MOLECULAR AND CELLULAR BIOLOGY, Aug. 1996, p. 4088–4094 Vol. 16, No. 8
0270-7306/96/$04.00+0

Deletion of an Amino-Terminal Sequence Stabilizes β-Catenin In Vivo and Promotes Hyperphosphorylation of the Adenomatous Polyposis Coli Tumor Suppressor Protein

SUSAN MUNEMITSU, IRIS ALBERT, BONNEE RUBINFELD, AND PAUL POLAKIS*

Onyx Pharmaceuticals, Richmond, California 94806

Received 14 February 1996/Returned for modification 9 April 1996/Accepted 2 May 1996

Regulation of cell adhesion and cell signaling by β-catenin occurs through a mechanism likely involving the targeted degradation of the protein. Deletional analysis was used to generate a β-catenin refractory to rapid turnover and to examine its effects on complexes containing either cadherin or the adenomatous polyposis coli (APC) protein. The results show that amino-terminal deletion of β-catenin results in a protein with increased stability that acts in a dominant fashion with respect to wild-type β-catenin. Constitutive expression in AtT20 cells of a β-catenin lacking 89 N-terminal amino acids (ΔN89β-catenin) resulted in severely reduced levels of the more labile wild-type β-catenin. The mutant β-catenin was expressed at endogenous levels but displaced the vast majority of wild-type β-catenin associated with *N*-cadherin. The ΔN89β-catenin accumulated on the APC protein to a level 10-fold over that of wild-type β-catenin and recruited a kinase into the APC complex. The kinase was highly active toward APC in vitro and promoted a sodium dodecyl sulfate gel band shift that was also evident for endogenous APC from cells expressing the mutant β-catenin. Unlike wild-type β-catenin, which partitions solely as part of a high-molecular-weight complex, the ΔN89 mutant protein also fractionated as a stable monomer, indicating that it had escaped the requirement to associate with other proteins. That similar N-terminal mutants of β-catenin have been implicated in cellular transformation suggests that their abnormal association with APC may, in part, be responsible for this phenotype.

Mutations in the adenomatous polyposis coli (APC) tumor suppressor gene have been identified in the vast majority of all cancers of the colon (6, 12, 14, 18, 23, 31). In the progression of sporadic tumors, APC mutations occur very early (31) and have been detected even in the earliest noticeable precursor to cancer, called aberrant intestinal crypt foci (11, 36). Individuals who inherit a mutant copy of APC present hundreds to thousands of colonic polyps by age 20 or 30, and without surgery, malignancies invariably ensue (4). The function of the APC gene is unknown, but it is presumed to possess growth-controlling properties that are lost because of mutations resulting in premature polypeptide chain termination (reviewed in reference 30). These deletions generally result in the loss of both the carboxyl-terminal region that interacts with microtubules (21, 37) and a portion of the centrally located region that can bind to and promote the down-regulation of β-catenin (21). As β-catenin is thought to control cell adhesion and possibly cell signaling events, loss of its regulation by APC may contribute to abnormal cell growth.

Several lines of evidence suggest that the regulation of β-catenin may be critical to cell growth control and differentiation. The *wnt-1* oncogene promotes tumor formation in murine mammary gland (38), and its ectopic expression in cell culture results in the stabilization and accumulation of γ- and β-catenins (3, 9). Mutated forms of β-catenin have also been identified in cancer cells (13, 24), and one deletion mutant, identified by expression cloning, was reported to transform NIH 3T3 cells (39). Studies with *Drosophila melanogaster* and *Xenopus laevis* suggest that β-catenin, or its homolog, armadillo, not only is required for cadherin-mediated cell adhesion but also plays a direct role in signal transduction essential to cell fate determination (5, 8, 28). In *D. melanogaster*, these differentiation events correlate tightly with the accumulation of armadillo in the cell cytoplasm (29). Thus, accumulation of β-catenin in mammalian cells may contribute to cellular transformation in a manner independent of its effects on cell adhesion.

We have shown previously that lysates from cells expressing wild-type APC contain β-catenin that exists entirely as part of a high-molecular-weight complex (19) and is unavailable for association with exogenously added APC protein (33). By contrast, cells containing mutant APC, such as the colorectal cancer cell line SW480, contain a fraction of uncomplexed β-catenin which probably represents a cytoplasmic pool (19). Introduction of wild-type APC into SW480 cells results in the elimination of the cytoplasmic β-catenin observed by immunofluorescence and in a reduction of the uncomplexed fraction detected by size exclusion chromatography (19). This reduction in free β-catenin levels following APC expression occurs through a posttranslational process that affects the half-life of the protein. A posttranslational process was also determined as the mechanism by which the *wnt-1* oncogene promotes the accumulation of β-catenin (9). Therefore, regulation of β-catenin protein stability may represent a mechanism by which the cell controls the intracellular distribution and amount of β-catenin and thereby its availability for interaction with protein targets. Proteins with high turnover rates are likely to be key regulators of intracellular functions that are controlled by specific targeted degradation of the regulatory protein. As β-catenin appears to fit this description, we sought to identify and eliminate the specific polypeptide structure that is required for its rapid turnover and to examine the consequences of such a mutant for β-catenin complexes containing cadherin and APC.

MATERIALS AND METHODS

Cell lines. The SW480 cell line was obtained from the American Type Culture Collection (ATCC CCL228) and is a human colon cancer cell line. AtT20 cells

* Corresponding author. Mailing address: Onyx Pharmaceuticals, 3031 Research Dr., Richmond, CA 94806. Phone: (510) 262-8723. Fax: (510) 222-9758.

(ATCC CCL89) are a murine pituitary tumor cell line and were obtained from Jackie Papkoff (Sugen Corporation).

Immunochemical procedures. (i) Antibodies. Antibodies to APC were raised in rabbits against two distinct purified recombinant fragments (APC2 and APC3) and then purified with the recombinant APC proteins immobilized on affinity supports (33). Three different antibodies to β-catenin were used as indicated: β-cat-C, rabbit polyclonal serum raised against a 15-amino-acid peptide based on the C-terminal sequence of β-catenin (9); β-cat-N, rabbit polyclonal serum raised against a glutathione *S*-transferase fusion protein containing amino acids 6 to 138 of *Xenopus* β-catenin (16); and β-cat-FL, affinity-purified rabbit polyclonal antibody raised against the purified full-length β-catenin protein produced with the baculovirus-sf9 cell system. Antibody to α-catenin is a rabbit polyclonal serum raised against full-length α-catenin protein produced with the baculovirus-sf9 cell system. Antibody to N-cadherin is a mouse monoclonal antibody raised against the human N-cadherin cytoplasmic domain and was generously provided by Margaret J. Wheelock (University of Toledo, Toledo, Ohio). Antibody to the Glu-Glu epitope tag is a mouse monoclonal antibody raised against the Glu-Glu peptide sequence derived from T antigen (7) and partially purified on DE-52 cellulose.

