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Phosphorylation of the Adenomatous Polyposis Coli Protein and Its Possible Regulatory Effects in Cells

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The adenomatous polyposis coli (APC) gene is etiologically associated with familial adenomatous polyposis and gastrointestinal malignancies, but its cellular function and role in tumorigenesis are unclear. Recent reports indicate that wild-type, but not mutant, APC gene product (APC) is associated with and promotes the assembly of cytoskeletal microtubules in vitro, suggesting that this mechanism has importance in tumor development. Because other microtubule-associated proteins (MAPs) undergo phosphorylation in their normal functioning, we postulated that APC is a phosphoprotein. HCT116 cells, containing full-length APC protein, were [^{32}P]-prelabeled, and a 300-kDa band corresponding to phosphorylated APC was immunoprecipitated using each of three different anti-APC antibodies. High voltage electrophoresis of [^{32}P]-labeled APC showed the presence of phospho-serine and phospho-threonine residues. Further immunoprecipitation analyses showed phosphorylation of i) full-length APC in human lymphoblastoid cells and ii) carboxyl-truncated APC in SW480 and DiFi colon carcinoma cells. Thus, APC is probably a phosphoprotein in normal and malignant tissues. We hypothesize a mechanism whereby phosphorylation of APC may play a regulatory role in its interaction with microtubules. This may involve phosphorylation of (Ser/Thr)-Pro amino acid motifs in APC's basic domain. We propose that deletion of this domain disrupts APC binding to microtubules, explaining how APC mutations are linked to cancer development.

Mutation of the adenomatous polyposis coli (APC) gene is responsible for familial adenomatous polyposis (FAP) and appears to be a critical initiating factor in development of tumors of the digestive tract. In a recent molecular analysis of sixteen colon cancer cell lines, more than 80% of them were found to have mutation of both APC alleles causing a total absence of full-length APC protein (1). Although the APC gene product (APC) thus appears to be important in the etiology of colorectal cancer, the cellular mechanisms whereby this mutation leads to tumors are unclear. Furthermore, our understanding of what APC actually does in cells is just beginning to be elucidated despite the fact that APC's expression appears to be ubiquitous (2, 3).

Initial evaluation of APC's amino acid sequence suggested that it may play a role as a structural protein in the cell. That is, computer analysis of the sequences of APC cDNA and its protein product

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Abbreviations: APC, adenomatous polyposis coli gene; APC, adenomatous polyposis coli protein; FAP, familial adenomatous polyposis; MAPs, microtubule associated proteins; SDS, sodium dodecyl sulfate.

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(APC) showed that the amino end of APC bears many short stretches (less than 20 amino acids) having sequence similarity to a number of cellular structural proteins such as myosin, keratin, neurofilament, and vimentin (2, 3). In fact, APC was recently shown to be associated with two structural components of the cytoskeleton, beta-catenin and microtubules (4-7). Moreover, immunocytochemistry shows that wild-type, but not mutant, APC associates with microtubules of the cytoskeleton and promotes their assembly in vitro (6, 7). This suggests that the interaction of APC with microtubules may be an important mechanism in cellular events and that disruption of this interaction may be important in tumor development.

The goal of the present study was to investigate the possibility that APC might be a phosphoprotein because phosporylation of other microtubule-associated proteins (MAPs), such as tau MAP2, and MAP4, appears to have functional significance in terms of how they interact with microtubules (8). Indeed, the APC sequence contains numerous residues of serine (n=435), threonine (n=167), and tyrosine (n=49) that are potential phosphorylation sites. We believe that this study provides the first evidence that APC is phosphorylated.

MATERIALS AND METHODS

APC Antibodies and Cell Lines

We used anti-APC polyclonal rabbit antibodies (APC-1 and APC-2), generated in our laboratory as described elsewhere (9, 10), to perform immunoprecipitation analysis. APC-1 and APC-2 antibodies target defined epitopes located in the carboxyl end and middle region of the APC protein (amino acids 1865-1881 and 1336-1350, respectively). In addition, anti-APC antibody Ab-1 (Oncogene Science, Cambridge, MA), directed against the amino-terminus of APC, was used.

