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The Tumor Suppressor Gene Product APC Is Hyperphosphorylated during the M Phase

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The APC gene is mutated in familial adenomatous polyposis and sporadic colorectal tumors. The product of this gene is a 300 kDa cytoplasmic protein and its overexpression results in the block of cell cycle progression from the G0/G1 to the S phase. In the present study, we studied the expression and phosphorylation of the APC protein through the cell cycle. The APC protein was found to be constantly expressed and phosphorylated at serine and threonine residues. Moreover, the APC protein immunoprecipitated from cells arrested in the M phase by nocodazole treatment migrated in SDS–PAGE more slowly than those from the G1 and S phases. Phosphatase treatment abolished this M phase-specific retarded migration, suggesting that APC is transiently hyperphosphorylated in the M phase.

Germline mutations of the *APC* gene have been found in most cases of familial adenomatous polyposis (FAP), an autosomal dominantly inherited disease that predisposes patients to multiple colorectal polyps and cancer (1–7). The *APC* gene is also somatically mutated in the majority of sporadic colorectal tumors (1–7). The product of the APC gene is a 300 kDa cytoplasmic protein and the majority of these mutations result in the loss of the carboxy-terminus of this protein (1–9). Although the predicted amino acid sequence of the APC protein has little sequence similarity to other proteins, APC is known to form a cytosolic complex with α-catenin and β-catenin (10–12). APC has also been reported to associate with microtubules and a novel protein, EB1, in cells overexpressing the exogenously transfected *APC* gene (13–15). Recently we have demonstrated that overexpression of APC blocks cell cycle progression from the G0/G1 to the S phase (16). In this report, we examined the expression and phosphorylation of APC through the cell cycle. We found that the level of APC phosphorylation specifically increases during the M phase.

MATERIALS and METHODS

Cell culture. Human osteosarcoma HOS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. For synchronization, HOS cells were cultured in the presence of 50 ng/ml nocodazole (Sigma) for 12 h and metaphase-arrested cells were collected by gentle pipeting and shaking. The mitotic cells were then washed and reseeded in fresh medium. The cell cycle was monitored by flow cytometric analysis with a FACScan/CellIIFIT DNA system (Becton Dickinson).

Immunoprecipitation and Western blotting. HOS cells labeled with [^{32}P]phosphate (250 μCi/ml) or [^{35}S]methionine (100 μCi/ml) (DuPont NEN) were lysed in solubilizing buffer (16) and subjected to immunoprecipitation with anti-APC antibodies (anti-APC-C-ter) raised against a synthetic peptide corresponding to the carboxy-terminal 14 amino acids as described previously (9, 16). The immunoprecipitates were analyzed by 5% SDS-PAGE followed by autoradiography. Western blotting analysis was also performed using 5% SDS-PAGE as described previously (9).

Phosphatase treatment. Immunocomplexes bound to protein A-Sepharose were incubated with 0.06 μ potato acid phosphatase (Boehringer Mannheim) in 100 μl of distilled water containing 1 mM PMSF, aprotinin (0.1 mg/ml) and BSA (2 mg/ml) for 10 min at 37°C.

Phosphoamino acid analysis was performed as described (17).

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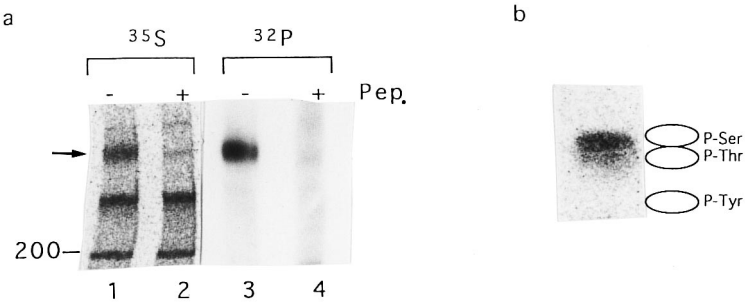

FIG. 1. APC is phosphorylated at serine and threonine residues in vivo. (a) APC was immunoprecipitated from [^{35}S]methionine-labeled (lanes 1 and 2) or [^{32}P]phosphate-labeled (lanes 3 and 4) HOS cells with anti-APC-C-ter (lanes 1 and 3) or anti-APC-C-ter that had been preabsorbed with peptide antigen (lanes 2 and 4). The immunoprecipitates were resolved by 5% SDS–PAGE followed by autoradiography. The arrow indicates APC. (b) Phosphoamino acid analysis of APC. [^{32}P]phosphate-labeled APC was extracted from the gel, trypsinized, and hydrolyzed in 6 N HCl. The labeled phosphoamino acids and unlabeled standards were mixed and separated by one dimensional electrophoresis (pH 3.5) on thin layer cellulose plates and were visualized by autoradiography and ninhydrin staining.

RESULTS AND DISCUSSION

To examine whether APC is phosphorylated *in vivo*, human osteosarcoma HOS cells were labeled with [^{32}P]phosphate, lysed and subjected to immunoprecipitation with anti-APC antibodies (anti-APC-C-ter). Anti-APC-C-ter immunoprecipitated a ^{32}P-labeled 300 kDa protein which migrated in SDS-PAGE with the same mobility as ^{35}S-labeled APC precipitated from [^{35}S]methionine-labeled HOS cells (Fig. 1a). Precipitation of this protein was prevented by preincubation of the antibodies with the antigenic peptide (Fig. 1a, lanes 2 and 4). Thus, APC was found to be phosphorylated in HOS cells. Phosphoamino acid analysis revealed that APC is phosphorylated mainly at serine and threonine residues (Fig. 1b).