(ii) Immunoblotting. All sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed with the Novex gel system (San Diego, Calif.) with either 6 or 8% polyacrylamide gels, except for the experiment whose results are shown in Fig. 9, in which a 4% gel was used. All electroblotting was performed in Tris-glycine (25 mM/192 mM) buffers for 1 to 2 h at 300 mA constant current with polyvinylidene difluoride filter membranes. The membranes were blocked with powdered milk solution and incubated overnight with the indicated antibodies diluted in phosphate-buffered saline containing 0.05% Tween 20 and 1% bovine serum albumin. All polyclonal sera were used at a 1/1,000 dilution, and purified antibodies were used at 0.2 μg/ml. Blots were washed and then developed with either the enhanced chemiluminescence (ECL) system (Amersham) or ^{125}I-protein A at 0.5 μCi/ml (Amersham) for 1 h at room temperature.

(iii) Immunoprecipitations. Cells were lysed in Triton X-100 lysis buffer (20 mM Tris-HCl [pH 8.0], 1.0% Triton X-100, 140 mM NaCl, 10% glycerol, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid], 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM sodium vanadate, 50 mM NaF, 1 mM Pefabloc, 10 μg each of aprotinin, pepstatin, and leupeptin per ml), and lysate containing 750 μg of total protein was incubated with the appropriate antibody for 2 h at 4°C. Antibodies were recovered with protein A- or G-Sepharose, and the beads were then washed three times with 1 ml each of buffer B (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5% Nonidet P-40) and finally eluted with SDS-PAGE sample buffer. For APC, 2 μg of affinity-purified anti-APC2 antibody was used for each immunoprecipitation, and for N-cadherin, 200 μl of hybridoma supernatant was used. Glu-Glu immunoprecipitations were carried out with anti-Glu-Glu antibody covalently coupled to protein G-Sepharose.

(iv) Immunofluorescence. The procedure for fixation and staining of cells with catenin antibodies has been described elsewhere (19). AtT20 cells were fixed with methanol. N-cadherin staining was performed with a mouse monoclonal antibody (Margaret Wheelock, University of Toledo) raised against the cytoplasmic domain of human N-cadherin. All micrographs presented were generated by a Zeiss Axiovert 100 TV microscope equipped with a cooled charge-coupled device camera.

(v) Quantitative immunoblotting. Radioactivity on immunoblots developed with ^{125}I-protein A was quantitated by β-scanning with an Ambis 4000 β scanner. Molar amounts were calculated from protein mass determined with a standard curve consisting of 1, 10, 50, and 100 ng of each purified catenin applied to the same gel and assuming a molecular mass of 95 kDa for β-catenin, 82 kDa for ΔN89β-catenin, and 102 kDa for α-catenin. In these experiments, the β-cat-C antibody was used.

Construction and expression of cDNAs. All β-catenin cDNA constructs were derived from the full-length cDNA cloned from a human fetal brain library as described previously (34). For mammalian expression, the β-catenin fragments were subcloned into CMV neo Bam Glu, a derivative of CMV neo Bam (2) in which the polylinker was replaced with a synthetic linker engineered to encode an initiating methionine, the Glu-Glu epitope tag, and a multiple cloning site containing several unique restriction enzyme sites. The following β-catenin fragments were subcloned in frame: the β-catenin *Acc*I-*Not*I fragment (for full length), the β-catenin ΔN_{89} *Xho*I-*Not*I fragment (codons 90 to 781), the β-catenin ΔN150 (*Acc*-*Not*I) fragment (codons 150 to 781), the β-catenin ΔC *Acc*I-*Eco*RV fragment (codons 1 to 710), the β-catenin 5′ *Acc*I-*Eco*RI fragment (codons 1 to 423), and the β-catenin 3′ *Eco*RI fragment (codons 423 to 781).

For transient transfection in SW480 cells, the Lipofectin (Bethesda Research Laboratories) procedure previously described for expression of APC constructs was followed (19). For the generation of stable cell lines, AtT20 cells were seeded in six-well plates at 3.0×10^5 cells per well and transfected with 10 μl of Lipofectamine reagent (Gibco BRL) in Dulbecco modified Eagle medium without serum according to the manufacturer's instructions. Two micrograms of the CMV neo Bam Glu plasmid containing cDNA coding for the indicated β-catenin construct or empty vector was used for each transfection. The following day, the medium was changed to Dulbecco modified Eagle medium with serum, and the cells were allowed to recover 1 day before analysis for transient expression or for selection in G418 (400 μg/ml) for production of stable cell lines. After 3 or 4 weeks in G418, the clones were isolated and analyzed for β-catenin expression.

Size exclusion chromatography. Size exclusion chromatography was performed with a Waters model 650E fast protein liquid chromatography system interfaced with a Superose 12 HR 10/30 column (Pharmacia) equilibrated in 25 mM Tris (pH 8.0)–0.25 M NaCl–0.1% Nonidet P-40–1 mM dithiothreitol–10 μg each of pepstatin and leupeptin per ml. Cells were harvested by scraping, pelleted by centrifugation, and lysed in a 5× volume of Triton X-100 lysis buffer. Following ultracentrifugation, the supernatants were adjusted to 2.5 mg of total protein per ml with lysis buffer and 300 μl was injected onto the column. Chromatography was performed at 4°C at a flow rate of 0.5 ml/min, and fractions of 0.5 ml each were collected. Twenty microliters of each fraction was analyzed by SDS-PAGE and immunoblotting as described above.

In vitro kinase reactions. Immunoprecipitations of β-catenin were performed with 2 μg of affinity-purified anti-β-catenin (β-cat-FL) and cell lysate containing approximately 1 mg of total protein. Immunoprecipitates were washed twice with 1 ml each of buffer B and once more with 1 ml each of kinase assay buffer (25 mM Tris [pH 7.5], 5 mM $MgCl_2$, 1 mM dithiothreitol, 4% glycerol) and then resuspended in 30 μl of kinase assay buffer containing 50 μM [γ-^{32}P]ATP (10,000 cpm/pmol) and 0.25 μg each of purified APC25 protein. This protein encodes amino acids 1342 to 2075 of wild-type APC and was produced and purified from recombinant sf9 cells by Glu-Glu immunoaffinity chromatography. The purified APC25 was first dephosphorylated with lambda phosphatase (New England Biolabs), then phosphorylated by protein kinase A, and finally repurified. Prephosphorylation by protein kinase A was essential to observe the band shift on phosphorylation by the immunoprecipitates. The reactions were incubated at 30°C for 30 min and terminated by the addition of 12 μl of 4× SDS-PAGE sample buffer followed by heating to 95°C for 5 min. Ten microliters of each sample was applied to the gel, and following electrophoresis, the proteins were electroblotted to polyvinylidene difluoride filters and the filters were exposed to X-ray film.