The human colon cancer cell lines used were HCT116, SW480, and DiFi. HCT116 cells are known to contain full length APC, whereas SW480 and DiFi cells are known to contain truncated APC with deletion of its carboxyl portion. HCT116 and SW480 cells were obtained from the American Type Culture Collection (ATCC, Gaithersburg, MD). The DiFi cell line was derived in our laboratory as described previously (11). All cell lines were grown either in L-15 (SW480), in a 1:1 mixture of RPMI:L-15 (DiFi) or in DMEM (HCT116) media containing 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO) in a humidified atmosphere of 95% air/5% CO_2 at 37° C.

Phospholabeling and Immunoprecipitation of APC

Semi-confluent HCT-116, SW480 or DiFi cells (approximately 3 X 10^6 cells in 25 cm^2 plastic tissue culture flasks), as well as suspension-cultured lymphoblastoid cells (10^7 Epstein-Barr virus-transformed normal human WBCs kindly supplied by Dr. H.T. Lynch), were metabolically labeled at 37° C for 4 h with 1 to 3 mCi of [^{32}P]-orthophosphate (Dupont NEN, Boston, MA) in 2 ml of DMEM-phosphate free medium (GIBCO, Grand Island, NY) containing 10% dialyzed fetal bovine serum. After incubation, medium was removed and cells were rinsed with 10 ml complete DMEM medium, washed twice with ice-cold phosphate-buffered saline (PBS, 10 ml), and scraped into 3 ml RIPA lysing buffer (0.1% SDS, 0.5% deoxycholate, 1% Nonidet P-40, 100 mM NaCl, 10 mM Tris [pH 7.4]) containing protease and phosphatase inhibitors (1 mM EGTA, 12 mM EDTA, 4.3 mM Na_2MoO_4, 1 mM Na_3VO_4, 50 mM phosphate [Na^+ salt], 0.5 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride [PMSF]). The cell lysate was vortexed, centrifuged in a microfuge (Eppendorf) at 13,000 rpm for 10 min at 4° C, and the supernatant was collected. To remove non-specific binding, the supernatants (200 µl aliquots) were incubated with preimmune rabbit serum (25 µl/ml) and a 50% slurry of Protein A-Sepharose CL 4B beads (15 µl/ml, Sigma) at 4° C for 1 h. The samples were centrifuged as above for 15 min, and the supernatant was retained for immunoprecipitation. Anti-APC sera (either APC-1, APC-2, or Ab-1) were then added to the lysates. Samples were incubated overnight at 4° C. Then Protein A-Sepharose 4B CL beads (15 µl/ml of a 50% slurry) were added, and samples were incubated on a rocking platform for 1 h at 4° C. The Protein A-Sepharose 4B CL mixture was pelleted by centrifugation as above for 3 min and washed once with RIPA lysis buffer containing 10 mM NaCl, six times with buffer containing 0.5 M NaCl, and once more with 100 mM NaCl buffer. The Protein A-Sepharose 4B CL pellet was resuspended in gel loading buffer (50 mM Tris-HCl [pH 6.8], 100 mM DTT, 2% SDS, 0.2% bromophenol blue, 20%

glycerol), boiled for 3 min, and centrifuged. The immunoprecipitated proteins contained in the supernatants were electrophoretically separated using 2.8% agarose gels, dried, and autoradiographed.

To further show that the [^{32}P]-labeled 300 kDa band represented APC that was specifically phosphorylated, some samples of [^{32}P]-labeled cell lysates were treated with potato acid phosphatase (20 U, Sigma) to induce dephosphorylation. To verify that only [^{32}P]-labeled APC was being immunoprecipitated, control procedures were performed including: 1) adding excess synthetic APC-1 or APC-2 polypeptides to each of our two anti-APC sera before incubation with [^{32}P]-labeled cell lysates (in separate experiments) to quench the immunoprecipitation of labeled native APC, and 2) incubation of [^{32}P]-labeled cell lysates with preimmune rabbit serum instead of an anti-APC antibody.

