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主論文

MCC, a Cytoplasmic Protein That Blocks Cell Cycle Progression from the G0/G1 to S Phase

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ABSTRACT

The MCC gene was isolated from the human chromosome 5q21 by positional cloning and was found to be mutated in several colorectal tumors. In this study, we prepared specific antibodies and detected the MCC gene product as a cytoplasmic 100 kDa phosphoprotein in mouse NIH3T3 cells. Immunoelectron microscopic analysis showed that the MCC protein is associated with the plasma membrane and membrane organelles in mouse intestinal epithelial cells and neuronal cells. The amount of the MCC protein remained constant during the cell cycle progression of NIH3T3 cells, while its phosphorylation state changed markedly in a cell cycle-dependent manner, being weakly phosphorylated in the G0/G1 and highly phosphorylated during the G1 to S transition. Overexpression of the MCC protein blocked the serum-induced cell cycle transition from the G1 to S phase, whereas a mutant MCC, initially identified in a colorectal tumor, did not exhibit this activity. These results suggest that the MCC protein may play a role in the signaling pathway negatively regulating cell cycle progression.

INTRODUCTION

Cell proliferation is controlled by both positive and negative regulators. A number of genes encoding positive regulators have been identified as oncogenes (1). Most of the oncogene products are deregulated forms of cellular proteins that participate in the signal transduction cascade from the cell membrane receptors to the nucleus. On the other hand, several genes encoding negative regulators have been identified as tumor suppressor genes. Like the oncogene products, the tumor suppressor gene products include proteins localized in the plasma membrane, cytoplasm and nucleus (2). The best studied tumor suppressor gene products, pRB, p53 and WT1, are nuclear transcription factors, while DCC (deleted in colorectal cancer) has the attributes of a cell surface receptor. NF (neurofibromatosis) 1 and 2 share structural similarities with GAP and the proteins of ERM family (3-5), respectively, presumably functioning at the cytoplasmic face of the plasma membrane. In contrast to these proteins, the predicted amino acid sequences of MCC (mutated in colorectal cancer) and APC (adenomatous polyposis coli) are unique and have little sequence similarity to other proteins, providing few clues to its mechanism of action (6-8).

The *MCC* gene was isolated from the human chromosome 5q21 by positional cloning and was found to be mutated in several colorectal tumors (6). In addition, this gene was first suggested to be implicated in the development of familial adenomatous polyposis (FAP). However, further studies revealed that inactivation of another gene isolated from 5q21, *APC*, is responsible for the genesis of FAP (7-10). Moreover, *APC* has been found to be somatically mutated in the majority of sporadic colorectal tumors (11,12).

The *MCC* and *APC* genes are predicted to encode proteins of 829 and 2843 amino acids, respectively, with little homology to other known proteins (6-8). Interestingly, both proteins contain several regions that have a high probability of forming coiled-coil structures. Indeed, the APC protein has been shown to form a stable homodimer via the amino-terminal part of the molecule (13,14). Cell fractionation experiments and immunohistochemical analysis suggested that APC is present as insoluble aggregates in the cytoplasm (15). Furthermore, it has recently been reported that APC is associated with an adherence junction protein β-catenin, suggesting that APC is involved in cell adhesion (16,17). On the other hand, the function of the MCC protein is still unknown. In the present study, we identified and characterized the product of the MCC gene. We found that the MCC gene product is a 100 kDa phosphoprotein localized in the cytoplasm. Furthermore, we show that this protein has the potential to negatively regulate the cell cycle transition from the G1 to S phase.

EXPERIMENTAL PROCEDURES

Antibodies. Anti-MCC antibodies were prepared by immunizing rabbits with synthetic peptides representing amino acid residues 817 to 829. Specific antibodies were purified by affinity chromatography using a column to which the synthetic peptide had been linked. Polyclonal antibodies against β-galactosidase were obtained from Cappel. Monoclonal anti-BrdU antibody was from Takara (Tokyo).

Plasmid construction and Transfection. The entire coding region of the *MCC* cDNA was subcloned into the vector pME18S carrying the SRα promoter, and then transfected into COS-7 cells transiently by use of Lipofectin (GIBCO, BRL). cDNAs encoding the full-length human β-galactosidase were also cloned into the pME18S vector. A mutant *MCC* encoding Gln in place of Arg-506 was generated by site-directed mutagenesis (18) using the synthetic oligonucleotide 5'-CATGACTGCCAGAAGACAGCT-3'. Another mutant encoding Thr and Ala in place of Lys-233 and Glu-234, respectively, was generated using the synthetic oligonucleotide 5'-CGGTGGGAGACGGCGCTGGCTGGG-3'.

Cell Culture. NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. For serum starvation, cells were left for 48 hr in DMEM containing 0.5% calf serum. Under these conditions, incubation with 50 μM BrdU for an additional 24 hr resulted in labeling of less than 5% of the nuclei. For synchronous induction of the growth cycle, fresh medium containing 10% calf serum with 50 μM BrdU was added to serum-starved cells. During cell cycle experiments, the cell cycle was monitored by flow cytometric analysis with a FACScan/CellIIFIT DNA System (Becton

Dickinson). SV40-transformed CV-1 (COS-7) cells were cultured in DMEM supplemented with 10% fetal calf serum.

In vitro Transcription and Translation. The full length *M C C* cDNA was subcloned into pBluescript II (Stratagene). ^{35}S-labeled MCC protein was synthesized by *in vitro* transcription and translation in the presence of [^{35}S]methionine (Amersham) using the TNT™ Coupled Reticulocyte Lysate System (Promega).

Immunoprecipitation. Cells were labeled for 4 h in methionine-free DMEM containing [^{35}S]methionine (100 μCi/ml, 1200 Ci/mmol, NEN). For ^{32}P-labeling, cells were labeled for 2 h in phosphate-free DMEM containing [^{32}P]orthophosphate (200 μCi/ml, Amersham). The labeled cells were lysed in solubilization buffer containing 10 mM Tris-HCl, pH 7.4, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA and 10 μg/ml of aprotinin (SIGMA). Anti-MCC antibodies were added to the labeled cell lysates or *in vitro* translation products and incubated for 1 h at 4°C. The immune complexes were adsorbed to protein A-Sepharose, washed extensively with lysis buffer, and then analyzed by SDS-polyacrylamide gel (8%) electrophoresis followed by fluorography or autoradiography.

V8 protease mapping. MCC was excised from SDS-polyacrylamide gels and homogenized in 100 μl of buffer A (125 mM Tris-HCl pH 6.8, 1 mM 2-mercaptoethanol, 1 mM EDTA, 0.1% SDS, 10% glycerol). Forty μl samples of the homogenates were added to the wells of a 15% polyacrylamide gel and proteins were digested with *Staphylococcus aureus* V8 protease and then separated by electrophoresis (19).

Western blotting analysis. The cell lysates were resolved on an 8% SDS-polyacrylamide gel and transferred to a poly(vinylidene difluoride) membrane filter (Immobilon-P, Millipore). The filters were incubated with the anti-MCC antibodies and subsequently with alkaline phosphatase-conjugated mouse anti-rabbit IgG (Promega). Alkaline phosphatase activity was developed according to the manufacturer's specifications.

Subcellular fractionation. NIH3T3 cells (2 x 10^7) were suspended in 2 ml of KMP buffer [5 mM KCl, 1 mM $MgCl_2$, 20 mM PIPES (1,4-piperazine diethanesulfonic acid), pH 7.0]. The cells were then allowed to swell for 10 min at 4°C, and disrupted with 20 strokes in a Potter-Elvehjem homogenizer (WHEATON). The nuclei were pelleted by centrifugation at 1000 x g for 5 min. The nuclear pellet and the supernatant were then processed separately. The nuclear pellet was resuspended in 2 ml of KMP buffer containing 0.1 % Triton X-100 and centrifuged at 1000 x g for 5 min. The pellet was resuspended in 20 mM Tris-HCl pH 7.5, 150 mM NaCl and 1% SDS, boiled for 5 min and then centrifuged at 15,000 x g for 10 min. The supernatant obtained was used as the nuclear extract. The supernatant obtained from the first centrifugation was overlaid on a 2 ml cushion of KMP containing 50% sucrose and centrifuged at 15,000 x g for 40 min. The supernatant and interface were further centrifuged in TL 100.3 rotor (Beckman) at 40,000 rpm for 60 min and the resulting supernatant was used as the cytosol fraction. The pellet was resuspended in solubilization buffer and used as the crude membrane fraction. The purity of each fraction was monitored by measuring the activities of the membrane marker alkaline phosphodiesterase I and cytosolic marker lactate

dehydrogenase (20). The presence of the nuclei was detected by staining with DAPI (4', 6-diamidino-2-phenylindole).

Immunofluorescence Analysis. Frozen sections of intestine and cerebellum of normal male ddY mice aged 4 weeks were prepared according to the method of Senda et al. (21). The sections were incubated at 4°C overnight with anti-MCC antibodies diluted 1:50 in PBS followed by FITC-conjugated goat anti-rabbit IgG antibodies diluted 1:100 in PBS at room temperature for 1 h, and then examined with a Nikon Fluorescence microscope. Control sections were incubated with normal rabbit serum instead of the anti-MCC antibodies.

Immunoelectron microscopy. Immunoelectron microscopic localization of the MCC protein in mouse intestinal epithelium and cerebellar cortex was determined by the post-embedding technique using colloidal gold-conjugated second antibodies. Lowicryl K4M ultrathin sections were prepared according to the method of Senda et al. (21). The Lowicryl sections were incubated at 4°C overnight with anti-MCC antibodies diluted 1:50 with PBS followed by 10 nm-colloidal gold-conjugated goat anti-rabbit IgG antibodies (Amersham) diluted 1:30 with PBS at room temperature for 1 h. Subsequently, the sections were stained with uranyl acetate and lead citrate, and analyzed with a JEOL 1200 EX transmission electron microscope. Control sections were incubated with normal rabbit serum instead of the MCC antibodies.

Microinjection. NIH 3T3 cells, which were grown on coverslips (2×10^5 cells/dish), were cultured in DMEM containing 0.5% calf serum for 24 h and then microinjected with the normal or mutated *M C C* expression plasmid or β-galactosidase expression plasmid (300 μg/ml

DNA). After incubating for 24 h in the same conditions, medium was replaced with fresh DMEM containing 10% calf serum and 50 μM BrdU, and then cells were incubated for another 18 h. Cells were fixed in 3.7% formaldehyde in PBS for 30 min, dehydrated with 100% methanol for 10 min and then treated with 4 N HCl for 10 min. MCC and β-galactosidase were detected with the affinity-purified antibodies followed by FITC-conjugated goat anti-rabbit IgG antibodies. BrdU was detected with an anti-BrdU monoclonal antibody BU-4 followed by RITC-conjugated goat anti-mouse IgG antibodies. To arrest the cells at G1/S, aphidicolin (50 ng/ml) was added 2 h after serum addition. The *MCC* expression plasmid was microinjected 8 h after addition of aphidicolin and BrdU was added after incubation for another 3 h.