In vitro binding analysis. For binding of purified proteins to proteins produced by in vitro translation, the indicated cDNAs were first transcribed and translated in vitro in the presence of [^{35}S]methionine with the TNT coupled wheat germ system (Promega). One microgram of purified recombinant α-catenin, rapGAP, or APC2 (33) was added to 25 μl of precleared lysate along with 10 μl of protein A-Sepharose and antibody specific to the Glu-Glu epitope present on each of the purified proteins. Following a 2-h incubation with rocking at 4°C, the beads were washed three times with 1 ml each of buffer B, eluted with 15 μl of SDS-PAGE sample buffer, and subjected to SDS-PAGE and fluorography.

RESULTS

Deletional analysis of β-catenin. To identify the region of β-catenin required for its degradation, we measured the relative ectopic expression levels of various β-catenin deletion mutants in two distinct cell lines. One of the cell lines, AtT20, exhibits a rapid turnover of endogenous β-catenin with a half-life of less than 1 h (9). In this cell line, a significant advantage in relative expression levels should be achieved if structure relevant to β-catenin degradation is deleted. By contrast, β-catenin in SW480 cells exhibits a half-life on the order of several hours (19), and therefore, deletions affecting its degradation should not affect its expression levels as significantly as in the AtT20 cells. The SW480 cell line provides a reference by which to compare the relative expression levels of β-catenin observed in the AtT20 cell line. Expression levels were examined by quantitative immunoblotting with a mixture of two β-catenin antibodies, one specific to the C terminus and the other specific to the N terminus. Relative to ΔN89β-catenin, the levels of wild-type catenin and ΔCβ-catenin were dramatically reduced when their expression in SW480 cells was compared with that in AtT20 cells (Fig. 1). Although detection of the 5′ product was marginal, its levels appeared not to deviate relative to ΔN89β-catenin. Expression of the 3′ construct resulted in barely detectable levels of product even in the SW480 cell line and was therefore not informative. These results suggested that ΔN89β-catenin, in particular, was relatively resistant to degradation in the AtT20 cell line compared with the full-length or ΔCβ-catenin proteins.

Stability and size fractionation of ΔN89β-catenin. To further investigate the turnover of ΔN89β-catenin, stable expression of the cDNA was established in the AtT20 cells. Previous

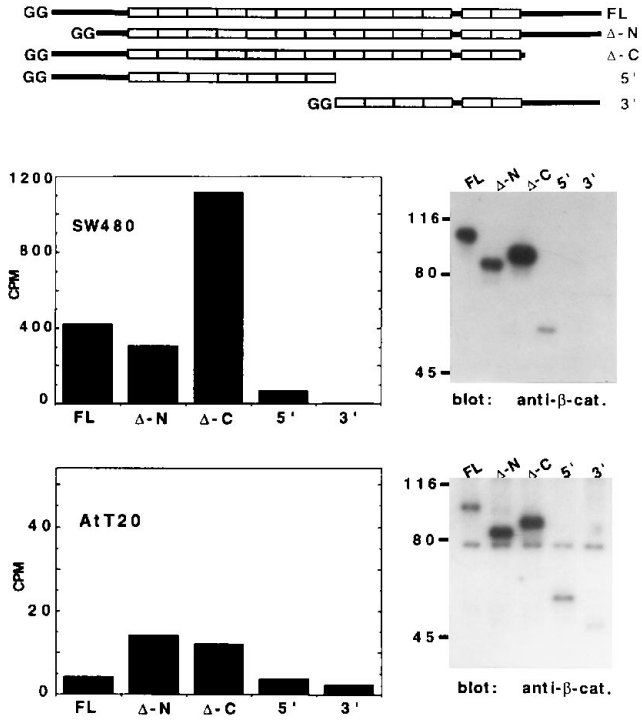

FIG. 1. Deletional analysis of β-catenin. Full-length β-catenin and four deletion mutants are represented schematically at top with armadillo repeat sequences shown as shaded rectangles and the engineered Glu-Glu epitope tag shown as GG. Following transient expression in either SW480 cells or AtT20 cells, the proteins were recovered from protein-normalized cell lysates by immunoprecipitation with anti-Glu-Glu and analyzed by SDS-PAGE and immunoblotting. Blots were developed with a mixture of two β-catenin antibodies, β-cat-C and β-cat-N (see Materials and Methods), and detection was with ^{125}I-protein A. Autoradiograms presented at right were quantitated by β-scanning to generate the corresponding histograms. Note: The mutants react differentially with the antibodies and cannot be compared quantitatively with each other in this experiment.

attempts at generating stable clones expressing wild-type β-catenin were unsuccessful, but three clones expressing ample amounts of ΔN89β-catenin were obtained (Fig. 2). All three of the clones exhibited low levels of wild-type β-catenin compared with vector controls. One of the clones expressing high levels of ΔN89β-catenin, clone 7, was examined further by pulse-chase analysis to assess the stability of β-catenin. Control

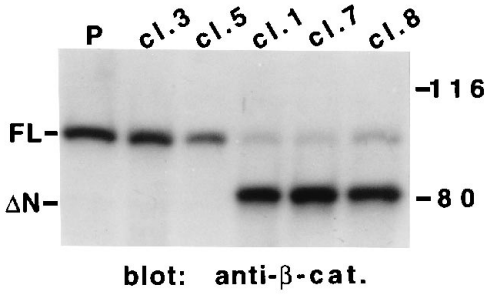

FIG. 2. Analysis of ΔN89β-catenin stably expressed in AtT20 cells. Three AtT20 cell lines stably expressing ΔN89β-catenin (clones 1, 7, and 8) were compared with the parent (P) and two cell lines carrying empty vector (clones 3 and 5) by Western blotting (immunoblotting) analysis of protein-equivalent amounts of cell lysate. The blot was reacted with the β-cat-C antibody, and ^{125}I-protein A was used for detection. The numbers at the right indicate positions and molecular sizes of standard proteins in kilodaltons.

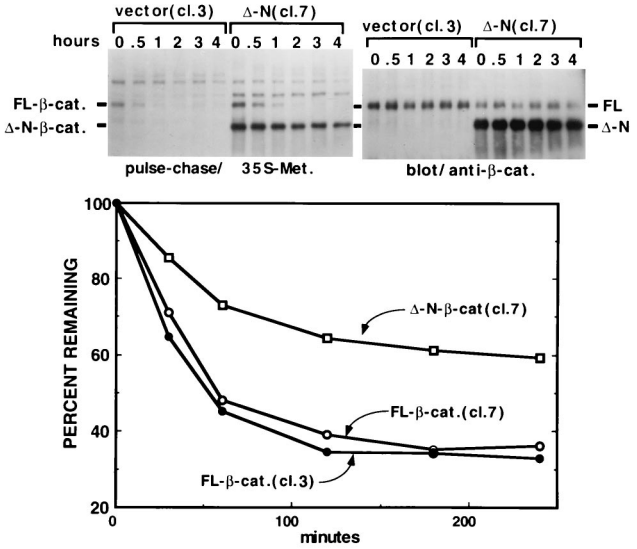

FIG. 3. Pulse-chase analysis. Cells carrying empty vector (cl.3) or expressing ΔN89β-catenin (cl.7) were labeled with [^{35}S]methionine and lysed at the indicated times following the addition of excess cold methionine. β-Catenin was recovered by immunoprecipitation with the β-cat-C antibody, and the precipitates were analyzed by autoradiography (left panel) or immunoblotting with anti-β-catenin (right panel). The autoradiogram was quantitated by β-scanning, and radioactive catenin was plotted as percent remaining relative to time zero.

