Phosphorylation of the Tumor Suppressor Adenomatous Polyposis Coli (APC) by the Cyclin-dependent Kinase p34<sup>cd2</sup>\*\*

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Mutations in the tumor suppressor gene APC invariably lead to the development of colorectal cancer. The vast majority of these mutations are nonsense or frame-shifts resulting in nonfunctional, truncated APC protein products. Eleven cyclin-dependent kinase (CDK) consensus phosphorylation sites have been identified in the frequently deleted carboxyl-terminal region of APC; loss of these phosphorylation sites by mutation could therefore compromise the ability of APC to inhibit cell growth. This report demonstrates that immunoprecipitates of full-length, but not truncated, APC protein include a mitosis-specific kinase activity in vivo. Biochemical and Western analysis of these immunoprecipitates confirms the presence of the CDK p34<sup>cd2</sup>, a cyclin B1. Modification of APC by p34<sup>cd2</sup> implicates phosphorylation as a mechanism for regulating APC function via a link to the cell cycle.

The tumor suppressor protein APC is inactivated by mutation in over 80% of all colorectal cancers, both sporadic and familial. Loss of APC function is considered the initial and rate-limiting mutation in the multistep model of colon carcinogenesis (1). APC is a cytoplasmic protein of 2844 amino acids with a predicted molecular mass of 312 kDa (2, 3) and without extensive homology to other known proteins. Functional roles for APC have been suggested by its association with other signaling proteins whose characteristics are better delineated. APC disrupts the ability of APC to regulate cytoplasmic levels of $\beta$-catenin (4, 5), a protein of unknown function designated EB1 (6), and the $\beta$-catenin (7). APC localizes to the microtubular network in vivo, while purified APC proteins promote the polymerization of tubulin into microtubules in vitro (8, 9). The regions that interact with these proteins have been localized to the carboxyl half of APC and include the regions deleted by somatic and germline mutations of APC (10). Such mutations disrupt the ability of APC to regulate cytoplasmic levels of $\beta$-catenin (11, 12) and consequently lead to the constitutive activation of $\beta$-catenin/Tcf signaling (13, 14). Other effects of APC mutation are less understood.

Germline alteration of APC results in an inherited predisposition to colorectal cancer known as familial adenomatous polyposis coli (2, 3). In one patient, a frameshift mutation has been identified at nucleotide 7935 of the APC cDNA that results in the loss of 200 amino acids from the carboxyl terminus of APC (15). Expression of a similarly truncated mutant protein in cells lacking normal APC protein is sufficient to down-regulate the $\beta$-catenin/Tcf signal (14). Interestingly, the patient with this germline mutation has a phenotype similar to that of other patients whose mutations have been shown to disrupt this down-regulation function. Therefore, this minimal region of APC must also harbor additional elements that facilitate the function or stability of this tumor suppressor. In addition to binding the DLG protein, these 200 amino acids in the predicted human APC protein sequence include two phosphorylation sites that are identical to the consensus target sequence for the cyclin-dependent kinase (CDK) family of serine and threonine kinases. Such kinases catalyze the addition of phosphate to their substrates in a cell cycle-specific manner. APC, a phosphoprotein, has been shown to be modified exclusively on serine and threonine residues (16, 17); therefore this modification could be the result of CDK activity. In this report, we investigated the relationship between APC phosphorylation and the CDKs.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HCT116 cells were obtained from the American Type Culture Collection (ATCC) and cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum. SW480 cells (ATCC) were cultured in Leibovitz's L15 medium supplemented with 10% fetal bovine serum. For synchronization of cells at the G1/S boundary, and in M-phase, nonconfluent cells were incubated for 24 h in 0.2 mM mimosine or 1.0 \mu M mimosine for 4 h in phosphate-free medium supplemented with 2.0 mCi of [32P]orthophosphate per 100-mm plate.