RESULTS

Identification of MCC in vivo. For the identification of the *MCC* gene product, we prepared antibodies against MCC by immunizing rabbits with a synthetic peptide corresponding to amino acid residues 817 to 829 of the predicted protein. The antibodies recognized [^{35}S]methionine-labeled MCC generated by *in vitro* transcription and translation of the *MCC* cDNA. The size of the synthesized protein was about 100 kDa, consistent with the calculated molecular weight of MCC (Fig. 1A, lanes 1-3). From [^{35}S]methionine- and [^{32}P]phosphate-labeled extracts of mouse fibroblast NIH3T3, the anti-MCC antibodies also immunoprecipitated a 100 kDa protein and precipitation of this protein was prevented by preincubation of the antibodies with an excess amount of the synthetic peptide used for immunization (Fig. 1A, lanes 7-12). To confirm that the 100 kDa protein immunoprecipitated from NIH3T3 cells is the MCC protein, we compared a partial proteolytic digestion of this protein with that obtained using in vitro translated MCC. As shown in Fig. 1B, the peptide mapping patterns of these proteins were very similar, suggesting that the 100 kDa protein detected in NIH3T3 cells is indeed the MCC protein. In addition, when similar experiments were performed with COS-7 cells transfected with the *MCC* cDNA expression plasmid, a large amount of the 100 kDa protein was detected in [^{35}S]methionine-labeled extracts, whereas this protein was barely detected in the parental COS-7 cells (Fig. 1A, lanes 4-6). Furthermore, MCC was detected as a band of 100 kDa, accompanied with additional slowly migrating faint bands, by Western blotting analysis (Fig. 4B). These results suggest that the 100 kDa protein identified by the anti-MCC antibodies is the *MCC* gene product and this protein is phosphorylated in living cells.

Subcellular Localization of MCC. To elucidate the subcellular localization of MCC, NIH3T3 cells labeled with [^{35}S]methionine were separated into nuclear, cytosol and crude membrane fractions. The purity of each fraction was monitored by measuring the activities of the membrane marker enzyme alkaline phosphodiesterase I and cytosolic marker enzyme lactate dehydrogenase and by staining the nuclei with DAPI. The results suggested that cells were properly separated into their subcellular fractions. When lysates of these fractions were subjected to immunoprecipitation with the anti-MCC antibodies, the MCC protein was detected mainly in the cytosol and crude membrane fractions (Fig. 2, lanes 1-8). The MCC localized in the crude membrane fraction could not be completely solubilized by 1% NP40 treatment (Fig. 2, lanes 9-14), suggesting that MCC is not a membrane protein but is complexed with an insoluble aggregate. MCC in this fraction also could not be completely extracted with 0.5 M NaCl.

Immunofluorescence and Immunoelectron Microscopic Analyses of MCC Localization. We next tried to further define the subcellular localization of MCC by immunofluorescence and electron microscopic analyses. However, the signals obtained using NIH3T3 cells stained with anti-MCC antibodies were weak, although strong cytoplasmic staining was observed in the same cells transfected with the *MCC*-expression plasmid (data not shown). Hence, we looked for mouse tissues expressing high levels of endogenous MCC and found that MCC is expressed in the intestinal epithelium and cerebellar cortex at a level detectable by immunofluorescence staining.

In the mouse intestinal epithelium, MCC was localized mainly along lateral cell borders of the epithelial cells (Fig. 3A). The cytoplasm of

the epithelial cell showed weak immunoreactivity for MCC, whereas the apical brush border and nuclei showed no significant labeling. Immunoelectron microscopic analysis using colloidal gold-conjugated second antibodies clearly demonstrated a close association of MCC with lateral plasma membranes of the intestinal epithelial cells (Fig. 3B). Some gold particles were scattered in the cytoplasmic matrix. By contrast, control sections stained with control rabbit serum showed no immunoreactivity either in the immunofluorescence (Fig. 3C) or immunoelectron microscopic (Fig. 3D) analyses.

In the mouse cerebellar cortex, the molecular layer showed high MCC expression (Fig. 3E). Purkinje cells and granular layer cells were also immunoreactive for the anti-MCC antibodies. Immunoelectron microscopic analysis showed that MCC is mainly associated with the plasma membrane and membrane organelles in the neuronal cell bodies and nerve fibers in the molecular layer and granular layer cells (Fig. 3F). Some gold particles were also distributed within the cytoplasmic matrix of the neuronal cells, whereas no MCC was detected in the nuclei of the cerebellar cortex. Sections stained with control rabbit serum showed no immunoreactivity either in the immunofluorescence (Fig. 3G) or immunoelectron microscopic (Fig. 3H) analyses.

Expression Levels and Phosphorylation of MCC during the Cell Cycle Progression. We next studied the expression and phosphorylation of MCC through the cell cycle. NIH3T3 cells were arrested in the G0 phase by incubation in medium containing 0.5% calf serum for 48 h and were then induced to proliferate by adding fresh medium supplemented with 10% calf serum. The synchrony of the cycling populations was confirmed by flow cytometric analysis as

shown in Fig. 4A. At the times indicated in Fig. 4B, the level of MCC was examined by Western blotting analysis with anti-MCC antibodies. The amount of MCC did not change markedly during cell cycle progression. In a parallel experiment, the phosphorylation state of MCC was examined by immunoprecipitating MCC from cells labeled with [^{32}P]phosphate (Fig. 4C). Phosphorylation of MCC was barely detectable in NIH3T3 cells arrested at G0, but was induced 6-8 h following restoration of serum.

The Effect of MCC Expression on Cell Cycle Progression. To assess the ability of MCC to regulate cell cycle progression, we performed a microinjection experiment. NIH 3T3 cells cultured in medium containing 0.5% calf serum for 24 h were microinjected with the *MCC* cDNA and further cultured under the same conditions for another 24 h. Fresh medium supplemented with 10% calf serum was then added to induce synchronous cell cycle progression, and BrdU was added to measure DNA synthesis. After an additional incubation for 18 h, cells were fixed and processed for immunofluorescence analysis; microinjected cells were identified by staining for the MCC protein and cells that had entered S phase were identified by staining for BrdU incorporation. As summarized in Fig. 5, incorporation of BrdU into cells injected with the *MCC* expression plasmid was inhibited, whereas microinjection of the β-galactosidase expression plasmid showed no such inhibitory effect. This effect was not observed when the *MCC* cDNA was microinjected into cells that had been arrested at G1/S by aphidicolin treatment. In contrast to normal *MCC*, a mutant *MCC* cDNA encoding Gln in place of Arg-506 (MCC-R506Q), originally identified in one case of colorectal cancer (6), barely inhibited cell cycle progression from the G0/G1 to S phase. Since MCC contains a small region (amino

acids 220-243) that possesses an amino acid similarity to the G protein-coupled m3 muscarinic acetylcholine receptor (6), we examined the effect of a mutant *MCC* cDNA encoding Thr and Ala in place of Lys-233 and Glu-234, respectively. As shown in Fig. 5, this mutant also failed to block cell cycle progression. These results suggest that overexpression of MCC specifically blocks cell cycle progression through the G1 phase.

DISCUSSION

In the present study, we detected the *MCC* gene product as a 100 kDa phosphoprotein localized in the cytoplasm. This finding is consistent with the expected characteristics of the predicted amino acid sequence of the MCC protein; i.e. MCC consists of 829 amino acids and does not have any obvious membrane-spanning region or nuclear localization signals (6). In several experiments, slowly migrating proteins were detected in addition to the main band of 100 kDa. It remains to be determined whether these are generated by modification or splicing, or if these are related but different proteins.

Cell fractionation experiments showed that most of the MCC protein is present in the cytosol, i.e. the 100,000 x g supernatant fraction, but a proportion of MCC was also detected in the crude membrane fraction. Immunoelectron microscopic analysis demonstrated that MCC is associated with the plasma membrane and membrane organelles in the mouse intestinal epithelial cells and neuronal cells. However, treatment of the insoluble crude membrane fraction with 1% NP40 did not solubilize MCC, suggesting that MCC is not a membrane protein, but rather is complexed in an insoluble aggregate. This is consistent with the fact that the MCC protein contains heptad repeats throughout almost the entire length of the molecule. These features are similar to those of the APC gene product; APC also possesses heptad repeats in the amino-terminal region and is present in an insoluble aggregate (15). Importantly, APC has been suggested to form a parallel, helical homodimer, as expected for a coiled coil (13). In addition, the wild type APC associates with truncated mutant APC proteins in colorectal cancer cells (14, 15). However, our preliminary experiments failed to detect homodimerization of MCC. Also, MCC did not heterodimerize with APC.

While the amount of MCC was constant during cell cycle progression, its phosphorylation state changed markedly in a cell cycle-dependent manner, being weakly phosphorylated in G0/G1 and highly phosphorylated during the G1 to S transition. These findings suggest that the function of MCC is regulated by phosphorylation, similar to the case of pRB whose function is inhibited by phosphorylation during the G1 to S phase transition (22-29). Although MCC possesses several consensus sequences for phosphorylation by protein kinase C and casein kinase II, identification of the responsible kinases remains to be achieved.

Our microinjection experiments showed that overexpression of MCC blocks serum-induced cell cycle progression from the G1 to S phase. Thus, MCC may play a role in the signaling pathway negatively regulating cell cycle progression. In this regard, it is interesting that the *MCC* gene was previously found to be mutated in several colorectal tumors (6), although mutation of the *APC* gene is far more frequent. In addition, it has been reported that the *MCC* loci frequently undergoes a loss of heterozygosity in esophageal and lung cancers and that this occurs without significant correlation to alterations in *APC* (30, 31). Intriguingly, a mutant version of MCC (MCC-R506Q), corresponding to that identified in a colorectal tumor (6), did not exhibit any cell cycle-blocking activity, suggesting the biological importance of this MCC mutation. Additionally, MCC-K233T-E234A, which was engineered to have mutations in a region possessing an amino acid similarity to the G protein-coupled m3 muscarinic acetylcholine receptor (6), also failed to induce cell cycle arrest. This finding suggests that this region of MCC may play an important role in regulating cell cycle progression.