cells carrying vector only, or the clone 7 cells expressing ΔN89β-catenin, were pulse-labeled with [^{35}S]methionine and then chased with cold methionine for the indicated times. Immunoprecipitation of total β-catenin was carried out on lysates from each of the time points, and radiolabeled β-catenin was detected by autoradiography of SDS-polyacrylamide gels. The wild-type endogenous β-catenin present in both the vector control cells and clone 7 cells decayed with approximately equivalent half-lives, whereas the ΔN89β-catenin was considerably more stable (Fig. 3). These results show that ΔN89β-catenin is less prone to degradation in vivo and that this is not simply due to its overexpression, because the decay of endogenous β-catenin in this same cell was unaffected. Immunoblotting analysis of these same samples was performed by ECL detection to demonstrate that each time point from a set of samples contained approximately equal amounts of β-catenin (Fig. 3).

We have previously noted that, in lysates from cells expressing wild-type APC, β-catenin was present only in a high-molecular-weight complex, presumably in association with cadherins (19). By contrast, monomeric β-catenin was detected in lysates from cells with mutant of APC (19). The AtT20 cells contain wild-type APC, and accordingly, their lysates contain only the complexed, high-molecular-weight pool of β-catenin (27). One interpretation is that the expression levels of wild-type β-catenin are dependent upon the levels of cadherin available for association and that β-catenin, when expressed in excess of cadherins, is rapidly degraded. It would follow that the escape from rapid degradation would permit the accumulation of a free pool of β-catenin. We therefore performed size fractionation on lysates from control cells or cells expressing ΔN89β-catenin to examine the distribution of β-catenin. As expected, the wild-type endogenous β-catenin was observed only in the high-molecular-weight fraction (Fig. 4). However, the ΔN89β-catenin was present both in a complexed and in a monomeric form. These results suggest that an uncomplexed pool of β-catenin can be achieved either by mutation of APC,

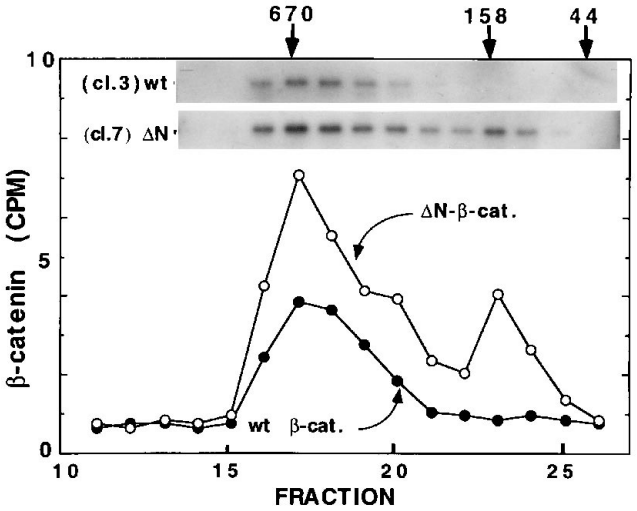

FIG. 4. Size fractionation of wild-type catenin and ΔN89β-catenin. Size exclusion chromatography was performed on lysates (750 μg of total protein) from cells carrying empty vector (cl.3, wt) or expressing ΔN89β-catenin (cl.7, ΔN), and the fractions were analyzed by quantitative immunoblotting with the β-cat-C antibody specific to β-catenin. Autoradiograms developed with ^{125}I-protein A are presented in the inset, and the radioactivity quantitated from catenin in each lane is shown plotted below the corresponding fractions on the chromatogram.

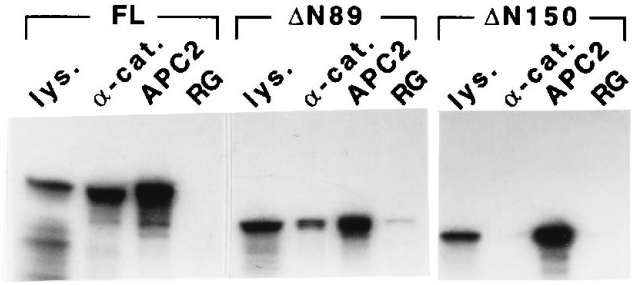

FIG. 6. In vitro binding analysis of full-length catenin and ΔN89β-catenin. cDNAs coding for full-length β-catenin (FL) and two different β-catenin deletion mutants lacking either amino acids 1 to 89 (ΔN89) or 1 to 150 (ΔN150) were transcribed and translated in vitro by the wheat germ lysate TNT system. Lysates containing the ^{35}S-labeled catenins were incubated with either purified α-catenin (α-cat.), an APC fragment (APC2), or, as a control, rapGAP (RG). The purified proteins were recovered by immunoprecipitation and analyzed for bound radioactive β-catenin by SDS-PAGE and fluorography.

which presumably affects the degradation system, or by circumventing degradation through removal of amino-terminal sequence from β-catenin.

APC and cadherin complexes from cells expressing wild-type catenin and ΔN89β-catenin. The appearance of ΔN89β-catenin in the high-molecular-weight complex observed by size exclusion chromatography suggests that it is fully competent to associate with N-cadherin. This is in agreement with previous reports on the association of mutant β-catenins with *E*-cadherin (1, 5, 10, 34). Accordingly, high levels of ΔN89β-catenin were detected in immunoprecipitates of N-cadherin (Fig. 5). However, the total amount of β-catenin (ΔN89 plus wild type) remained relatively constant compared with controls, resulting in a significant reduction in the amount of wild-type β-catenin associated with N-cadherin. As α-catenin binds indirectly to cadherin through its association with β-catenin (1, 5, 10, 34), we also measured the amounts of α-catenin in the cadherin complexes. Little difference in the amount of α-catenin coimmunoprecipitated by N-cadherin antibodies was seen between control and ΔN89β-catenin cells (Fig. 5). This suggests that, although the binding site for α-catenin resides in the amino-terminal region preceding the armadillo repeats (1, 10, 34), it is not deleted by removal of the first 89 residues. That ΔN89β-catenin retains its ability to bind α-catenin was confirmed by in vitro binding analysis. In this experiment, full-length β-catenin and two different mutants deleted at the amino terminus, ΔN89 and ΔN150, were translated in vitro and tested for binding to purified α-catenin. Their ability to bind APC protein and, as a control, rapGAP protein, was also tested. α-Catenin affinity precipitated both the full-length catenin and ΔN89β-catenin, but as previously demonstrated with the *Saccharomyces cerevisiae* two-hybrid format (34), the ΔN150β-catenin did not associate with α-catenin (Fig. 6). The APC protein fragment associated with all of the β-catenins. These results demonstrate that the amino acid residues between 90 and 150 of β-catenin are critical for its binding to α-catenin.

