitation; WB, immunoblotting.

Immunoblot analysis of the DLG immunoprecipitates with antibodies to β-catenin revealed that β-catenin also coprecipitates

with DLG and APC. Because DLG does not interact directly with β-catenin (*12*), these findings suggest that APC, β-catenin, and DLG are contained in the same complex in vivo.

Both APC and DLG are highly expressed in the central nervous system, including the hippocampus, olfactory bulb, and cerebellum (*11*, *13*). When cultured rat hippocampal neurons were double-labeled with anti–APC-COOH and an antibody directed against synaptotagmin (*14*), a synaptic vesicle membrane protein, the two exhibited identical distribution patterns, concentrating at synaptic sites along dendrites in addition to the cell bodies (Fig. 3, A through C). Immunostaining patterns of DLG also showed extensive overlap with those of synaptotagmin (Fig. 3, D through F), which is consistent with the observation that DLG is localized in the presynaptic nerve terminals (*11*). Both APC and DLG were expressed in colon epithelial cells (Fig. 4, A through C). Double-labeling immunoelectron microscopy showed colocalization of APC and DLG along the lateral plasma membrane (Fig. 4, D through F).

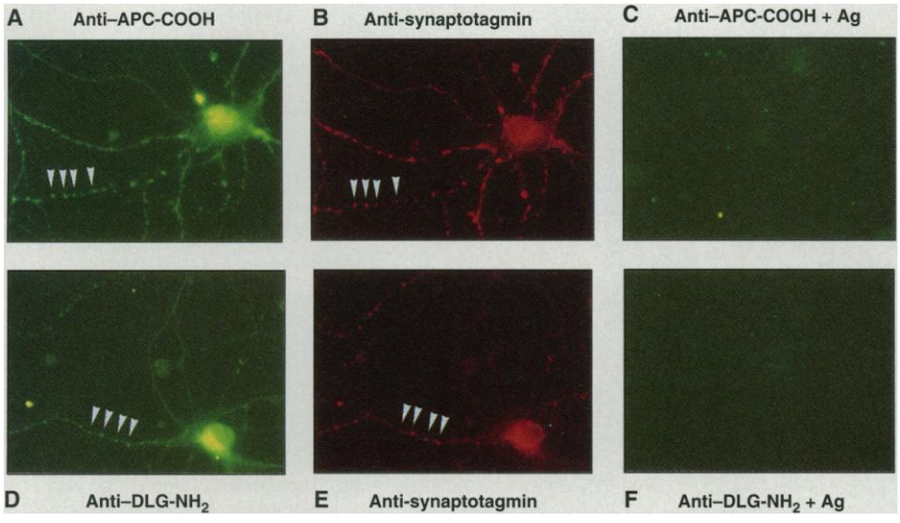

Fig. 3. Colocalization of APC and DLG in cultured rat hippocampal neurons. Hippocampal neurons were double-labeled with anti–APC-COOH (**A**) and anti-synaptotagmin (**B**) or with anti–DLG-NH_2 (**D**) and anti-synaptotagmin (**E**) (*21*). Anti–DLG-NH_2 recognizes the region specific for DLG and does not cross-react with PSD-95. Control experiments were performed with anti–APC-COOH and anti–DLG-NH_2 that had been preabsorbed with antigen (Ag) (**C** and **F**, respectively). White arrowheads indicate representative sites of double labeling.

In *Drosophila*, DLG is localized to the septate junctions in imaginal disc epithelia, and its mutation causes epithelial cells to lose polarity and undergo neoplastic proliferation (*10*). *Drosophila* DLG is also localized at neuronal synaptic boutons and is required for normal synaptic structure. In mammalian cells, the postsynaptic density protein PSD-95/SAP90 (*15*), which is very closely related to DLG, has been shown to interact with the COOH-terminal portion of the *N*-methyl-D-aspartate (NMDA) receptor subunits through its DHR domain (*16*). Subunits of the voltage-gated K^+ channel have also been demonstrated to associate with the DHR domains of PSD-95 and DLG (*17*). The DHR-2 domain was shown to be important for the formation of these complexes, which is consistent with our present results. Furthermore, both the NMDA receptor and the K^+ channel contain a COOH-terminal S/TXV motif (S/T, serine or threonine; X, any amino acid; V, valine) that is essential for their interaction with PSD-95 and DLG. The COOH-terminus of APC also possesses a S/TXV motif, namely TEV (E, glutamic acid). It is therefore possible that APC competes with the NMDA receptor or the K^+ channel for binding to DLG–PSD-95 or is contained within the same complex, and thus may play a role in neuronal cell function as well as in epithelial cell proliferation. Furthermore, because the DHR domain is contained in a number of proteins, including the tight junc-