Among the known tumor suppressor gene products, p53 (32, 33), pRB (34, 35) and WT1 (36, 37) have been shown to have a potential to

negatively regulate cell proliferation. These are all nuclear proteins and are believed to regulate the expression of the genes whose products are important for negative growth control. Recently, one of the important targets of p53 has been identified as the gene encoding the CDK inhibitor p21 (38-42). In addition to these tumor suppressor gene products, several different classes of negative regulators of cell growth have also been reported, including secreted proteins such as the transforming growth factor betas (reviewed in reference 43), a growth arrest-specific membrane protein Gas1 (44), Prohibitin (45) and a ras-related transformation suppressor gene product K-Rev-1/Rap 1 (46). Compared to these negative regulators, MCC is unique in its structure and subcellular localization. Additionally, MCC is highly expressed in differentiated murine tissues (our unpublished observation) such as neuronal cells (Fig. 3), suggesting that MCC may play a role besides cell cycle regulation. Thus, detailed analysis of the function of MCC may give new insight into the mechanism of the regulation of cell growth and differentiation.

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LEGENDS TO FIGURES

FIG 1. Identification of MCC. (A) Lanes 1-3, *in vitro* translation of *MCC*. A plasmid vector containing the *MCC* cDNA downstream of the T7 promoter was transcribed and translated *in vitro* in the presence of [^{35}S]methionine as described under the "Experimental Procedures". *In vitro* translation products of *MCC* are shown in lane 1. MCC was then immunoprecipitated with anti-MCC antibodies (lane 2) or anti-MCC antibodies that had been preadsorbed with an excess of peptide antigen (lane 3). Lanes 4-12, identification of MCC *in vivo*. COS-7 cells transfected with the expression vector alone (pME18S vector) (lane 4) or *MCC* expression plasmid (pME18S-MCC) (lanes 5 and 6) were labeled with [^{35}S]methionine for 4 h. NIH3T3 cells (lanes 7-12) were also labeled with [^{35}S]methionine (lanes 7-10) or [^{32}P]phosphate (lanes 11 and 12) for 4 h. MCC was immunoprecipitated from cell lysates with anti-MCC antibodies. Lanes 6, 8, 10, and 12, anti-MCC antibodies were preadsorbed by an excess of the antigenic peptide. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. (B) Partial peptide mapping analysis of MCC. MCC which had been immunoprecipitated from [^{35}S]methionine-labeled NIH3T3 cells (lanes 1-3) and that generated by *in vitro* translation (lanes 4-6) were excised from the gel, partially digested with V8 protease (lanes 1 and 4, 2 μg/ml; lanes 2 and 5, 20 μg/ml; lanes 3 and 6, 200 μg/ml) and analyzed by SDS-polyacrylamide gel (15%) electrophoresis.

FIG. 2. Subcellular localization of MCC. NIH 3T3 cells labeled with [^{35}S]methionine (lanes 1 and 2) were separated into crude membrane (lanes 3 and 4), cytoplasmic (lanes 5 and 6) and nuclear

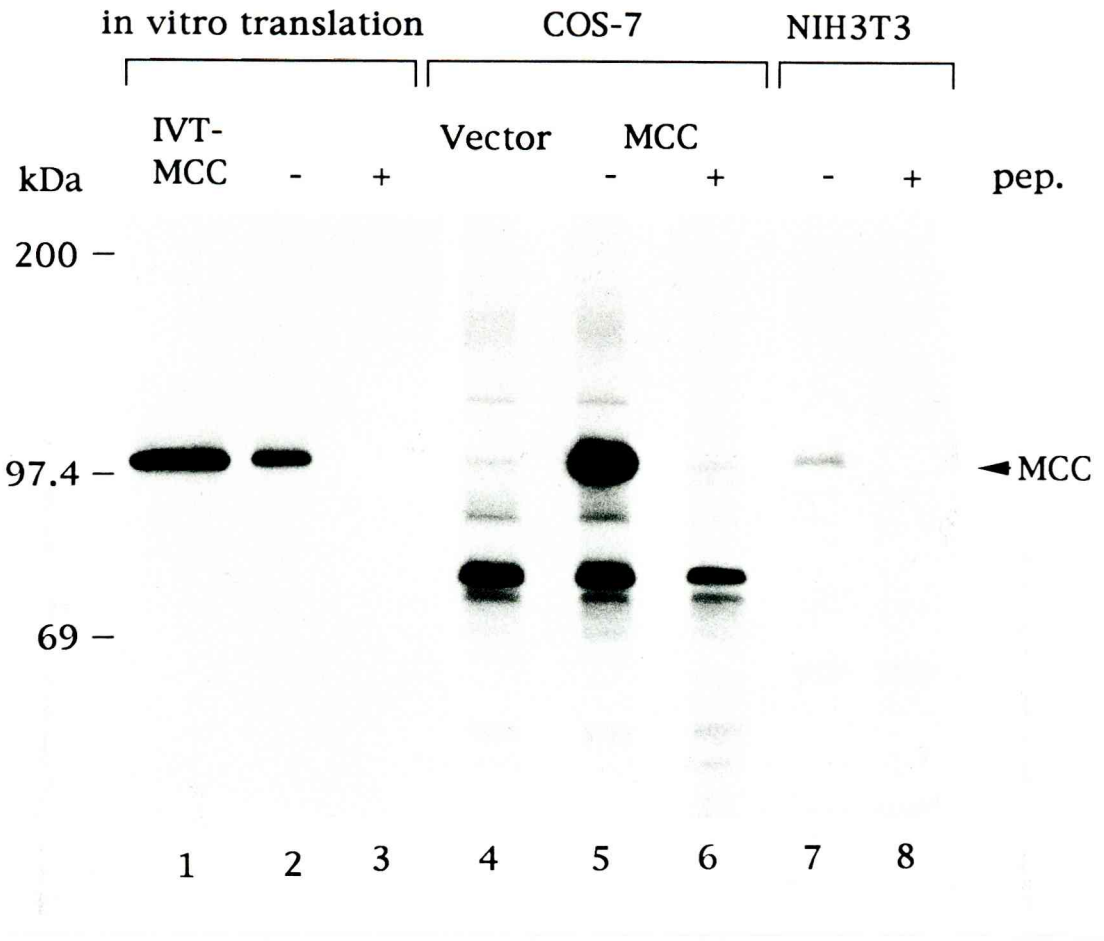
(lanes 7 and 8) fractions as described under the "Experimental Procedures", and lysates of these fractions were subjected to immunoprecipitation with anti-MCC antibodies. The crude membrane fraction derived from subcellular fractionation was resuspended in 0.1% SDS (lanes 9 and 10), 1% NP-40 (lanes 11 and 12), or 0.5 M NaCl (lanes 13 and 14). After 30 min on ice, the insoluble material was pelleted by centrifugation, and the supernatants (lanes 9, 11 and 13) and pellets (lanes 10, 12 and 14) were subjected to immunoprecipitation with anti-MCC antibodies.

FIG. 3. Localization of MCC in the mouse intestinal epithelium (A-D) and cerebellar cortex (E-H). (A, C, E and G) Immunofluorescence microscopic analyses. (B, D, F and H) Immunoelectron microscopic analyses using colloidal gold-conjugated secondary antibodies. (A) Lateral cell borders of the intestinal epithelial cells showed intense immunoreactivity for MCC. The cytoplasm of the epithelial cell is weakly positive. (B) A number of gold particles were associated with the interdigitated lateral plasma membranes of the epithelial cells. Some gold particles were scattered in the cytoplasmic matrix. (E) MCC is highly expressed in the molecular layer (m) of the cerebellar cortex. Purkinje cells (arrows) and cells in the granular layer (g) were also immunopositive. (F) Immunogolds were associated with the plasma membrane (arrows) and membrane organelles (arrowheads) of Purkinje cell (p) and nerve fibers (n). (C, D, G and H) As a negative control, normal rabbit serum was used for staining instead of anti-MCC antibodies. Scale bars: 10 μm in A, C, E and G; 100 nm in B, D, F and H.

FIG. 4. Level of expression and phosphorylation of MCC

during cell cycle progression. (A) NIH3T3 cells were growth arrested by culturing in DMEM containing 0.5% calf serum for 48 h, and then stimulated with DMEM containing 10% calf serum to induce synchronous cell cycle progression. At the times indicated after serum stimulation, 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)** NIH3T3 cells synchronized as in (A) were harvested at the indicated times and subjected to Western blotting analysis with anti-MCC antibodies. (C) NIH3T3 cells synchronized as in (A) were labeled with [^{32}P]phosphate for 2 h before harvesting. MCC was immunoprecipitated with anti-MCC antibodies and analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. Randomly growing NIH3T3 cells were also analyzed.

FIG. 5. Effect of overexpression of MCC on cell cycle progression. Serum-starved NIH3T3 cells were microinjected with the *MCC* or β-galactosidase expression plasmid. Cells were re-stimulated with serum and then visualized for MCC, β-galactosidase or BrdU incorporation. The histogram shows the percentage of BrdU-positive NIH3T3 cells expressing the injected normal or mutant MCC cDNA. At least 90 cells were scored for each experiment. MCC-R506Q, the mutant MCC cDNA encoding Gln in place of 506-Arg; MCC-K233T-E234A, the mutant MCC cDNA encoding Thr and Ala in place of Lys-233 and Glu-234, respectively. The MCC cDNA was also microinjected into cells that had been arrested at G1/S by aphidicolin treatment.


in vitro translation
COS-7
NIH3T3
IVT-MCC
Vector
MCC
kDa
-
+
-
+
-
+
pep.
200
97.4
69
MCC
1
2
3
4
5
6
7
8