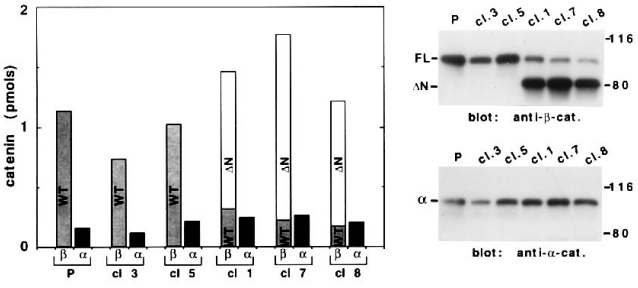

FIG. 5. Analysis of N-cadherin–catenin complexes. Immunoprecipitation of N-cadherin was performed on protein-equivalent lysates from parent (P) AtT20 cells, two cell lines carrying empty vector (clones 3 and 5), and three cell lines stably expressing ΔN89β-catenin (clones 1, 7, and 8). Immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with antibodies specific to α-catenin or the β-cat-C antibody to β-catenin. The molar amounts of α-catenin (black bars), wild-type β-catenin (shaded bars), and ΔN89β-catenin (white bars) were estimated by quantitative immunoblotting (see Materials and Methods) with the blots presented at the right.

The above results suggest that, in spite of the overexpression of ΔN89β-catenin, the cadherin present in these cells may still be competent to interact with the cytoskeleton through its association with α-catenin. Examination of the subcellular localization of α-catenin and N-cadherin by immunofluorescence also indicated that α-catenin and N-cadherin were localized to sites of cell-to-cell contact in cells expressing ΔN89β-catenin (Fig. 7).

We next examined the effects of ΔN89β-catenin on APC-catenin complexes. In contrast to cadherin complexes, there was no effect on the amount of wild-type β-catenin associated with APC, which appeared quite low in both control cells and those expressing ΔN89β-catenin (Fig. 8). However, the total amount of β-catenin (ΔN89 plus wild type) associated with APC was increased over 10-fold in the cells expressing ΔN89β-catenin compared with controls. Interestingly, this did not result in any increase in the amount of α-catenin associated with APC, even though ΔN89β-catenin binds α-catenin. We also noted that the APC immunoprecipitated from cells expressing ΔN89β-catenin appeared to exhibit a lower mobility on SDS-polyacrylamide gels. This was readily apparent when lysates were electrophoresed on 4% polyacrylamide gels (Fig. 9). As alterations in gel mobility are frequently correlated with phosphorylation, we immunoprecipitated the APC protein and treated it with protein phosphatase in vitro. This treatment increased the mobility of APC recovered from both the cells

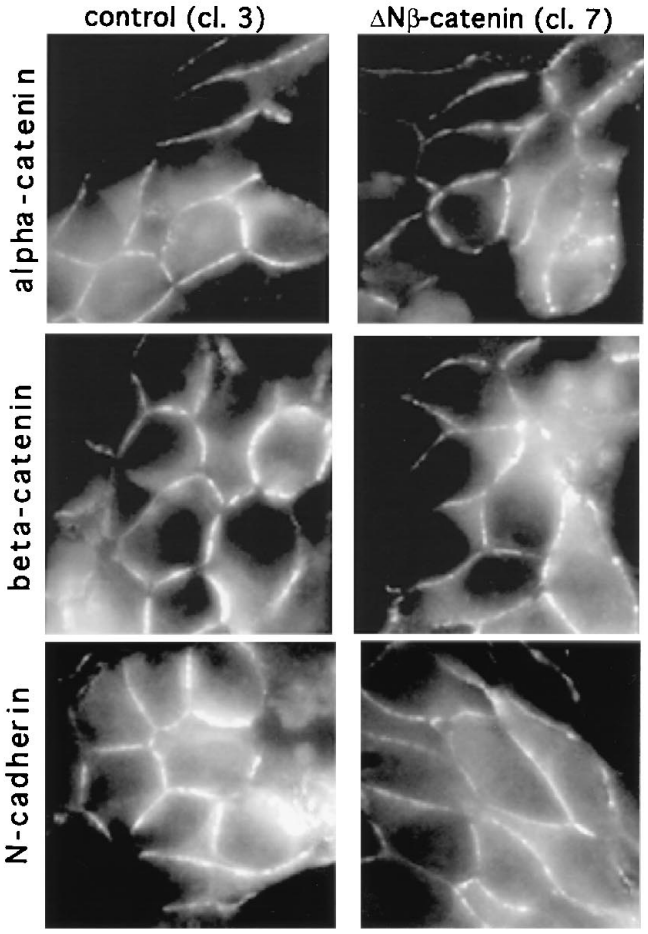

FIG. 7. Immunofluorescent staining of catenins and N-cadherin. AtT20 cells carrying empty vector (cl.3) or expressing ΔN89β-catenin (cl.7) were fixed and stained with antibody specific to either α-catenin, β-catenin (β-cat-FL), or N-cadherin. Detection was with fluorescein isothiocyanate-conjugated goat anti-rabbit antibody for α- and β-catenins and donkey anti-mouse antibody for N-cadherin. Each frame width is approximately 80 μm.

expressing ΔN89β-catenin and control cells, such that their mobilities appeared approximately equal (Fig. 9).

In vitro phosphorylation of APC by β-catenin immunocomplexes. The hyperphosphorylation of APC in the ΔN89β-catenin cells may be a direct consequence of the accumulation of

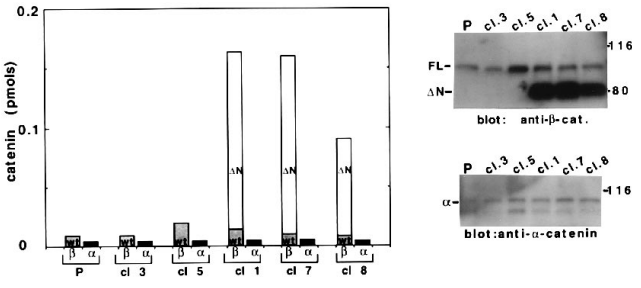

FIG. 8. Analysis of APC-catenin complexes. The molar amounts of catenins coimmunoprecipitated with antibody specific to APC are shown in the histogram. The corresponding immunoblots are presented as the autoradiograms at right. The analysis was performed exactly as described for Fig. 5 for N-cadherin immunoprecipitates.