High Voltage Electrophoresis

Phosphoamino acid determination was performed as we have described previously (12). Immunoprecipitated [^{32}P]-labeled APC from HCT116 cells was resolved on 4% polyacrylamide gels containing SDS. The region of the wet gel that corresponded to the position of the [^{32}P]-labeled APC band identified by autoradiography was excised, crushed with the flame-sealed end of a Pasteur pipette and heated to 95° C for 5 min in buffer containing 0.05 M NH_4CO_3 (pH 8.5), 0.1% SDS and 5% beta-mercaptoethanol. The radiolabeled proteins were extracted overnight at 37° C while being mechanically shaken. The solution was cleared of gel pieces by centrifugation at 3000 rpm for 15 min and then extracted with the same ammonium bicarbonate buffer for 2 h at 37° C. The pooled supernatants were filtered through glass wool, 50 μg of carrier bovine serum albumin (BSA, Sigma) was added, and the entire sample was adjusted to a 15% trichloroacetic acid level. The proteins were precipitated at 4° C for 4 h and were pelleted by centrifugation at 10,000 rpm for 30 min at 4° C. Samples were washed successively with absolute ethanol and then with ether:ethanol (1:1) at -20° C, and dried in a vacuum oven. Acid hydrolysis was performed by dissolving the protein in 50 μl of 6N HCl at 100° C for 1 min, sealing this acid solution in glass capillaries, and hydrolyzing at 110° C for 2 h. Hydrolysates were cooled, dried under vacuum, and redissolved in 10 μl of a mixture containing excess unlabeled phospho-serine, phospho-threonine, and phospho-tyrosine (1:1:1) each at 2 mg/ml in water. Samples were applied to pre-coated thin layer chromatography plates (Sigma Cell Type 100 cellulose polyester) and were electrophoresed in acetic acid:pyridine:water (50:5:945, pH 3.5) at 2000 volts for 3 h. After high voltage electrophoresis, the plates were dried, stained with ninhydrin, and autoradiographed on Kodak X-Omat film with the aid of an intensifying screen. The stained spots (that were identified by detection of unlabeled phosphoserine, phosphothreonine, and phosphotyrosine added to the sample) were excised from the plates and enumerated in a scintillation counter to determine the relative amount of phosphorylation of each amino acid residue in the APC sample.

RESULTS

Immunoprecipitation analysis of proteins was carried out on [^{32}P]-prelabeled HCT116 cells. In separate experiments, three different antibodies against APC's amino, middle, and carboxyl regions each showed a distinct [^{32}P]-labeled band at 300 kDa corresponding to APC's known molecular weight (Fig. 1). Immunoprecipitated [^{32}P]-labeled protein was also observed at the top of each lane in the loading wells, which is expected of high molecular weight protein aggregates that are not electrophoretically mobile. When the [^{32}P]-labeled proteins were dephosphorylated using the potato acid phosphatase treatment, radioactivity in the 300 kDa band disappeared. No radioactivity was detected in those experimental controls in which immunoprecipitation of phospholabeled cellular APC was quenched by co-incubation with excess synthetic APC-1 or APC-2 polypeptide and in those where preimmune rabbit serum was substituted for anti-APC antibody.