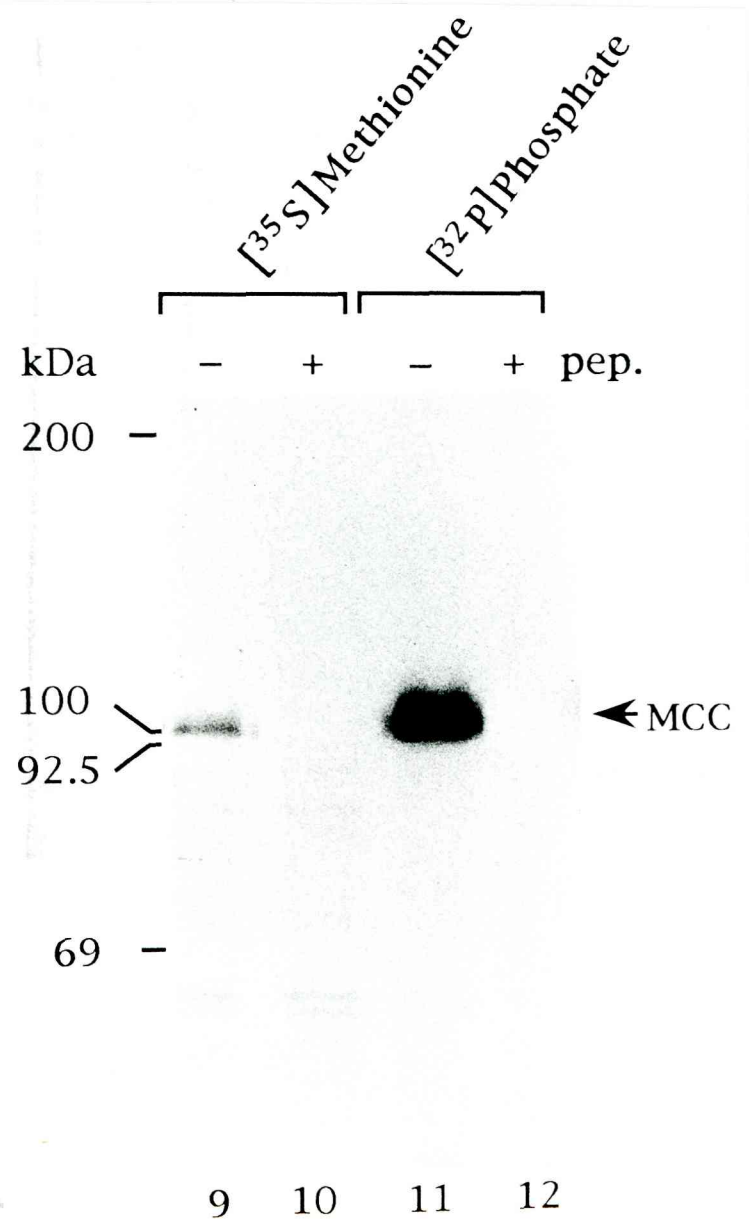

[35S]Methionine
[32P]Phosphate
kDa
–
+
–
+
pep.
200
100
92.5
69
MCC
9
10
11
12

Fig. 1 (A)

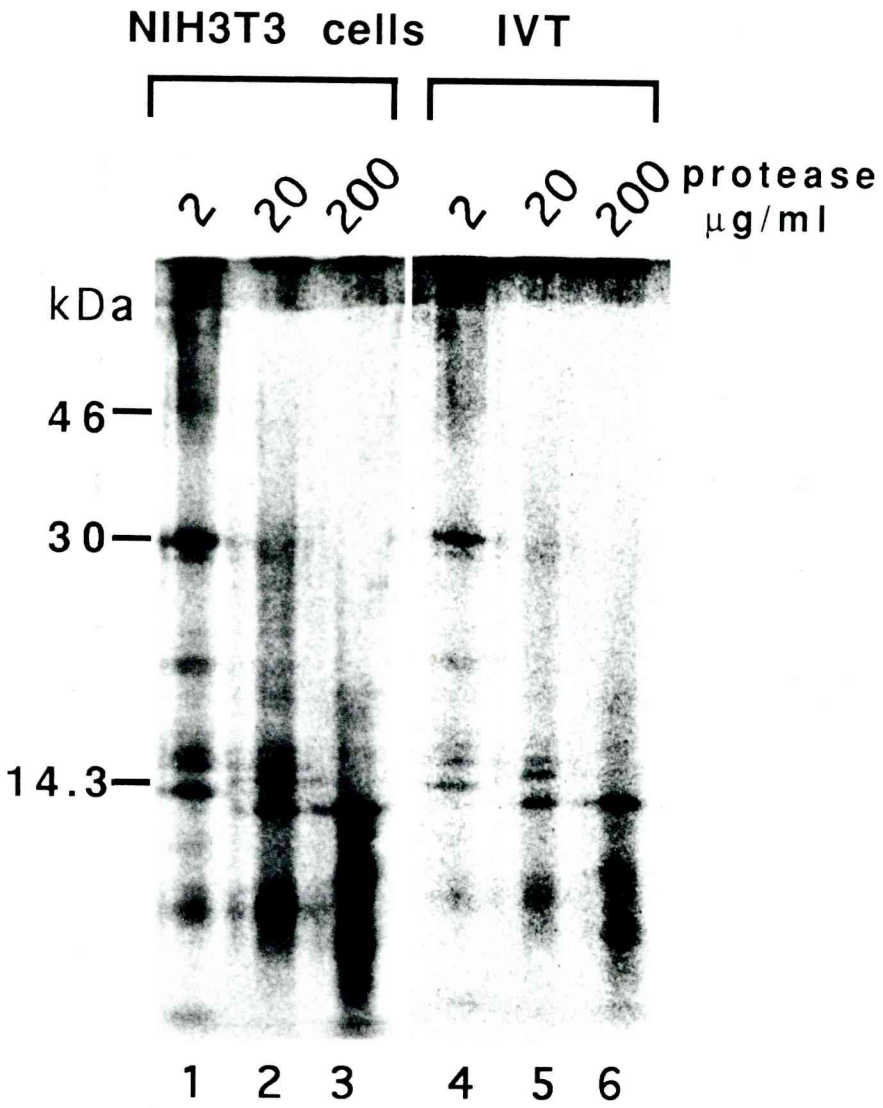
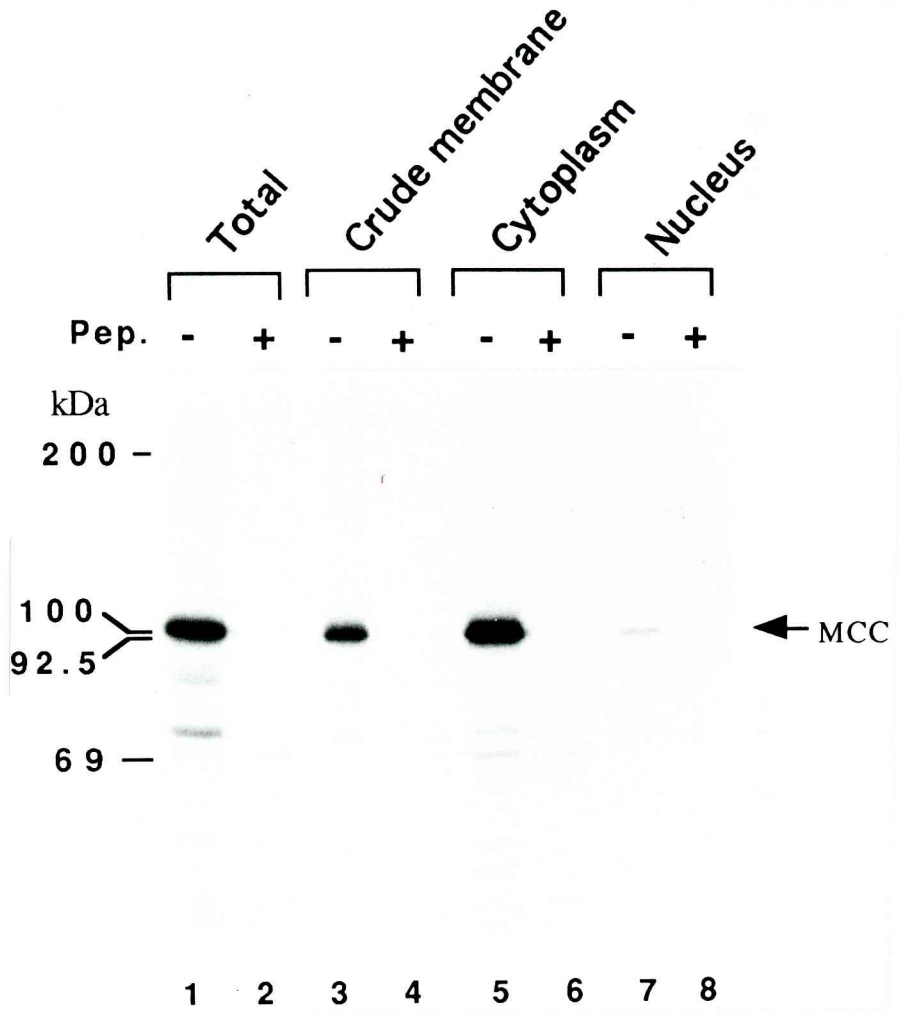

NIH3T3 cells
IVT
2
20
200
2
20
200
protease
μg/ml
kDa
46
30
14.3
1
2
3
4
5
6

Fig. 1 (B)


Total
Crude membrane
Cytoplasm
Nucleus
Pep. - + - + - + - +
kDa
200 –
100
92.5
69 —
MCC
1 2 3 4 5 6 7 8