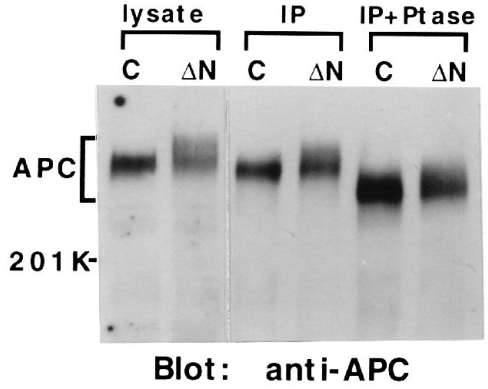

FIG. 9. Electrophoretic mobility of APC protein in cells expressing ΔN89β-catenin. Triton X-100 lysates (100 μg of total protein) from the clone 3 control AtT20 cells (C) or the clone 7 cells expressing ΔN89β-catenin (ΔN) were directly subjected to SDS-PAGE and immunoblotting (lysate). Immunoprecipitates of APC from the same lysates were analyzed either directly (IP) or following incubation with lambda phosphatase (IP + Ptase). The blot was developed with antibody specific to APC, and detection was by ECL.

β-catenin on the APC protein. We reasoned that this could be due to the recruitment of an active APC-kinase into the complex. To test this, we immunoprecipitated β-catenin and examined the immunocomplexes for kinase activity toward a fragment of purified recombinant APC. This fragment, termed APC25, represents the central region of the full-length protein and undergoes phosphorylation in vivo (20). Immunoprecipitates from cells expressing ΔN89β-catenin phosphorylated both the wild-type APC contained in the immunoprecipitate and the APC25 fragment added as substrate to the reaction mixture (Fig. 10). Phosphorylation of wild-type APC was not detected with immunoprecipitates from control cells, although this might be expected given the lower levels of β-catenin bound to APC in these cells (Fig. 7). However, it was apparent from the phosphorylation of the exogenous APC25 substrate that the immunocomplexes from cells expressing ΔN89β-catenin contained significantly more APC kinase activity than those from controls (Fig. 10). Also, the APC25 substrate was band-shifted slightly following phosphorylation by immunocomplexes from the ΔN89β-catenin cells relative to controls. These results demonstrate that an APC kinase is associated with the

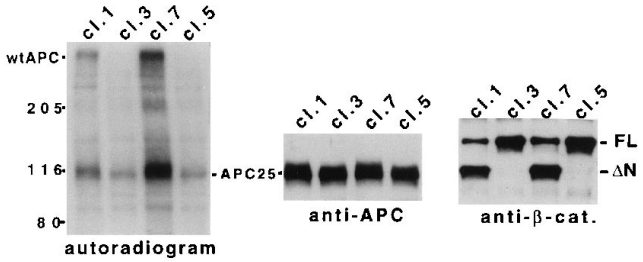

FIG. 10. In vitro kinase assay of β-catenin immunoprecipitates. β-Catenin immunoprecipitates from cells expressing ΔN89β-catenin (clones 1 and 7) or carrying empty vector (clones 3 and 5) were washed and then incubated in kinase buffer containing [γ-^{32}P]ATP and 0.25 μg each of a purified APC protein fragment (APC25). Reactions were terminated by addition of SDS-PAGE sample buffer, and the samples were subjected to electrophoresis and then electroblotted onto a polyvinylidene difluoride filter membrane. Following autoradiography (autoradiogram), the membranes were reacted with antibody to APC (anti-APC) and β-catenin (anti-β-cat.) and then developed by ECL. The numbers at far left indicate positions and molecular sizes of standard proteins in kilodaltons. Wild-type APC (wtAPC) is also indicated.

β-catenin–APC complex and suggest that this kinase is responsible for the hyperphosphorylation of endogenous APC observed in the cells expressing ΔN89β-catenin.

DISCUSSION

It is becoming apparent that β-catenin, originally regarded as a cell adhesion molecule (17, 22, 25), may have additional functions that involve its association with substrates other than cadherins. In development of *Xenopus* embryos, downregulation of β-catenin by antisense expression interferes with the induction of mesoderm (8). This cannot be easily explained by inferring a reduction in cadherin-catenin complexes because overexpression of full-length cadherin itself produces this same phenotype (8). Additional studies with *X. laevis* have shown that β-catenin mutants which lack the capacity to bind α-catenin, and therefore should not function in junctional complexes, retain the ability to signal for dorsalization in the development of the embryo (5). In *Drosophila* development, the accumulation in the cytoplasm of the β-catenin homolog armadillo is an outcome of wingless signaling leading to transcription and cell fate determination (29, 35). In this system, as in *X. laevis*, deletion mutants of armadillo–β-catenin that cannot bind α-catenin are nonetheless competent to signal for cell fate determination (28). Finally, ectopic expression of an amino-terminal deletion mutant of β-catenin in NIH 3T3 fibroblasts results in their transformation (39). These observations, along with the findings that the *wnt-1* proto-oncogene promotes the stabilization and accumulation of β-catenin (9) and its homolog plakoglobin (3), suggest that the control of β-catenin levels and its distribution is an important event in cell growth control. That the artificially stabilized ΔN89β-catenin can mimic some of the *wnt-1* effects has already been noted for mammalian epithelial cells stably expressing this mutant. These cells assume an altered morphology resembling that observed on expression of *wnt-1* (26).

In this study, we attempted to create a setting in which β-catenin is immune to rapid turnover by eliminating specific protein structure required for this process. One of our objectives was to determine whether a stabilized mutant β-catenin would display any of the characteristics noted for wild-type β-catenin when present in a cell either with mutant APC or stimulated by the *wnt-1* oncogene. These characteristics include a prolonged half-life, the distribution of the protein into an uncomplexed pool, and its accumulation on the APC protein (9, 19, 27). All of these criteria were met by the ΔN89β-catenin. We propose that the presence of β-catenin in an uncomplexed form is an indication that its level has exceeded that of cadherin and α-catenin and also that it is no longer subject to rapid turnover. This, in turn, leads to its increased accumulation on protein targets in addition to cadherins. One of these targets is the APC protein. Accordingly, cells expressing ΔN89β-catenin contained 10-fold-higher levels of β-catenin (wild type plus ΔN89) associated with APC than did control cells. By contrast, the total level of β-catenin associated with cadherin was not appreciably changed by expression of ΔNβ-catenin. This indicates that the capacity of cadherin to bind catenin may be near saturation in the control AtT20 cells, whereas specialized circumstances, presumably involving the stabilization of monomeric β-catenin, are required for the maximal loading of APC with β-catenin.