High voltage electrophoretic analysis revealed that [^{32}P] incorporated into this 300 kDa immunoprecipitated protein is located in phosphoserine and phosphothreonine (but not phosphotyrosine) amino acid residues (Fig. 2). To test for APC phosphorylation in other cells, immunoprecipitation analysis was performed on [^{32}P]-prelabeled lymphoblastoid, DiFi, and SW480 cells (Fig. 3). In the lymphoblastoid cells we observed a [^{32}P]-labeled 300 kDa band co-migrating with the one seen in HCT116 cells that corresponds to full length APC. In comparison, lower

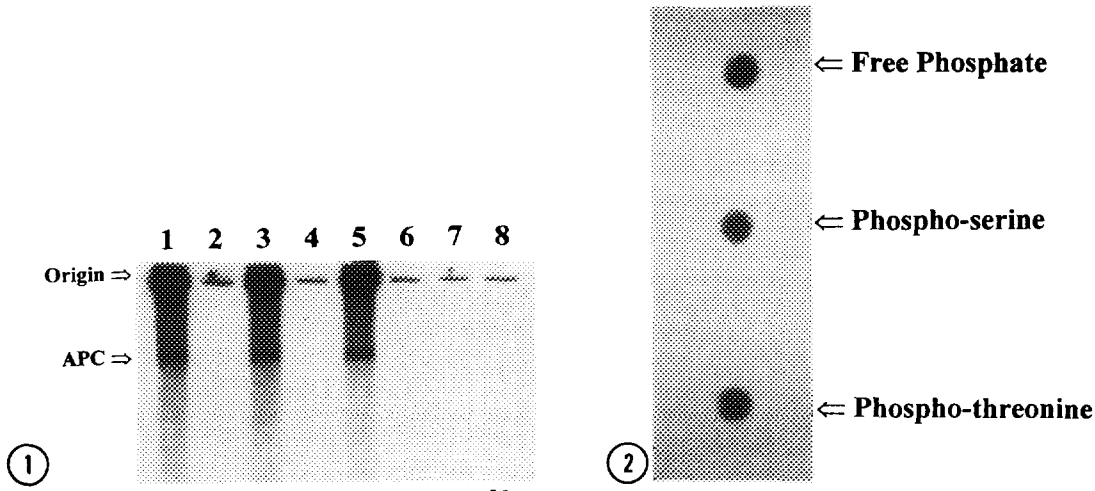

Fig. 1. *Immunoprecipitation of APC from [^{32}P]-labeled HCT116 cells using antibodies against epitopes in APC's amino-terminal, middle, and carboxyl-terminal regions (Ab-1, APC-1, and APC-2, respectively). Lanes show immunoprecipitation using 1) APC-1 antibody, 2) preimmune rabbit serum, 3) APC-2 antibody, 4) preimmune rabbit serum, 5) Ab-1 antibody, 6) APC-1 antibody after quenching with excess synthetic APC-1 polypeptide, 7) APC-2 antibody after quenching with excess synthetic APC-2 polypeptide, and 8) APC-2 after pretreatment of cell lysates with potato acid phosphatase.*

Fig. 2. *High voltage electrophoresis determination of the phosphoamino acid composition of immunoprecipitated [^{32}P]-labeled APC from HCT116 cells. Serine and threonine residues are phosphorylated in APC. No radioactivity was detected in the electrophoretic mobility range corresponding to phosphotyrosine.*

molecular weight phosphorylated bands were detected in DiFi and SW480 cells corresponding to the 120 kDa and 147 kDa truncated APC known to be present in these two cell lines, respectively.

DISCUSSION

Our results provide evidence that endogenous APC protein is phosphorylated in both HCT116 and normal human lymphoblastoid cells which each contain full-length APC. Several findings corroborate this observation: i) The same autoradiographic APC protein pattern was observed regardless of which of three different anti-APC antibodies was used. This indicates that it is unlikely that another protein having antigenic cross-reactivity is being immunoprecipitated with any of the APC antibodies used in our experiments. ii) Dephosphorylation experiments using potato acid phosphatase abolished the [^{32}P]-labeling in the 300 kDa band. This indicates that APC's phosphorylated state can be enzymatically reversed, as is expected of any endogenous process that is carefully controlled by phosphorylation and suggests that the [^{32}P] in phosphorylated APC is linked to amino acid residues as compared to being non-specifically trapped by the precipitation. iii) No detectable radioactivity was found in the experimental controls employing quenching with excess synthetic APC polypeptide and substitution of preimmune rabbit serum for anti-APC antibody. This denotes antibody specificity for APC and shows that the [^{32}P]-labeled 300 kDa band is not non-specifically immunoprecipiated.