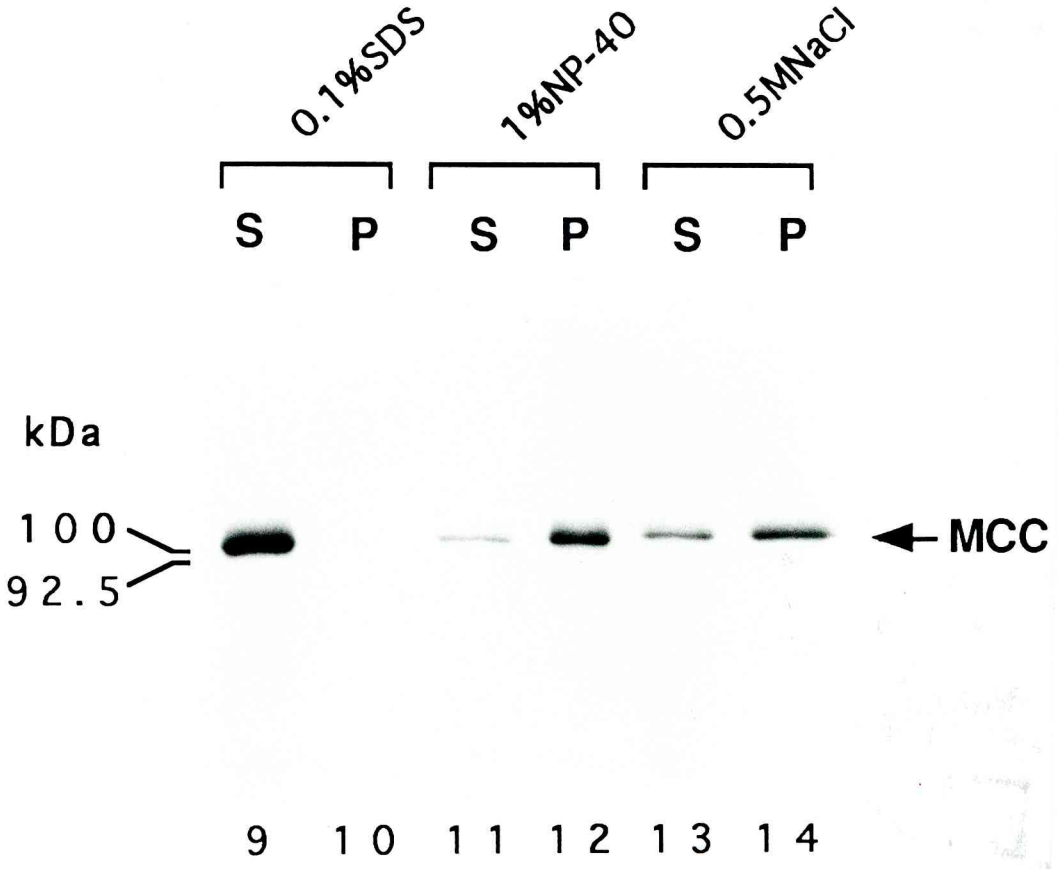
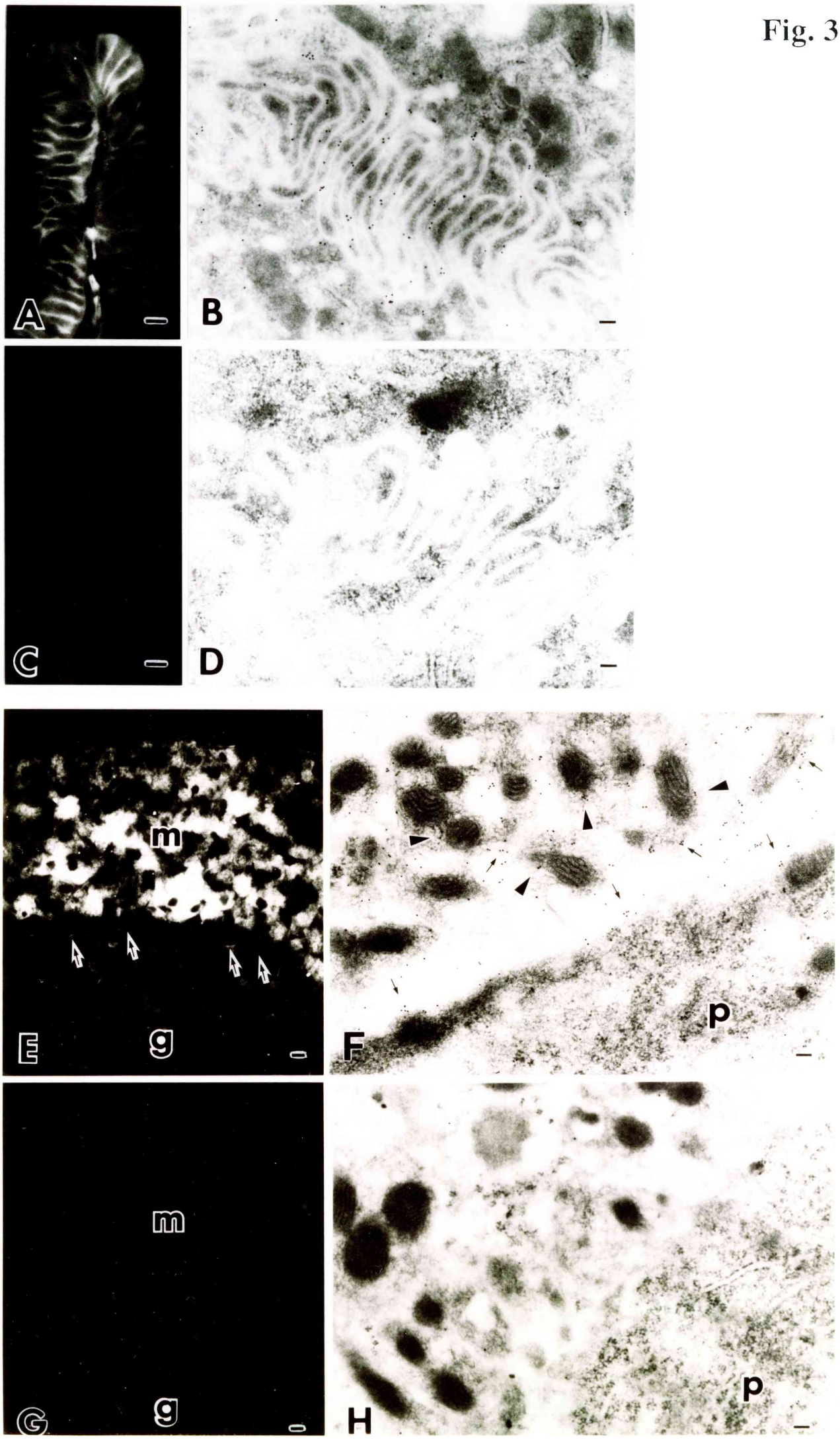
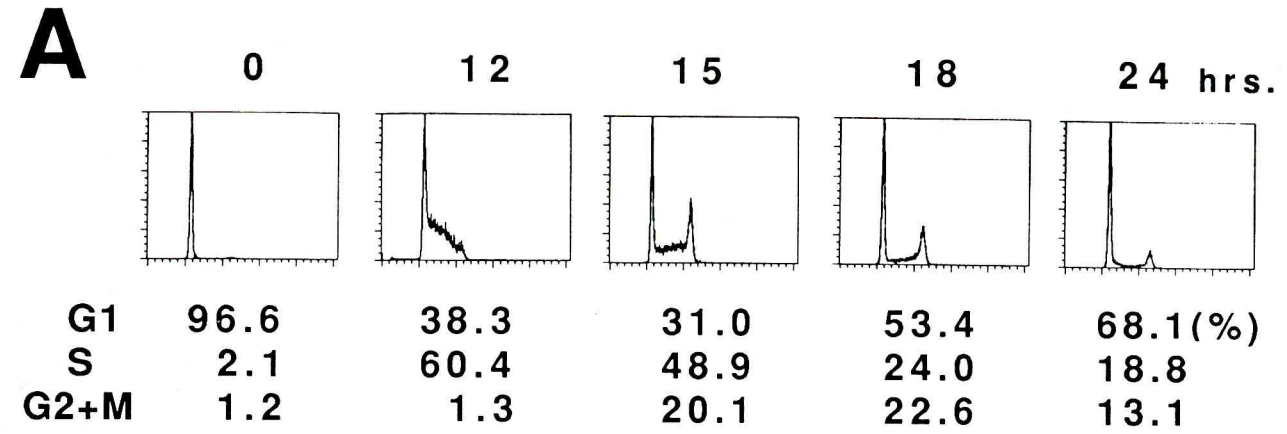
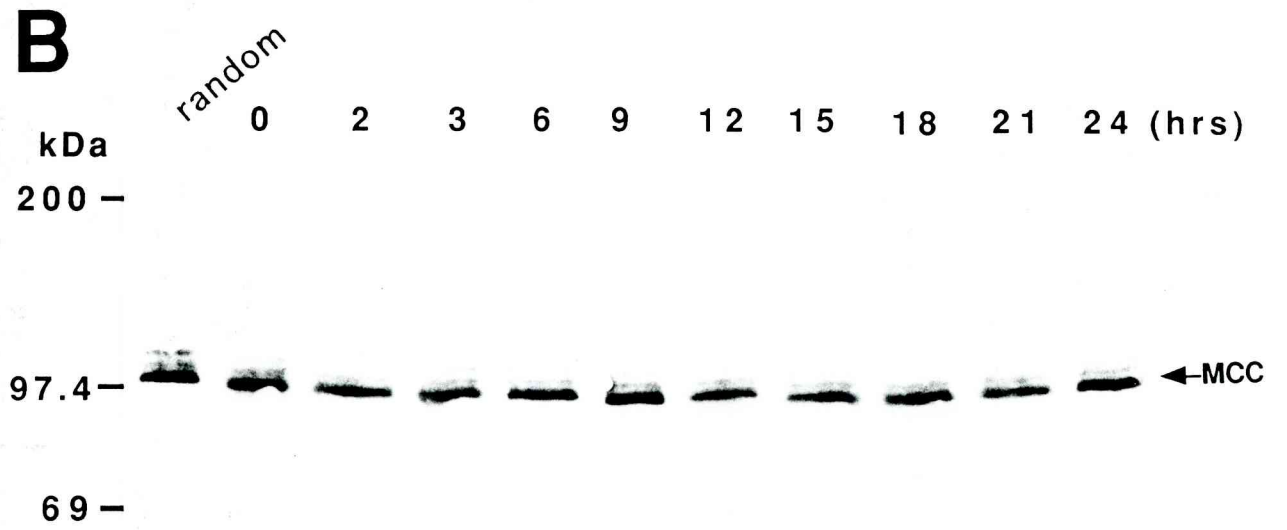

0.1%SDS
1%NP-40
0.5MNaCl
S P S P S P
kDa
100
92.5
MCC
9 10 11 12 13 14

Fig. 2

Fig. 3




A
0
12
15
18
24 hrs.
G1 96.6 38.3 31.0 53.4 68.1(%)
S 2.1 60.4 48.9 24.0 18.8
G2+M 1.2 1.3 20.1 22.6 13.1