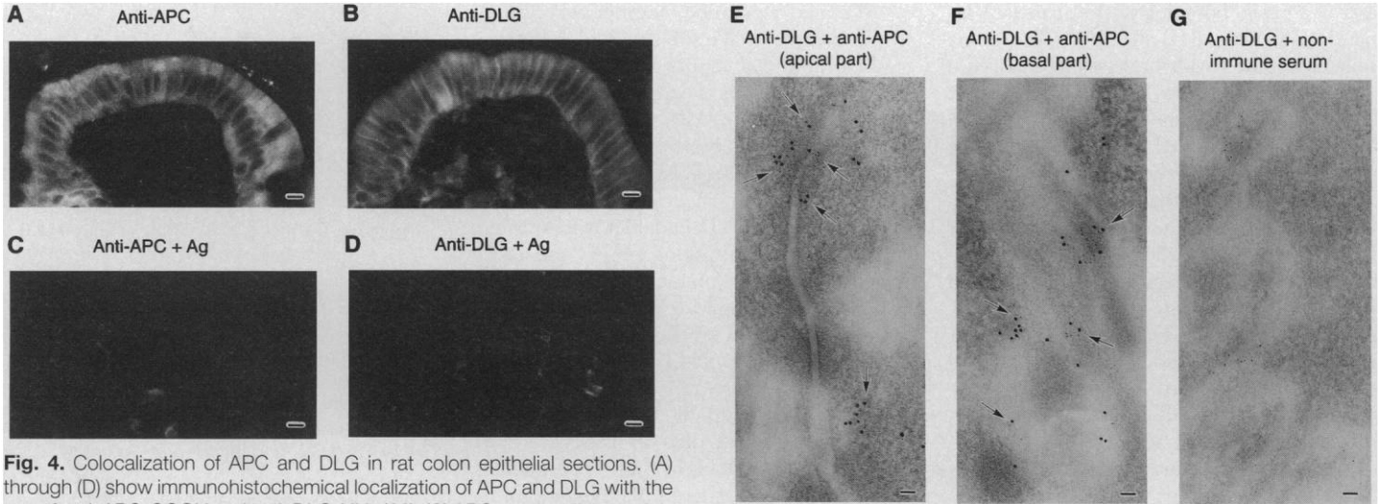

Fig. 4. Colocalization of APC and DLG in rat colon epithelial sections. (A) through (D) show immunohistochemical localization of APC and DLG with the use of anti–APC-COOH and anti–DLG-NH_2 (*22*). (**A**) APC was localized mainly in the cytoplasm of the epithelial cells. (**B**) DLG was localized along lateral cell borders, with some cytoplasmic staining. Control experiments were performed with anti–APC-COOH and anti–DLG-NH_2 that had been preabsorbed with antigen (**C** and **D**, respectively). (E) through (G) show double labeling-immunoelectron microscopy of APC and DLG (*23*). (**E** and **F**) Close association of 10-nm gold particles (APC) and 5-nm gold particles (DLG) was observed along the lateral plasma membranes [arrows in (E) and (F); (E), apical part; (F), basal part]. (**G**) Only 5-nm gold particles were detected when nonimmune rabbit serum was used in place of anti–APC-COOH. Also, only 10-nm particles were detected when nonimmune rabbit serum was used in place of anti–DLG-NH_2 (*24*). No particles were detected when the control rabbit serum was used for staining in place of anti–APC-COOH and anti–DLG-NH_2. Scale bars in (A) through (D), 5 μm; in (E) through (G), 50 nm.

tion proteins ZO-1 and ZO-2 and erythroid p55 (*18*), it is possible that APC plays various physiologic roles by forming complexes with these DHR proteins.