Multiple sites for β-catenin binding are present throughout the central region of the wild-type APC protein and could conceivably allow for superstoichiometric binding. However, some of these sites map to regions of APC previously shown to promote the downregulation of β-catenin when introduced into the SW480 colon cancer cell line (19). Therefore, under normal circumstances β-catenin may not be able to stably associate with all potential binding sites, since some of these might promote its turnover. However, when presented with a mutant β-catenin refractory to degradation, these binding sites may serve only as binding sites and thus permit the stable accumulation of high levels of β-catenin on APC. One consequence of this high-level association is the hyperphosphorylation of APC. Our results indicate that this is the outcome of the direct recruitment of an APC kinase activity into the APC–β-catenin complex. We have recently found that glycogen synthase kinase 3 β binds APC in vivo and that phosphorylation of the central region of APC by glycogen synthase kinase 3 β in vitro promotes the binding of β-catenin (32). This suggests that the hyperphosphorylation of APC observed in the ΔN89β-catenin-expressing cells is responsible for the high levels of associated β-catenin and that glycogen synthase kinase 3 β may be involved in this response. This is particularly interesting as the wingless gene, the *Drosophila* homolog of *wnt-1*, is thought to regulate armadillo (β-catenin) levels through a signaling pathway involving a glycogen synthase kinase 3 homolog (28, 35).

Under normal circumstances, how does the cell establish the appropriate steady-state level of β-catenin? One clue might lie in the observation that all of the β-catenin present in the control cells exists in a high-molecular-weight complex. This suggests that the stabilization of an otherwise labile monomeric β-catenin could occur through its association with other proteins such as cadherin and α-catenin. Indeed, the steady-state levels of both β-catenin and its homolog plakoglobin were dramatically increased by ectopic expression of cadherins in cells otherwise lacking these proteins (15). Therefore, the steady-state levels of β-catenin are in part dependent upon cadherin expression. α-Catenin may also stabilize β-catenin, particularly since its binding site lies in close proximity to the region that we have found to be important for rapid turnover. Thus, the stability of β-catenin may be determined both by the amount of cadherin and α-catenin available for association and by the relative activity of the mechanism employed for its targeted degradation. This model predicts that uncomplexed β-catenin will not reach significant levels in the cell without prior inactivation of the degradation mechanism. This could be accomplished either by mutation of APC, stimulation of cells by *wnt-1*, or deletion of specific amino-terminal sequence from β-catenin. In any of these settings, β-catenin will be stabilized, and if its expression level exceeds the capacity of cadherin available to it, it will accumulate as a free protein.

The above scenario implies that a deletion mutant of β-catenin lacking amino-terminal structure would be particularly hazardous with respect to growth control. Accordingly, transfection of NIH 3T3 cells with library cDNA, followed by selection of transformed colonies, resulted in the recovery of a cDNA encoding a β-catenin lacking 173 amino-terminal residues (39). Moreover, a mutant β-catenin, lacking amino acids 28 to 134, was recently identified in the cell line HSC-39, established from a stomach cancer (13, 24). Although these mutations likely compromise α-catenin binding, our results predict that they would also stabilize the resulting mutant β-catenin and thereby promote its accumulation on the APC protein. Therefore, the implications for mutation in β-catenin, as it relates to cancer, may go beyond a simple loss-of-function model in which cell adhesion is compromised.

ACKNOWLEDGMENTS

We thank Jackie Papkoff, Barry Gumbiner, and Margaret Wheelock for providing the β-cat-C, β-cat-N, and N-cadherin antibodies, respec-

tively. We also thank David Lowe and Brian Souza for the generation and production of recombinant sf9 cells.