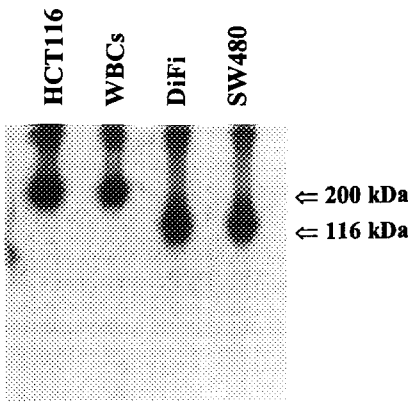

Fig. 3. Immunoprecipitation of [^{32}P]-labeled APC from [^{32}P]-prelabeled HCT116, lymphoblastoid (WBCs), DiFi, and SW480 cells using anti-APC antibody Ab-1 (Oncogene Science). HCT116 and lymphoblastoid cells contain full-length APC, whereas DiFi and SW480 cells contain truncated APC, having the carboxyl-terminal portion deleted.

The presence of electrophoretically immobile immunoprecipitated [^{32}P]-protein at the top of each lane in the gel sample loading wells is consistent with APC's known association with insoluble aggregates of proteins (1). Based on the known insolubility of microtubule proteins, one would expect to find them and any associated APC in an insoluble aggregate at the electrophoretic origin.

Results from phosphoamino acid determinations indicate that APC is phosphorylated at serine and threonine, but not tyrosine, amino acid residues. Finding this APC phosphorylation pattern is consistent with APC's putative role as a MAP because regulation of the interaction of other MAPs, such as tau, with microtubules appears to involve phosphorylation of their serine and threonine amino acid residues, especially those that are on the amino side of a proline (13). Another MAP, MAP4, is also regulated by phosphorylation of amino acid residues, especially those in its proline-rich regions (14). This phosphorylation of MAP4 is cell cycle regulated and thought to be a primary event in the destruction of cytoplasmic microtubule arrays prior to the M phase (15). Although it is uncertain whether APC is phosphorylated by kinases targeting serine or threonine residues preceding a proline, it is interesting to note that APC's sequence, like tau, is rich in serine, threonine, and proline and contains numerous Ser-Pro (n=35) and Thr-Pro (n=21) motifs.

Perhaps APC, also like tau, undergoes multiple phosphorylations. For example, tau is phosphorylated at multiple serine and threonine residues, and the degree of its phosphorylation affects the affinity of tau for microtubules (16). Interestingly, aberrantly phosphorylated tau, which is unable to bind microtubules, appears to be responsible for the disease-related modification of tau protein associated with neurofibrillary pathology in Alzheimer's disease (17). Moreover, the phosphorylation of tau appears to be developmentally regulated by proline-directed protein kinases, distinct kinases that phosphorylate proline-directed Ser/Thr residues (13).

Our findings also provide evidence that mutant APC protein is phosphorylated in two human colorectal carcinoma cell lines, DiFi and SW480. Because these cell lines contain truncated APC protein having deletion of its carboxyl region, our findings indicate that phosphorylation can occur in

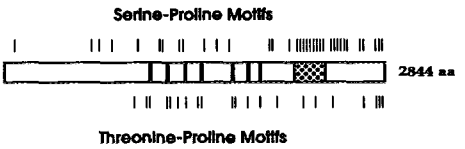

Fig. 4. Locations of the Ser-Pro (n = 35) and Thr-Pro (n = 21) amino acid motifs in the sequence of the APC protein are represented by hash marks above and below the APC schematic drawing, respectively. The heavy vertical bars within the APC schematic denote the seven repeats of 20 amino acids that are thought to be critical for beta-catenin binding (4, 5). The spotted region denotes APC's basic domain (residues 2200-2400) contained within its carboxyl-terminal region (2). We hypothesize that APC binding to microtubules occurs via APC's basic domain in a manner similar to other MAPs containing a basic domain that is essential for their binding to microtubules (17, 18). It is proposed that phosphorylation of (Ser/Thr)-Pro motifs in APC's 20 amino acid repeat sequences and its basic domain may modulate APC's interaction with beta-catenin and with microtubules.