B
random
0 2 3 6 9 12 15 18 21 24 (hrs)
kDa
200
97.4
69
MCC

C
random
pep.
- +
0 2 3 6 9 12 15 18 21 24 (hrs)
kDa
200
97.4
69
MCC

Fig. 4

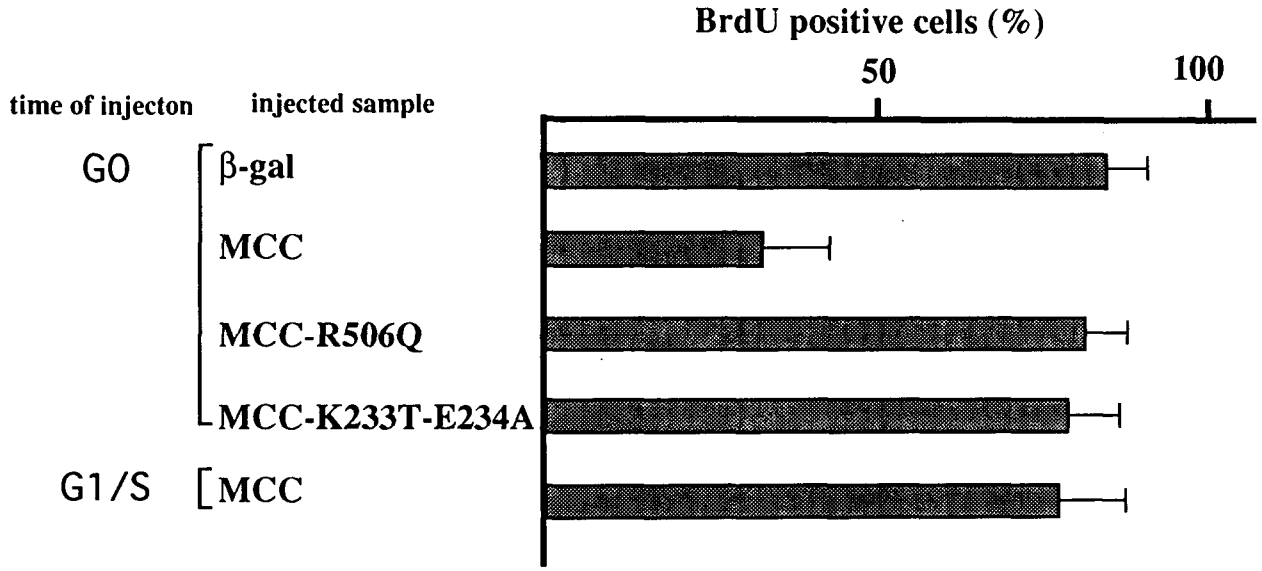

BrdU positive cells (%)
50
100
time of injecton
injected sample
GO
β-gal
MCC
MCC-R506Q
MCC-K233T-E234A
G1/S
MCC

Fig. 5

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The tumour suppressor gene product APC blocks cell cycle progression from G_0/G_1 to S phase

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[5]Corresponding author

The APC gene is mutated in familial adenomatous polyposis (FAP) as well as in sporadic colorectal tumours. The product of the APC gene is a 300 kDa cytoplasmic protein associated with the adherence junction protein catenin. Here we show that overexpression of APC blocks serum-induced cell cycle progression from G_0/G_1 to the S phase. Mutant APCs identified in FAP and/or colorectal tumours were less inhibitory and partially obstructed the activity of the normal APC. The cell-cycle blocking activity of APC was alleviated by the overexpression of cyclin E/CDK2 or cyclin D1/CDK4. Consistent with this result, kinase activity of CDK2 was significantly down-regulated in cells overexpressing APC although its synthesis remained unchanged, while CDK4 activity was barely affected. These results suggest that APC may play a role in the regulation of the cell cycle by negatively modulating the activity of cyclin–CDK complexes.

Keywords: APC/CDK/cell cycle/cyclin/tumour suppressor gene

Introduction

Familial adenomatous polyposis (FAP) is a dominantly inherited disease that results in the development of multiple adenomatous polyps in the colon of affected individuals (Burt and Samowitz, 1988). Some of these benign tumours, if not surgically removed, invariably progress to carcinomas. Additionally, FAP is associated with extracolonic manifestations such as benign soft tissue and bony tumours, dental abnormalities, desmoid tumours and gastric and small intestinal polyps (Gardner and Richards, 1953; Herrera, 1990).

Cytogenetic and linkage studies showed that the human chromosome 5q21 harbours the gene responsible for FAP (Bodmer *et al.*, 1987; Leppert *et al.*, 1987). In this region, the tumour suppressor gene APC was recently isolated by positional cloning and was found to be mutated in most cases of FAP (Groden *et al.*, 1991, 1993; Joslyn *et al.*, 1991; Kinzler *et al.*, 1991; Nishisho *et al.*, 1991; Miyoshi *et al.*, 1992a; Nagase *et al.*, 1992; Olschwang *et al.*, 1993; Varesco *et al.*, 1993). In addition, mice with germline mutations of the APC gene have been reported to manifest a phenotype similar to that of FAP (Su *et al.*, 1992; Fodde *et al.*, 1994). Furthermore, recent studies have shown that the APC gene is also somatically mutated in the majority of sporadic colorectal tumours (Miyoshi *et al.*, 1992b; Powell *et al.*, 1992). Mutation of APC is believed to precede mutations in the *ras*, *p53* and *DCC* genes, the latter which lead to further clonal expansion and progression from the benign to malignant state (Fearon and Vogelstein, 1990; Vogelstein and Kinzler, 1993). Both germline and somatic mutations are almost exclusively either nonsense or frame-shift mutations that result in the truncation of the APC gene product.

The product of the APC gene is a 300 kDa protein consisting of 2843 amino acids (Groden *et al.*, 1991; Kinzler *et al.*, 1991; Smith *et al.*, 1993). Although the APC protein has little sequence similarity to other known proteins, it contains several regions that have a high probability of forming coiled-coil structures. Consistent with this prediction, the APC protein has been shown to form a stable homodimer via the N-terminal part of the molecule, raising the intriguing possibility that the typical C-terminally truncated mutant APC protein might associate with the normal APC protein and inactivate it in a dominant negative manner (Joslyn *et al.* 1993; Su *et al.*, 1993a). Cell fractionation experiments and immunohistochemical analysis have suggested that APC is present as insoluble aggregates in the cytoplasm (Smith *et al.*, 1993). Furthermore, it has been reported that APC is associated with the adherence junction protein catenin, suggesting that APC is involved in cell adhesion (Rubinfeld *et al.*, 1993; Su *et al.*, 1993b; Shibata *et al.*, 1994). More recently, APC has been reported to associate with microtubules in cells overexpressing the exogenously transfected APC gene (Munemitsu *et al.*, 1994; Smith *et al.*, 1994).

In the present study, we investigated the function of the APC protein in the regulation of cell proliferation. We found that APC negatively regulates cell growth by inhibiting cell cycle transition from the G_1 to S phase. Furthermore, we show that the overexpression of certain CDK–cyclin proteins abrogates the cell-cycle blocking activity of APC. Consistent with this result, CDK2-associated kinase activity was found to be down-regulated in cells overexpressing APC.

Results

Expression of the full-length APC gene product in NIH 3T3 cells

To investigate the function of the APC protein in the regulation of cell proliferation, we constructed an APC expression plasmid utilizing a tetracycline-regulable promoter (Gossen and Bujard, 1992) and examined its ability

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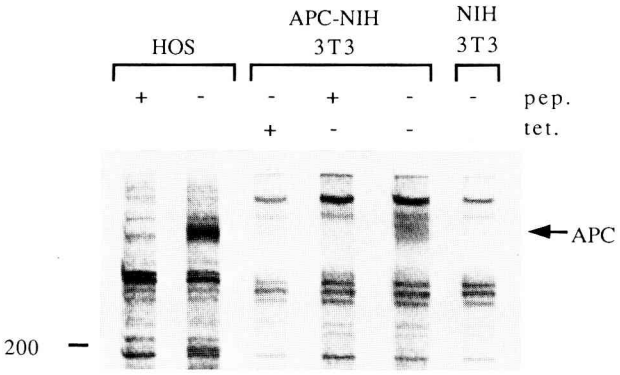

Fig. 1. Transient expression of APC in NIH 3T3 cells. HOS cells (lanes 1 and 2), NIH 3T3 cells transfected with APC cDNA (lanes 3–5) and parental NIH 3T3 cells (lane 6) were labelled with [^{35}S]methionine for 4 h. In lane 3, cells were cultured in the presence of tetracycline (1 μg/ml). APC was immunoprecipitated with anti-APC-C-ter (lanes 2, 3, 5 and 6) or the same antibody pre-absorbed with an excess of antigen used for immunization (lanes 1 and 4). The immunoprecipitates were separated on SDS–PAGE (5%) followed by fluorography. APC is indicated by the arrowhead.

to synthesize the full-length gene product. Antibodies against the C-terminal portion of the human APC protein (referred to as anti-APC-C-ter) detected the ectopically expressed 300 kDa APC protein in NIH 3T3 cells that had been transfected with the APC expression plasmid as well as the endogenously expressed APC protein in human osteosarcoma HOS cells (Figure 1). By contrast, APC was barely detected in both the parental NIH 3T3 cells and the transfectant cultured in the presence of tetracycline, which represses the activity of the promoter utilized in the expression vector. Precipitation of APC was blocked by pre-incubation of the antibodies with the antigenic peptide. In addition, cDNA expression vectors were generated encoding a mutant APC lacking its N-terminal region (amino acids 6–1210) (referred to as APC-ΔN) and mutants in which stop codons replaced Arg302 (APC-302), Ser932 (APC-932) and Gln1338 (APC-1338). These latter mutations recreate those previously identified in FAP and/or sporadic colorectal tumours (Miyoshi *et al.*, 1992a,b; Nagase *et al.*, 1992). When these constructs were transfected into NIH 3T3 cells and expression induced by culture in the absence of tetracycline, proteins of ~40, 100, 150 and 170 kDa size were produced (data not shown) consistent with the calculated molecular weights of APC-302, -932, -1338 and -ΔN, respectively. These results suggest that the normal and mutant APC expression constructs are able to direct the synthesis of the full-length and truncated APC proteins in living cells.

Colony formation assay

Using these expression constructs, we performed a colony formation assay with the monkey kidney fibroblast cell line CV-1. CV-1 cells transfected with the normal APC expression plasmid formed 10-fold fewer geneticin-resistant colonies than those transfected with the control expression vector (Figure 2). APC-1338 showed less of an inhibitory effect on colony formation, while APC-ΔN had no inhibitory activity. These results suggest that normal APC possesses the ability to inhibit cell proliferation.

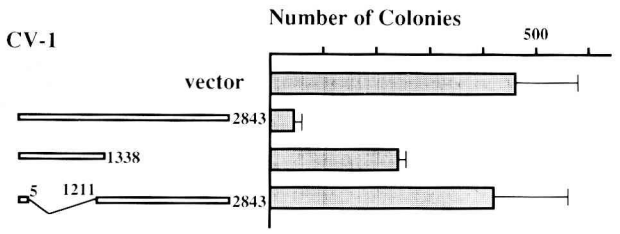

Fig. 2. Effect of APC on colony formation. CV-1 cells were transfected with the indicated plasmids, and cultured in the presence of 600 μg/ml of geneticin for 3 weeks. The histogram shows the number of geneticin-resistant colonies. Schematic representations of the normal and mutant APCs are given on the left side of the histogram. Values are the mean ± SD of three experiments.