We next studied the expression and phosphorylation of APC through the cell cycle. HOS cells arrested at the metaphase by nocodazole treatment were concentrated by a shake-off procedure and refed with fresh medium to allow progression through mitosis and into the G1 phase. The syn-

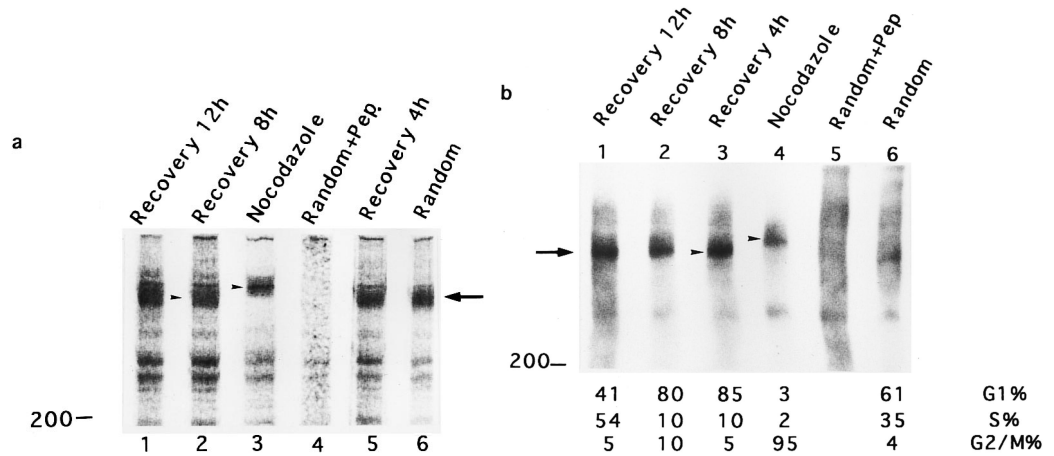

FIG. 2. APC is hyperphosphorylated in nocodazole-arrested HOS cells. HOS cells were arrested at metaphase by nocodazole treatment for 12h and then refed with fresh medium lacking nocodazole. Cells were labeled with [^{35}S]methionine (a) or [^{32}P]phosphate (b) for 2 h before harvesting at the times indicated and subjected to immunoprecipitation with anti-APC-C-ter. The cell cycle profile was determined by FACS analysis of cellular DNA content. The relative percentages of G1, S, and G2 or M cells are listed at the bottom (b). Arrows and arrowheads indicate APC.

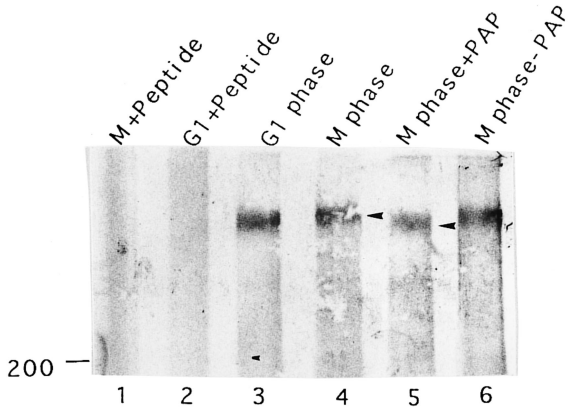

FIG. 3. Phosphatase treatment of APC. HOS cells arrested at metaphase and those which had progressed into the G1 phase (4 h after withdrawal of nocodazole) were collected and subjected to immunoprecipitation with anti-APC-C-ter. The immunoprecipitates were incubated in the presence (+PAP, lane 5) or absence (−PAP, lane 6) of potato acid phosphatase. The immunoprecipitates were then subjected to Western blotting analysis with anti-APC-C-ter. Arrowheads indicate APC.

chrony of the cycling populations was confirmed by flow cytometric analysis as shown in Fig. 2. Cells were labeled with [^{35}S]methionine or [^{32}P]phosphate for 2 h before harvesting at the times indicated in Figs. 2A and B, and subjected to immunoprecipitation with anti-APC-C-ter. The amount of APC did not change markedly during cell cycle progression, but the band of APC that was immunoprecipitated from nocodazole-arrested cells migrated more slowly than those from cells which had progressed into the G1 and S phase. Even 2 h after withdrawal of nocodazole, retarded migration of APC was no longer observed (data not shown). When the immunoprecipitate prepared from nocodazole-arrested cells was treated with potato acid phosphatase, the APC band migrated with a mobility similar to those from cells in the G1 and S phases (Fig. 3). These results suggest that APC is transiently hyperphosphorylated in the M phase.

The results presented in this report suggest that the function of APC is regulated by phosphorylation in the M phase. While APC is thought to play a role in the regulation of the G0/G1 to S transition, nothing is known about its function in the M phase. In this regard, it is interesting to note that APC possesses consensus sequences for phosphorylation by CDC2 kinase, a master key enzyme of the M phase. Although the subcellular locations of APC and CDC2 kinase are normally different, the two proteins may be able to interact in the M phase owing to nucleolar disassembly. In particular, most of the phosphorylation sites for CDC2 kinase are located in the basic region of APC which was reported to be responsible for its interaction with microtubules. Thus, it may be possible that phosphorylation of the basic region of APC may block its interaction with microtubules, as has been reported for the interaction between microtubules and microtubule-associated proteins (17, 18). Recently we also found that APC is a good substrate for glycogen synthase kinase-3 (GSK-3) *in vitro* (our unpublished observation). The *Drosophila* homologue of GSK-3, Zeste-White-3, is known to be involved in the Wingless pathway, a pathway in which the *Drosophila* homologue of β-catenin, Armadillo, is also involved (19, 20). It is therefore interesting to speculate that GSK-3 may phosphorylate APC in vivo. Identification of the APC kinase and elucidation of the physiological significance of APC phosphorylation may provide us new insights into the understanding of the function of APC in the cell cycle.

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