APC's amino-terminus to middle portions. However, the degree to which APC's carboxyl region is phosphorylated cannot be determined from these data.

Because immunohistochemical evidence indicates that interaction of APC with the microtubule cytoskeleton occurs via its carboxyl terminus, one might ask how phosphorylation of APC's amino- to middle regions could be involved in such an interaction. Perhaps phosphorylation of APC at one or more intramolecular serine and threonine residues modulates APC's conformational structure and thus regulates the extent of interaction of its carboxyl terminus with microtubules. Alternatively, phosphorylation within the APC carboxyl terminus region itself may be involved in its binding to microtubules. For example, other MAPs contain a sub-region involved in microtubule binding, which is a basic domain contained within the carboxyl third of these proteins (18). APC contains a similar basic domain in its carboxyl third (Fig. 4; 2). In other MAPs this basic region is essential for binding, and the underlying mechanism appears to involve electrostatic interaction of basic residues in MAPs with acidic residues of the carboxyl portion of microtubules. In addition, phosphorylation of multiple (Ser/Thr)-Pro motifs in this basic domain affects the charge-charge interaction of MAPs with microtubules, and phosphorylation thereby regulates the MAPs' affinity toward microtubules (17, 18).

Analysis of the COOH-third of APC shows that it is enriched in Ser-Pro and Thr-Pro motifs (50% occur between residues 2145-2843). Moreover, 20% of all these motifs, particularly the Ser-Pro type, reside in APC's basic domain (Fig. 4). It can be hypothesized that phosphorylation of these motifs regulates APC's interaction with microtubules in a manner similar to the known phosphorylation-mediated regulation of other MAPs' interactions with microtubules. Thus, it is predicted that increased phosphorylation of APC would lower its microtubule binding affinity.

There is also reason to speculate that this same phosphorylation-mediated regulatory mechanism may apply to the binding of APC to another cytoskeletal component, beta-catenin. For instance, analysis of APC's catenin binding domain shows that the seven repeats of a 20 amino acid sequence in this region (2), which are thought to be critical to catenin binding (4, 5) each contain a Thr-Pro motif (Fig. 4). Thus, beta-catenin binding to APC may also involve a phosphorylation-mediated regulatory mechanism involving APC's Thr-Pro motifs. Perhaps tissues can even selectively regulate APC binding to beta-catenin as opposed to binding to microtubules. Possibly, cells could do this by selectively activating specific proline-directed kinases. In one case, the cell could phosphorylate Thr-

Pro motifs in the 20 amino acid repeats, motifs that regulate beta-catenin binding. Alternatively, the cell might phosphorylate Ser-Pro motifs in the basic domain that controls microtubule binding.

Finally, one could ask what phosphorylation of these regions might have to do with APC's role in the development of tumors. Analysis of APC mutations in colonic tumors shows that the beta-catenin binding region is deleted in many (but not all) truncated proteins caused by these mutations (19). In comparison, the basic domain (and any of its phosphorylation sites) is totally deleted in the vast majority (probably 100%; 19) of truncated APC proteins contained in colonic tumors. The APC mutation at codon 1941 is located on the amino side of the basic region of APC and represents the mutation (among those identified to date in a colonic tumor) that is closest to the carboxyl terminus (6). Thus, it appears that generation of a mutant APC protein having its basic region intact is a rarity in colonic tumors. If it turns out that microtubules bind to the basic domain of APC, then its loss could disrupt APC binding to microtubules and become a critical factor in tumor development.

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