REFERENCES

1. **Aberle, H., S. Butz, J. Stappert, H. Weissig, R. Kemler, and H. Hoschuetzky.** 1994. Assembly of the cadherin catenin complex in vitro with recombinant proteins. J. Cell Sci. **107:**3655–3663.
2. **Baker, S. J., S. Markowitz, E. R. Fearon, J. K. Wilson, and B. Vogelstein.** 1990. Suppression of human colorectal carcinoma cell growth by wildtype p53. Science **249:**912–915.
3. **Bradley, R. S., P. Cowin, and A. M. C. Brown.** 1993. Expression of *Wnt-1* in PC12 cells results in modulation of plakoglobin and E-cadherin and increased cellular adhesion. J. Cell Biol. **123:**1857–1865.
4. **Burt, R. W.** 1989. Polyposis syndromes, p. 1674–1696. *In* T. Yamada, D. H. Alpers, C. Oqyang, D. W. Powell, and F. E. Silverstein (ed.), Textbook of gastroenterology. J. P. Lippincott, Philadelphia.
5. **Funayama, N., F. Fagotto, P. McCrea, and B. M. Gumbiner.** 1995. Embryonic axis induction by the armadillo repeat domain of β-catenin: evidence for intracellular signaling. J. Cell Biol. **128:**959–968.
6. **Groden, J., A. Thliveris, W. Samowitz, M. Carlson, L. Gelbert, H. Albertsen, G. Joslyn, J. Stevens, L. Spirio, M. Robertson, L. Sargeant, K. Krapcho, E. Wolff, R. Burt, J. P. Hughes, J. Warrington, J. McPherson, J. Wasmuth, D. LePaslier, H. Abderrahim, D. Cohen, M. Leppert, and R. White.** 1991. Identification and characterization of the familial adenomatous polyposis gene. Cell **66:**589–600.
7. **Grussenmyer, T., K. H. Scheidtmann, M. A. Hutchinson, and G. Walter.** 1985. Complexes of polyoma virus medium T antigen and cellular proteins. Proc. Natl. Acad. Sci. USA **82:**7952–7954.
8. **Heasmen, J., A. Crawford, K. Goldstone, P. Garner-Hamrick, B. Gumbiner, P. McCrea, C. Kitner, C. Y. Noro, and C. Wylie.** 1994. Overexpression of cadherins and underexpression of b-catenin inhibit dorsal mesodermal induction in early xenopus embryos. Cell **79:**791–803.
9. **Hinck, L., W. J. Nelson, and J. Papkoff.** 1994. Wnt-1 modulates cell-cell adhesion in mammalian cells by stabilizing β-catenin binding to the cell adhesion protein cadherin. J. Cell Biol. **124:**729–741.
10. **Hulsken, J., W. Birchmeier, and J. Behrens.** 1995. E-cadherin and APC compete for the interaction of β-catenin with the cytoskeleton. J. Cell Biol. **127:**2061–2069.
11. **Jen, J., S. M. Powell, N. Papadopolous, K. Smith, S. R. Hamilton, B. Vogelstein, and K. Kinzler.** 1994. Molecular determinants of dysplasia in colorectal lesions. Cancer Res. **54:**5523–5526.
12. **Joslyn, G., M. Carlson, A. Thliveris, H. Albertsen, L. Gelbert, W. Samowitz, J. Groden, J. Stevens, L. Spirio, M. Robertson, L. Sargeant, K. Krapcho, E. Wolff, R. Burt, J. P. Hughes, J. Warrington, J. McPherson, J. Wasmuth, D. Le Paslier, H. Abderrahim, D. Cohen, M. Leppert, and R. White.** 1991. Identification of deletion mutations and three new genes at the familial polyposis locus. Cell **66:**601–613.
13. **Kawanishi, J., J. Kato, K. Sasaki, S. Fujii, N. Watanabe, and Y. Niitsu.** 1995. Loss of E-cadherin-dependent cell-cell adhesion due to mutation of the β-catenin gene in a human cancer cell line. Mol. Cell. Biol. **15:**1175–1181.
14. **Kinzler, K. W., M. C. Nilbert, L.-K. Su, B. Vogelstein, T. M. Bryan, D. B. Levy, K. J. Smith, A. C. Preisinger, P. Hedge, D. McKechnie, R. Finniear, A. Markham, J. Groffen, M. S. Boguski, S. F. Altschul, A. Horii, H. Ando, Y. Miyoshi, Y. Miki, I. Nishisho, and Y. Nakamura.** 1991. Identification of FAP locus genes from chromosome 5q21. Science **253:**661–664.
15. **Kowalczyk, A. P., H. L. Palka, H. H. Luu, L. A. Nilles, J. E. Anderson, M. J. Wheelock, and K. J. Green.** 1994. Posttranslational regulation of plakoglobin expression. Influence of the desmosomal cadherins on plakoglobin metabolic stability. J. Biol. Chem. **269:**31214–31223.
16. **McCrea, P. D., W. M. Brieher, and B. M. Gumbiner.** 1993. Induction of secondary axis in *Xenopus* by antibodies to β-catenin. J. Cell Biol. **123:**477–484.
17. **McCrea, P. D., C. W. Turck, and B. Gumbiner.** 1991. A homolog of the *armadillo* protein in *Drosophila* (plakoglobin) associated with E-cadherin. Science **254:**1359–1361.
18. **Miyoshi, Y., H. Nagase, H. Ando, S. Ichii, S. Nakatsura, T. Aoki, Y. Miki, T. Mori, and Y. Nakamura.** 1992. Somatic mutations of the APC gene in colorectal tumors: mutation cluster region in the APC gene. Hum. Mol. Genet. **1:**229–223.
19. **Munemitsu, S., I. Albert, B. Souza, B. Rubinfeld, and P. Polakis.** 1995. Regulation of intracellular β-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein. Proc. Natl. Acad. Sci. USA **92:**3046–3050.
20. **Munemitsu, S., and P. Polakis.** Unpublished data.
21. **Munemitsu, S., B. Souza, O. Muller, I. Albert, B. Rubinfeld, and P. Polakis.** 1994. The APC gene product associates with microtubules in vivo and promotes their assembly in vitro. Cancer Res. **54:**3676–3681.
22. **Nagafuchi, A., and M. Takeichi.** 1988. Cell binding function of E-cadherin is regulated by the cytoplasmic domain. EMBO J. **7:**3679–3684.
23. **Nishisho, I., Y. Nakamura, Y. Miyoshi, Y. Miki, H. Ando, A. Horii, K. Koyama, J. Utsunomiya, S. Baba, P. Hedge, A. Markham, A. J. Krush, G. Petersen, S. R. Hamilton, M. C. Nilbert, D. B. Levy, T. M. Bryan, A. C. Preisinger, K. J. Smith, L. K. Su, K. W. Kinzler, and B. Vogelstein.** 1991. Mutations of chromosome 5q21 genes in FAP and colorectal patients. Science **253:**665–669.
24. **Oyama, T., Y. Kanai, A. Ochiai, S. Akimoto, T. Oda, K. Yanagihara, A. Nagafuchi, S. Tsukita, S. Shibamaoto, F. Ito, M. Takeichi, H. Matsuda, and S. Hirohashi.** 1994. A truncated β-catenin disrupts the interaction between E-cadherin and α-catenin: a cause of loss of intracellular adhesiveness in human cancer cell lines. Cancer Res. **54:**6282–6287.
25. **Ozawa, M., H. Baribault, and R. Kemler.** 1989. The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. EMBO J. **8:**1711–1717.
26. **Papkoff, J. (Megabios Corp., Burlingame, Calif.).** Personal communication.
27. **Papkoff, J., B. Rubinfeld, B. Schryver, and P. Polakis.** 1996. Wnt-1 regulates free pools of catenins and stabilizes APC-catenin complexes. Mol. Cell. Biol. **16:**2128–2134.
28. **Peifer, M.** 1995. Cell adhesion and signal transduction: the armadillo connection. Trends Cell Biol. **5:**224–229.
29. **Peifer, M., D. Sweeton, M. Casey, and E. Wieschaus.** 1994. *wingless* and zeste-white 3 kinase trigger opposing changes in the intracellular distribution of armadillo. Development **120:**369–380.
30. **Polakis, P.** 1995. Mutations in the APC gene and their implications for protein structure and function. Curr. Opin. Genet. Dev. **5:**66–71.
31. **Powell, S. M., N. Zilz, Y. Beazer-Barclay, T. M. Bryan, S. R. Hamilton, S. N. Thibodeau, B. Vogelstein, and K. W. Kinzler.** 1992. APC mutations occur early during colorectal tumorigenesis. Nature (London) **359:**235–237.
32. **Rubinfeld, B., I. Albert, E. Porfiri, C. Fiol, S. Munemitsu, and P. Polakis.** 1996. Binding of GSK3β to the APC-β-catenin complex and regulation of complex assembly. Science **272:**1023–1026.
33. **Rubinfeld, B., B. Souza, I. Albert, O. Muller, S. C. Chamberlain, F. Masiarz, S. Munemitsu, and P. Polakis.** 1993. Association of the APC gene product with β-catenin. Science **262:**1731–1734.
34. **Rubinfeld, B. R., B. Souza, I. Albert, S. Munemitsu, and P. Polakis.** 1995. The APC protein and E-cadherin form similar but independent complexes with α-catenin, β-catenin, and plakoglobin. J. Biol. Chem. **270:**5549–5555.
35. **Siegfried, E., E. L. Wilder, and N. Perrimon.** 1994. Components of the wingless signalling pathway. Nature (London) **367:**76–80.
36. **Smith, A. J., H. S. Stern, M. Penner, H. Kazy, A. Mitri, B. V. Bapat, and S. Gallinger.** 1994. Somatic APC and K-ras codon 12 mutations in aberrant crypt foci from human colons. Cancer Res. **54:**5527–5530.
37. **Smith, K. J., D. B. Levy, P. Maupin, T. D. Pollard, B. Vogelstein, and K. W. Kinzler.** 1994. Wild-type but not mutant APC associates with the microtubule cytoskeleton. Cancer Res. **54:**3672–3675.
38. **Tsukamoto, A. S., R. Grosschedl, R. C. Guzman, T. Parslow, and H. E. Varmus.** 1988. Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. Cell **55:**619–625.
39. **Whitehead, I., H. Kirk, and R. Kay.** 1995. Expression cloning of oncogenes by retroviral transfer of cDNA libraries. Mol. Cell. Biol. **15:**704–710.