APC arrests cells at the G_1 phase

Given the ability of APC negatively to regulate cell growth, we tested whether APC blocks cell cycle progression at a specific point. To examine the effect of APC expression on cell cycle progression from the G_1 to S phase, we micro-injected the APC cDNA into NIH 3T3 cells that had been brought to quiescence by serum starvation. After cells were stimulated with serum to restart synchronous cell cycle progression, micro-injected cells were identified by staining for the APC protein, and cells that entered the S phase were identified by staining for BrdU incorporation. Cells micro-injected with the APC cDNA showed significantly lower BrdU incorporation than those injected with the β-galactosidase cDNA (Figure 3A and B). By contrast, BrdU incorporation was not inhibited when the micro-injected cells were cultured in the presence of tetracycline, which represses the activity of the promoter utilized in the expression vector (Figure 3B, indicated by '+Tet') (Gossen and Bujard, 1992). These results suggest that APC can block the progression of cells from the G_0 to the S phase.

Cell-cycle blocking activity of APC mutants

In a similar assay, the mutant APCs, APC-302, -932 and -1338, showed only weak inhibitory effects, while APC-ΔN barely inhibited cell cycle progression (Figure 3B). Interestingly, C-terminally truncated mutant APCs have been shown to form heterodimers with the normal APC via an N-terminal dimerization domain and it has been suggested that this inactivates the normal APC in a dominant-negative fashion (Smith *et al.*, 1993; Su *et al.*, 1993a). Indeed, cells micro-injected with normal APC along with APC-302, -932 or -1338 showed higher BrdU incorporation than those injected with the normal APC alone (Figure 3B), suggesting that these mutant APCs can inhibit the cell-cycle blocking activity of the normal APC.

Effect of APC at different time points through the G_1 to S phases

We next tried to define more precisely the time point at which APC exerts its inhibitory activity. NIH 3T3 cells were injected with the APC cDNA or β-galactosidase cDNA at various times after addition of serum, and DNA synthesis was scored by measuring BrdU incorporation. Under the conditions used in our experiment, the APC protein synthesized from the injected cDNA was detectable in the cytoplasm from at least 2 h after micro-injection. A time-course experiment with uninjected cells showed

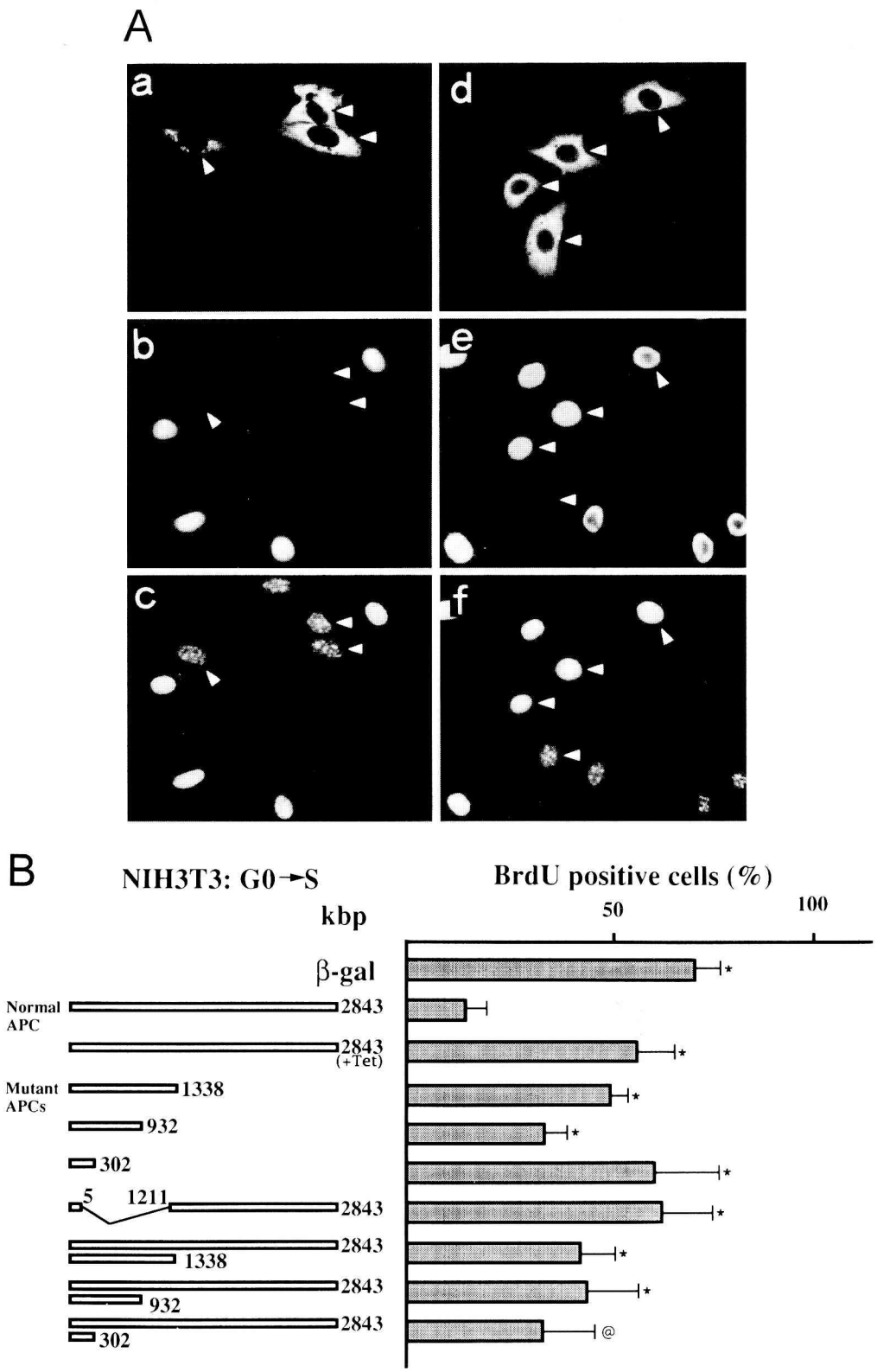

Fig. 3. Effect of overexpression of the normal and mutant APCs on cell cycle progression. (**A**) Micro-injection of APC cDNA inhibits the serum-induced G_0/G_1 to S transition. Serum-deprived NIH 3T3 cells micro-injected with the APC (a, b and c) or β-galactosidase (d, e and f) expression plasmids were re-stimulated with serum and then visualized for APC (a), β-galactosidase (d) or BrdU incorporation (b and e). In c and f, cells were stained with DAPI. White arrowheads point to representative cells. (**B**) Quantification of BrdU-positive cells. Schematic representations of the normal and mutant APCs are given on the left side of the histogram. The histogram shows the percentage of BrdU-positive NIH 3T3 cells injected with the normal or mutant APCs. For experiments with NIH 3T3 cultured in the presence of tetracycline (+Tet), β-galactosidase cDNA was co-micro-injected with APC cDNA and micro-injected cells were identified by staining for β-galactosidase, since APC is barely expressed in the presence of tetracycline. Effects of mutant APC expression on the cell-cycle blocking activity of normal APC are also shown; serum-deprived NIH 3T3 cells were micro-injected with the normal APC along with APC-302, -932 or -1338. At least 90 cells were scored for each experiment. Values are the mean ± SD of three experiments. $*P < 0.005$ as compared with normal APC; $@P < 0.05$ as compared with normal APC.

that the number of BrdU-positive cells began to increase ~12 h after serum addition. Injection of the APC cDNA at 8 h after serum addition could still inhibit BrdU incorporation, but injection at 10 h was ineffective (Figure 4). By contrast, injection of the β-galactosidase cDNA did not inhibit BrdU incorporation at any time point. These results suggest that APC acts in the mid to late G_1 phase.

Cyclin–CDK inhibits cell-cycle blocking activity of APC

To further elucidate the mechanism by which APC blocks cell cycle progression, we examined whether overexpression of molecules important for cell cycle regulation could overcome the inhibitory effect of APC. We first co-micro-injected the SV40 large T antigen or HPV E6 and/or E7 genes together with APC, but found no effect on APC inhibitory activity (Figure 5). However, the T antigen and E6/E7 expression constructs alleviated the cell-cycle blocking activity of pRB on human osteosarcoma SaosII cells and that of p53 on both NIH 3T3 cells and SaosII cells, respectively (data not shown). Thus neither the inhibition of pRB nor p53 seems to be important for the action of APC. Also neither the Rous sarcoma virus *src* gene nor an active version of the *raf* gene (Mitsunobu *et al.*, 1989) inhibited the activity of APC, although these expression constructs were capable of transforming NIH 3T3 cells (data not shown). In addition, APC exerted an inhibitory activity on v-*src*- and activated *raf*-transformed NIH 3T3 cell lines (data not shown). However, co-micro-injection of the CDK2 cDNA along with cyclin E, or CDK4 along with cyclin D1 abrogated APC inhibition (Figure 5). Micro-injection of either cyclin E or cyclin D1 alone also had a partial effect in alleviating APC inhibition, whereas neither CDK2 nor CDK4 alone could reverse the effect of APC.

Effect of APC on cyclin–CDK-associated kinase activity

To determine whether cyclin–CDK complexes function upstream or downstream of APC, we asked whether cyclin–CDK-associated kinase activity is down-regulated by the action of APC. For these experiments, we used cells transiently overexpressing APC, since we could not establish cell lines in which APC was inducibly expressed. Under the conditions used, ~10% of the cells took up and expressed the transfected APC plasmid. We therefore separated the transfected cells by flow cytometry using the decay accelerating factor (DAF) as a marker protein (Inoue *et al.*, 1993). Serum-deprived NIH 3T3 cells which had been transfected with the APC cDNA along with DAF cDNA were induced to enter the cell cycle by the addition of 10% serum and, after 12 h, the transfected cells were separated by flow cytometry based on DAF immunofluorescence. Under these conditions, <30% of the cells expressing the transfected APC incorporated BrdU, while almost all of the untransfected cells or cells transfected with DAF and the vector took up BrdU. From the separated DAF+ cells, CDK2 and CDK4 were immunoprecipitated and assayed for their abilities to phosphorylate histone H1 and bacterially produced pRB, respectively. DAF+ cells co-transfected with APC displayed a very low level of CDK2-associated kinase activity,

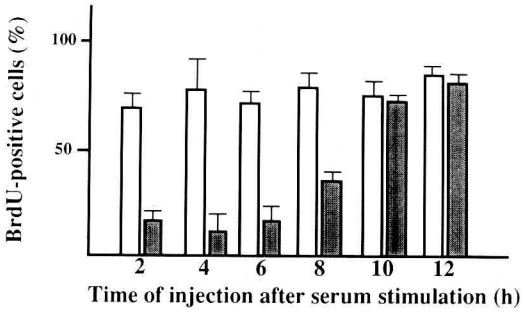

Fig. 4. Effect of APC expression at different time points through G_1 to S phase. Serum-starved NIH 3T3 cells were micro-injected with APC cDNA (filed column) or β-galactosidase cDNA (open column) at the indicated times after stimulation with 10% calf serum. At least 90 cells were scored for each experiment. Values are the mean ± SD of three experiments.