**Immunoprecipitation**—Cells were rinsed with phosphate-buffered saline and lysed in Nonidet P-40 wash buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40) supplemented with protease inhibitors (2.0 \mu M each of pepstatin A, leupeptin, and aprotinin, 1 \mu M EDTA, and 0.2 mM phenylmethylsulfonyl fluoride), and protein was quantitated using a detergent-compatible assay (Bio-Rad). Cell lysates were precleared with normal mouse IgG (Sigma) and immunoprecipitated as described (18) using normal mouse IgG, the monoclonal antibody AB5 (Oncogene Science), or the monoclonal antibody cdc2-Ab2 (Oncogene Science). Immunocomplexes were isolated with Protein A-agarose, washed liberally with wash buffer, and used in the H1 kinase assays or Western analyses.

**Kinetin Assays**—In vitro H1 kinase assays were performed using immunoprecipitates in 30.0 \mu L of kinase buffer (50 mM HEPES (pH 7.0), 10 \mu M MgCl<sub>2</sub>, 5 \mu M MnCl<sub>2</sub>, 1 \mu M dithiothreitol, 100 \mu M ATP, and 5.0 \muCi of [γ-32P]ATP using 10.0 \mu g of histone H1 (Boehringer Mannheim) as a substrate. For olomoucine inhibition reactions, H1 kinase assays were performed with the concentration of ATP decreased to 15 \muM (19). Olomoucine (Sigma) was added from a 10 mM stock to the indicated concentrations prior to incubation. As a control, the activity of 1.0 unit

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Fig. 1. Location of CDK consensus phosphorylation sites in APC. A, sequence of the final 244 amino acids of APC. The sequences that match the consensus site for CDK phosphorylation (STPXR(K)/R) are underlined and bold. The star designates the site of the most carboxyterminal truncating mutation of APC, a four-nucleotide deletion that results in a novel reading frame (lower sequence) and the creation of a stop codon 14 residues downstream (15). B, map of APC protein. The numbers above the protein indicate the residue number in increments of 500 amino acids; the black arrows indicate the locations of the CDK consensus phosphorylation sites. The majority of these sites are located in the region of APC frequently deleted by mutation (10). N, amino terminus; C, carboxy terminus. C, APC is a phosphoprotein. (32P)orthophosphate-labeled asynchronous (AS), G1/S boundary (G1/S), or mitotic (M) HCT116 cell lysates harvested from 100-mm plates were used for immunoprecipitations with normal mouse IgG (IgG) or an APC-specific antibody (APC) followed by resolution on an SDS-denaturating agarose gel. The left panel displays autoradiography, the right a Western blot of the same gel using the monoclonal antibody APC-Ab1 as a probe. There is no quantitative difference in the phosphorylation of APC in the three different cell populations. Numbers indicate approximate molecular masses in kDa.

RESULTS AND DISCUSSION

The final 200 carboxy-terminal residues of APC contain two consensus phosphorylation sites usually targeted by the CDKs (Fig. 1A). Analysis of the peptide sequence of the full-length APC molecule revealed an additional nine consensus sites, for a total of 11 (Fig. 1B); each of these is conserved in the predicted mouse and rat APC sequences (data not shown). The majority of these sites are located in the region that is frequently deleted in mutant APC proteins. Populations of asynchronous, G1/S boundary-synchronized, and M-phase-synchronized HCT116 colon carcinoma cells (which express a normal, full-length APC protein) were labeled with [32P]orthophosphate in vivo to look for cell cycle specificity in the incorporation of the isotope in immunoprecipitated APC protein. APC was found to be phosphorylated in each of these cell populations (Fig. 1C), with no apparent cell cycle-specific incorporation of phosphate into the protein.