REFERENCES AND NOTES

1. W. F. Bodmer *et al.*, *Nature* **328**, 614 (1987).
2. K. W. Kinzler *et al.*, *Science* **253**, 661 (1991); I. Nishisho *et al.*, *ibid.*, p. 665; J. Groden *et al.*, *Cell* **66**, 589 (1991); G. Joslyn *et al.*, *ibid.*, p. 601; Y. Miyoshi *et al.*, *Hum. Mol. Genet.* **1**, 229 (1992); S. M. Powell *et al.*, *Nature* **359**, 235 (1992).
3. B. Vogelstein and B. W. Kinzler, *Trends Genet.* **9**, 138 (1993).
4. K. J. Smith *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2846 (1993).
5. I. Miyashiro *et al.*, *Oncogene* **11**, 89 (1995).
6. B. Rubinfeld *et al.*, *Science* **262**, 1731 (1993); L.-K. Su, B. Vogelstein, K. W. Kinzler, *ibid.*, p. 1734; T. Shibata, M. Gotoh, A. Ochiai, S. Hirohashi, *Biochem. Biophys. Res. Commun.* **203**, 519 (1994).
7. K. J. Smith *et al.*, *Cancer Res.* **54**, 3672 (1994); S. Munemitsu *et al.*, *ibid.*, p. 3676; L.-K. Su *et al.*, *ibid.* **55**, 2972 (1995).
8. G.-H. Baeg *et al.*, *EMBO J.* **14**, 5618 (1995).
9. P. L. Bartel, C. T. Chien, R. Sternglanz, S. Fields, *Using the 2-Hybrid System to Detect Protein-Protein Interactions in Cellular Interactions in Development: A Practical Approach* (Oxford Univ. Press, Oxford, 1993), pp. 153–179.
10. D. F. Woods and P. J. Bryant, *Cell* **66**, 451 (1991); R. A. Lue, S. M. Marfatia, D. Branton, A. H. Chishti, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9818 (1994).
11. B. M. Muller *et al.*, *J. Neurosci.* **15**, 2354 (1995).
12. A. Matsumine *et al.*, unpublished data.
13. R. V. Bhat *et al.*, *J. Neurosci.* **14**, 3059 (1994).
14. W. D. Matthew, L. Tsavaler, L. F. Reichardt, *J. Cell Biol.* **91**, 257 (1981).
15. K.-O. Cho, C. A. Hunt, M. B. Kennedy, *Neuron* **9**, 929 (1992); U. Kistner *et al.*, *J. Biol. Chem.* **268**, 4580 (1993).
16. H.-C. Kornau, L. T. Schenker, M. B. Kennedy, P. H. Seeburg, *Science* **269**, 1737 (1995).
17. E. Kim *et al.*, *Nature* **378**, 85 (1995).
18. E. Willott *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7834 (1993); M. Itoh *et al.*, *J. Cell Biol.* **121**, 491 (1993); L. A. Jesaitis and D. A. Goodenough, *ibid.* **124**, 949 (1994); P. Ruff, D. W. Speicher, C. A. Husain, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6595 (1991).
19. [^{35}S]methionine-labeled truncated DLGs, containing the indicated regions, were synthesized by in vitro transcription-translation (Promega) with the use of templates obtained by linearizing the *dlg* cDNA constructs with restriction enzymes or by polymerase chain reaction. We generated GST fusion proteins by subcloning the cDNAs into pGEX5X-1 (Pharmacia), expressing the fusion proteins in *Escherichia coli*, and isolating them by absorption to glutathione-Sepharose [D. B. Smith and K. S. Johnson, *Gene* **67**, 31 (1988)]. GST fusion proteins immobilized to glutathione-Sepharose were mixed with in vitro–translated proteins in buffer A [50 mM tris-HCl (pH 7.5), 140 mM NaCl, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, leupeptin (5 μg/ml), and aprotinin (5 μg/ml)] containing 0.1% Triton X-100 for 1 hour at 4°C and then were washed extensively with buffer A containing 0.5% NP-40.
20. Antibodies to APC were prepared as in (*8*). Antibodies to the NH_2-terminus and the DHR region of DLG were prepared by immunization of rabbits with peptides containing amino acids 6 to 205 and 203 to 518 of DLG, respectively. Antibody to β-catenin was obtained from Transduction Laboratories. Embryonic mouse brain (embryonic day 18) was lysed with a dounce homogenizer in buffer A (*19*) containing 1% Triton X-100, and the lysates were incubated with indicated antibodies for 1 hour at 4°C. The immunocomplexes were adsorbed to protein A–Sepharose 4B and washed extensively with buffer A containing 0.1% Triton X-100. Samples were resolved by 5% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to immunoblotting.
21. Cell cultures were prepared from the hippocampus of 18-day-old fetal rats [T. L. Fletcher, P. Cameron, P. De Camilli, G. Banker, *J. Neurosci.* **11**, 1617 (1991)]. Staining patterns obtained with anti-APC and anti-DLG were visualized with fluorescein isothiocyanate (FITC)–labeled secondary antibodies to rabbit immunoglobulin G (IgG) (Cappel); those obtained with anti-synaptotagmin (Wako) were visualized with Texas Red–labeled secondary antibodies to mouse IgG (Amersham).
22. For fluorescence immunohistochemistry, serial frozen sections of rat colon were stained with anti-APC-COOH or anti-DLG-NH_2 and then with FITC-conjugated goat antibody to rabbit IgG (Seikagaku Kogyo) (*5*).
23. For double-labeling immunoelectron microscopy. Lowicryl ultrathin sections were incubated with anti-DLG-NH_2 and then with 5-nm colloidal gold-conjugated goat antibody to rabbit IgG (Amersham). After blocking with a goat antibody to rabbit IgG, the sections were stained with anti-APC-COOH and then with 10-nm colloidal gold-conjugated goat antibody to rabbit IgG (Amersham) (*5*) [H. J. Geuze, J. W. Slot, P. A. van der Ley, R. C. Scheffer, *J. Cell Biol.* **89**, 653 (1981)].
24. T. Senda *et al.*, unpublished data.

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Binding of GSK3β to the APC–β-Catenin Complex and Regulation of Complex Assembly

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The adenomatous polyposis coli gene (*APC*) is mutated in most colon cancers. The APC protein binds to the cellular adhesion molecule β-catenin, which is a mammalian homolog of ARMADILLO, a component of the WINGLESS signaling pathway in *Drosophila* development. Here it is shown that when β-catenin is present in excess, APC binds to another component of the WINGLESS pathway, glycogen synthase kinase 3β (GSK3β), a mammalian homolog of *Drosophila* ZESTE WHITE 3. APC was a good substrate for GSK3β in vitro, and the phosphorylation sites were mapped to the central region of APC. Binding of β-catenin to this region was dependent on phosphorylation by GSK3β.

The *APC* tumor suppressor gene is mutated in most human colon tumors and in the germ line of individuals with familial adenomatous polyposis coli (*1*, *2*). How *APC* dysfunction contributes to colon cancers is unknown but may involve its ability to interact with β-catenin. Both wild-type (WT) and truncated mutant forms of APC associate with β-catenin (*3*, *4*), but only WT APC down-regulates β-catenin when expressed ectopically in colon cancer cells (*5*). β-Catenin was originally identified as a cell adhesion protein by virtue of its association with cadherins (*6*). In addition, in both *Drosophila* and *Xenopus* β-catenins have been implicated in cell fate determination through a mechanism that is apparently independent of their interaction with cadherins (*7*). In both of these systems, interference with the serine-threonine kinase GSK3β results in a cell fate determination identical to that resulting from overexpression of β-catenin (*8*, *9*), which suggests that GSK3β is required for β-catenin down-regulation. These observations prompted us to examine the relation between GSK3β, APC, and β-catenin in mammalian cells.

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To investigate whether GSK3β and the APC–β-catenin complex interact, we overexpressed Myc-tagged GSK3β in SW 480 colon cancer cells, immunoprecipitated either Myc-GSK3β or β-catenin, and then analyzed the precipitates for the presence of APC, β-catenin, and Myc-GSK3β. Both APC and β-catenin coimmunoprecipitated with antibody specific to the Myc epitope and, conversely, Myc-GSK3β was detected in the β-catenin immunoprecipitates (Fig. 1). These associations were observed with WT but not with kinase-dead Myc-GSK3β (a catalytically inactive mutant of GSK). To examine this association in the absence of ectopic cDNA expression, we performed GSK assays on β-catenin immunoprecipitates from four different cell lines. Specific GSK activity was detected in the β-catenin immunocomplexes from COLO 205 and SW 480 human colon cancer cells, but not from 293 human embryonic kidney cells or AtT20 murine pituitary tumor cells (Fig. 2A). In addition, immunoprecipitates of endogenous GSK3β contained β-catenin and the truncated mutant APC (*10*) endogenous to SW 480 cells (Fig. 2B). Similar results were obtained with COLO 205 cells, also mutant for APC; but WT APC could not be coimmunoprecipitated from either 293 or AtT20 cell lysates by the antibody to GSK3β (*11*). To verify that GSK3β entered into a complex with