We subsequently employed the histone H1 kinase assay to evaluate potential CDK modification of APC. Histone H1 is an in vivo and in vitro substrate of CDKs, with the amount of phosphate incorporated in the protein a quantitative indicator of CDK activity. Immunoprecipitates from lysates of asynchronous populations of HCT116 cells contain an H1 kinase activity that coprecipitates with APC-specific antibodies but not with the control antibody (Fig. 2). To ascertain whether this kinase activity is cell cycle-regulated, HCT116 cells were synchronized into G1/S boundary and M-phase populations, and the experiments were repeated. The APC-associated kinase activity was most pronounced during M-phase, with only a low level of activity found in G1/S. Such mitosis-restricted H1 kinase activity is most characteristic of the CDK p34cdc2/cyclin B complex. In vitro kinase assays using p34cdc2-specific immunoprecipitates isolated from similar cell lysates revealed that the kinase activity associated with APC and that of p34cdc2 are most active in the same cell cycle periods. This cell cycle-specific kinase activity is not the result of differential expression of the APC protein, since the protein is present in equivalent quantities throughout the cell cycle as shown by anti-APC immunoblots (Fig. 2B), nor is it a consequence of a generalized reduction in cellular H1 kinase activity in the examined populations of cells (data not shown). A similar series of H1 kinase assays were performed using immunoprecipitates from the colon carcinoma cell line SW480. This cell line produces an APC protein truncated at residue 1338 (20) and is predicted to contain only one CDK consensus site. The SW480 IgG- and p34-specific immunoprecipitates display associated H1 kinase activity profiles similar to those of HCT116. However, the immunoprecipitates of truncated APC molecules show greatly reduced levels of kinase activity when isolated from asynchronous and mitotic cell lysates (Fig. 2A), establishing a positive correlation between the presence of APC-associated H1 kinase activity and the carboxy-terminal region of APC, which contains 10 additional CDK consensus phosphorylation sites.

The p34cdc2/cyclin B complex is active during M-phase; its association with APC may be one source of the H1 kinase activity associated with APC in this segment of the cell cycle. To test this, immunoprecipitates from nocodazole-synchronized HCT116 cell lysates were incubated with increasing concentrations of olomoucine, a nucleotide analog that specifically and competitively inhibits several CDKs, including p34cdc2 (14). As expected, olomoucine inhibited the phosphorylation of H1 histone catalyzed by the p34cdc2 immunoprecipitates and a purified recombinant p34cdc2/cyclin B1 enzyme complex (Fig. 3). The APC-associated kinase activity also was inhibited by olomoucine at a concentration equivalent to that required for inhibition of p34cdc2 (Fig. 3), suggesting the physical association of an active and inhibitable CDK with APC.
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**FIG. 2.** Association of APC with a cell cycle-regulated kinase activity. A, *in vitro* histone H1 kinase assays. Phosphorylation of histone H1 was used to measure the level of kinase activity associated *in vivo* with the complexes immunoprecipitated from APC and p34\(^{cd2}\) from HCT116 or SW480 cell lysates. AS, asynchronous; G1/S, mimoseine-treated cells; M, nocodazole-treated cells. B, Western blot of APC immunoprecipitated from the same three cell populations from cell lines HCT116 and SW480 using APC-Ab1 as a probe. All immunoprecipitations were performed on normalized quantities of protein, 0.50 mg per assay. APC, full-length APC protein; APC\(^{mut}\), mutant truncated APC protein. Numbers indicate approximate molecular masses in kDa.

**FIG. 3.** Olomoucine inhibits APC-associated kinase activity. Recombinant p34\(^{cd2}\)-cyclin B1 enzyme (p34 enzyme) or p34-specific immunoprecipitates (p34 IP) and APC-specific immunoprecipitates (APC IP) from HCT116 mitotic cell lysates were used in H1 kinase assays in the presence of increasing concentrations of olomoucine. Each data point represents the mean ± S.D. of five independent kinase experiments and is derived from the quantitation of H1 autoradiography relative to an uninhibited control (represented as 0 µM olomoucine). Values for each point are: p34 enzyme, 100, 94 ± 14, 78.6 ± 5.1, 65.4 ± 14.1, 49.7 ± 28.2, 26.8 ± 14.3, 24.5 ± 8.8, 11.6 ± 2.4; p34 IP, 100, 86.7 ± 18.7, 73.4 ± 26.2, 55.9 ± 17.1, 46.5 ± 19.1, 29.6 ± 14.6, 34.8 ± 3.5, 23.6 ± 9.5; APC IP, 100, 82.6 ± 22.2, 66.2 ± 19.7, 74.2 ± 25.4, 45.1 ± 18.4, 39.7 ± 3.6, 30.9 ± 6.7, 24.0 ± 12.9. Quantitation was performed with a Molecular Dynamics PhosphorImager.

Finally, to determine whether APC and p34\(^{cd2}\) are associated within the same complex, APC protein was immunoprecipitated from lysates of asynchronous, G1/S boundary, and M-phase HCT116 cells and analyzed by Western blotting for associated p34\(^{cd2}\) (Fig. 4). Immunoprecipitations with a control antibody (IgG) from mitotic lysates or APC-specific antibodies from asynchronous and G1/S lysates failed to detect associated p34\(^{cd2}\). Immunoprecipitation of APC from M-phase lysates, however, revealed that p34\(^{cd2}\) coimmunoprecipitated with APC. The presence of p34\(^{cd2}\) consensus sites in APC, its association with an olomoucine-inhibitable H1 kinase activity, and the physical interaction of APC and p34\(^{cd2}\) strongly indicate that the H1 kinase activity present in APC immunoprecipitates is attributable to p34\(^{cd2}\) kinase.

These data implicate APC as a catalytic target of p34\(^{cd2}\), although it is possible that other proteins interacting with APC could also be phosphorylated by p34\(^{cd2}\) in addition to, or instead of, APC. Microtubules, for example, associate both with APC (8, 9) and p34\(^{cd2}\) (21, 22). Alternatively, the presence and activity of p34\(^{cd2}\) may be required for the activation of another coinmunoprecipitated kinase which may modify APC. Therefore, the capacity of APC to act as a direct substrate of the human p34\(^{cd2}\)-cyclin B1 active kinase complex was evaluated. A fragment of APC encoding the residues 2141–2844 was cloned in-frame with a FLAG epitope (FLAG-APC) and introduced into bacteria. This region of APC contains 8 of the 11 putative CDK consensus sites (Fig. 5A). Bacterially expressed FLAG-APC protein was recovered by immunoprecipitation and incubated with recombinant human p34\(^{cd2}\)-cyclin B1. The FLAG-APC protein was phosphorylated by the kinase (Fig. 5, B and C).

Although p34\(^{cd2}\) is best recognized for its role in the G2/M transition, we have now shown that its activity is associated with APC during M-phase of the cell cycle. This kinase not only phosphorylates APC *in vitro* but likely targets this tumor suppressor as a substrate *in vivo*. Our data (Fig. 1C), as well as that of another recent study (17), have demonstrated that APC is phosphorylated throughout the cell cycle and that APC becomes hyperphosphorylated during M-phase, suggesting that additional kinases may be responsible for phosphorylating APC. Indeed, APC is known to be phosphorylated by two other kinases in *vitro*: protein kinase A and glycogen synthase kinase-3β (23). Phosphorylation of APC by these kinases regulates the binding, turnover, and subsequent degradation of β-catenin in association with APC and therefore controls β-catenin-transduced signaling. The interaction of β-catenin with APC is unchanged in the G1/S boundary and M-phase populations of HCT116 cells, suggesting that modification of APC by p34\(^{cd2}\) does not directly affect this association quantitatively although the rate of APC-mediated β-catenin degradation may be influenced. It also remains possible that phosphorylation of APC at CDK consensus sites alters its ability to interact with other proteins, and that a cell cycle-specific change in such interactions mediates some of the tumor-suppressing effects of APC.

2 C. Trzepacz and J. Groden, unpublished data.
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A

B

C

**Fig. 5.** In vitro phosphorylation of APC by p34\(^{cdk2}\). Control antibody (IgG) and FLAG-PROBE (FLAG) immunoprecipitates from uninduced (-) and isopropyl-1-thio-\(\beta\)-D-galactopyranoside-induced (+) lysates of bacteria harboring the FLAG-APC expression construct were incubated with recombinant human p34\(^{cdk2}\)-cyclin B1 complex in the presence of [\(\gamma\)-\(\text{32P}\)]ATP and resolved by SDS-PAGE on an 8% gel.

Substrates of the CDK family include diverse proteins, both nuclear and cytoplasmic, that are critically involved in the regulation or mechanics of the cell cycle (24). Furthermore, the phosphorylation of APC by p34\(^{cdk2}\) may result in similar cell cycle-regulated modulations of APC-mediated tumor suppression by altering a domain of APC that is frequently deleted by mutation. It remains to be determined whether the loss of APC consensus phosphorylation sites is sufficient to disrupt APC function.

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