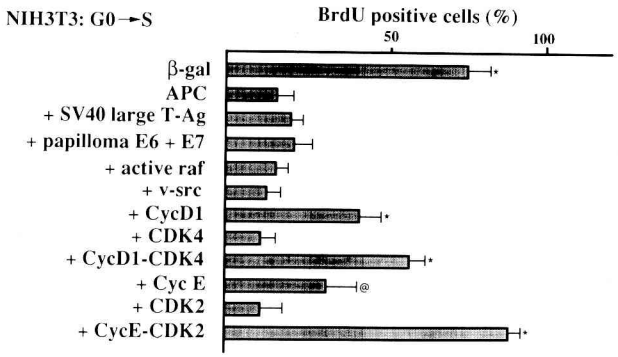

Fig. 5. Effect of expression of cell-cycle regulating genes on APC-mediated cell cycle arrest. Serum-deprived NIH 3T3 cells were micro-injected with APC cDNA along with the genes indicated. The experiments were performed as in Figure 3. At least 90 cells were scored for each experiment. Values are the mean ± SD of three experiments. $^*P$ <0.005 as compared with normal APC; @P <0.05 as compared with normal APC.

compared with both untransfected cells and DAF+ cells co-transfected with the vector (Figure 6A). In untransfected cells CDK2 kinase activity was enhanced by the addition of serum. On the other hand, CDK4-associated kinase activity was not affected by the overexpression of APC and was enhanced by serum stimulation. Although in APC-transfected cells CDK2 activity was suppressed, neither CDK2 nor CDK4 protein levels were significantly affected (Figure 6B); both CDK2 and CDK4 protein levels were enhanced by the addition of serum. In both the APC-expressing cells and control cells the faster migrating, active form of CDK2 was detected, while the amount of slower migrating, inactive form was decreased. We could not examine the level of cyclin E, since anti-cyclin E antibodies suitable for the detection of cyclin E were not available to us.

Discussion

The colony formation assay showed that APC inhibits the growth of monkey kidney fibroblast CV-1 cells. Micro-injection experiments further showed that APC blocks serum-induced cell cycle progression into S phase in mouse NIH 3T3 cells. In contrast, mutant APCs corresponding to those identified in FAP and/or colorectal tumours were less inhibitory in these assays. These findings are consistent

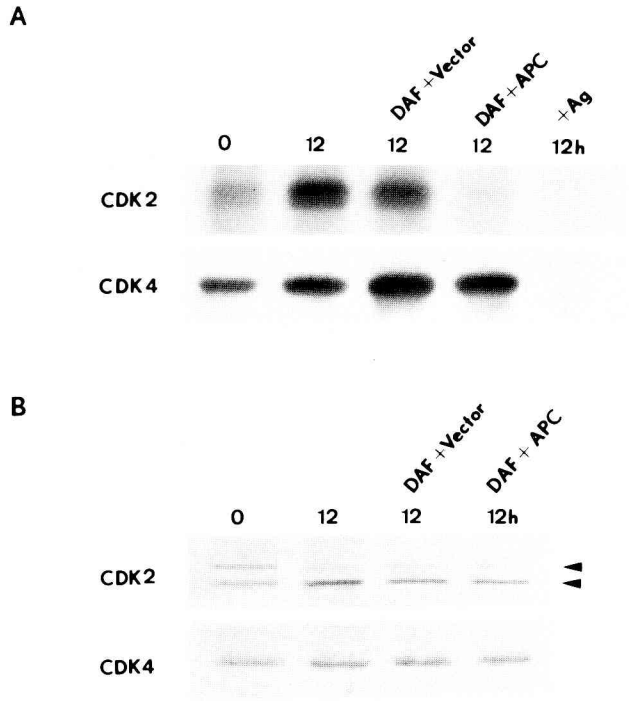

Fig. 6. (**A**) Effect of APC on the activity of cyclin–CDK. NIH 3T3 cells were transfected with the control expression vector or the APC expression construct along with the DAF expression construct. Cells were serum-starved for 48 h and then induced to enter the cell cycle by the addition of 10% serum and, after 12 h, the transfected cells were separated by flow cytometry based on DAF immunofluorescence. CDK2 and CDK4 were immunoprecipitated from the separated cells (1×10^5 cells) and subjected to a kinase assay using histone H1 or bacterially produced pRB. Kinase assays were also performed with control (untransfected) NIH 3T3 cells harvested at 0 h and 12 h after serum addition. When the antibodies that had been pre-absorbed with antigens were used, kinase activity was not detected in the precipitates prepared from control NIH 3T3 cells cultured in the presence of 10% serum for 12 h. (**B**) Effect of APC on the levels of CDK2 and CDK4. Cells used for the kinase assay in (A) were also subjected to Western blotting analysis using anti-CDK2 or CDK4 antibodies. Arrowheads indicate inactive and active CDK2, respectively.

with the observation that inactivation of APC results in the development of adenomatous polyps in the colon. Additionally, several C-terminally truncated mutant APCs partially alleviated the cell-cycle blocking activity of the normal APC, suggesting that the function of the normal APC may be inhibited by the formation of heterodimers with the mutant APCs via an N-terminal dimerization domain (Nishisho *et al.*, 1991; Joslyn *et al.*, 1993; Su *et al.*, 1993a).

Our micro-injection experiments showed that the cell-cycle blocking activity of APC was not inhibited by the SV40 large T antigen or papillomavirus E6/E7, suggesting that the APC pathway may not involve pRB or p53. Although we cannot rule out the possibility that the levels of expression of these transforming proteins were not sufficient to abrogate the effect of APC, it should be noted that our expression constructs were capable of relieving the inhibitory effects of p53 and pRB on cell cycle progression (data not shown). In a similar series of experiments, we also examined the effects of co-expression of v-Src or the N-terminal truncated active Raf. v-Src was previously reported to phosphorylate β-catenin on tyrosine residues and inactivate cadherin-mediated cell–cell interaction (Hamaguchi *et al.*, 1993). However, overexpression of v-Src did not abrogate the effect of APC. Raf, which is activated immediately after serum stimulation, and is known to play an important role in the transduction of signals from growth factor-stimulated tyrosine kinases and Ras to the MAP kinase cascade (Nishida and Gotoh, 1993; Marshall, 1994). Nevertheless, the activity of APC was not inhibited by the overexpression of the active Raf. It should also be noted that the v-*src* and active *raf* constructs used in these experiments are able to transform NIH 3T3 cells (data not shown). Additionally, molecular events occurring during the first 8 h after serum stimulation are apparently not essential for the APC-mediated cell cycle block, since micro-injection of the APC cDNA at this time point could still partially abrogate cell cycle progression to the S phase.

One of the most intriguing findings in this study is that overexpression of cyclin E plus CDK2 or cyclin D1 plus CDK4 abrogates the APC-mediated G_1 growth arrest. Our finding suggests that cyclin–CDK may function upstream or downstream of APC. Cyclin E–CDK2 and cyclin D1–CDK4 are believed to regulate cell cycle progression through the G_1 to S phase by phosphorylating key substrate proteins (Hunter and Pines, 1994; Sherr, 1994). However, unlike pRB, whose activity is down-regulated by cyclin–CDK-mediated phosphorylation (Akiyama *et al.*, 1992; Hinds *et al.*, 1992; Dowdy *et al.*, 1993; Ewen *et al.*, 1993a; Kato *et al.*, 1993), direct phosphorylation of APC by cyclin–CDK seems unlikely given their different subcellular locations. Another possibility is that cyclin–CDK complexes are involved in mediating the function of APC. For example, the growth-suppressive signal mediated by the cytoplasmic APC may be transduced into the nucleus and consequently down-regulate the activity of cyclin–CDKs. Indeed, we found that CDK2-associated kinase activity remains low in the late G_1 phase of APC-overexpressing cells. These results suggest that APC may block cell cycle progression by inhibiting the activity of CDK2.

Unlike CDK2, CDK4-associated kinase activity was not suppressed but was normally enhanced in the APC-overexpressing cells concomitant with cell cycle progression through the G_1 phase. However, overexpression of CDK4 along with cyclin D1 abrogated the activity of APC, though the effect was not as significant as that of CDK2 plus cyclin E. In this regard, it is interesting to note that it has been suggested that CDK4 acts upstream of CDK2, and its activation may precede and promote the activation of CDK2 (Ewen *et al.*, 1993b). Hence, it is possible that overexpression of CDK4 along with cyclin D1 may alleviate the cell-cycle blocking activity of APC by activating CDK2.

Although the activity of CDK2 is suppressed in APC-overexpressing cells, this does not appear to be the result of a change in the level of CDK2 expression. Thus, a change in the level of cyclin E might be one possible mechanism for down-regulation of CDK2 activity, though anti-cyclin E antibodies available to us were not suitable for the detection of mouse cyclin E. An alternative mechanism for suppression of CDK2 activity may involve the recently discovered class of small CDK inhibitor proteins such as p21 (also known as WAF1, Cip1, CAP20, Sdi1 and Pic1), $p16^{INK4}$, $p15^{INK4B}$ and $p27^{Kip1}$ (Hunter

and Pines, 1994; Sherr, 1994). Interestingly, the TGFβ-mediated cell cycle block has been shown to involve inhibitors of cyclin–CDKs, $p15^{INK4B}$ and $p27^{Kip1}$ (Hannon and Beach, 1994; Polyak *et al.*, 1994a,b; Toyoshima and Hunter, 1994), in addition to decreasing the expression of CDK4 and cyclin E (Ewen *et al.*, 1993b; Geng and Weinberg, 1993). Also, p53 has been suggested to block the cell cycle by inducing the expression of p21 (El-Deiry *et al.*, 1993; Gu *et al.*, 1993; Harper *et al.*, 1993; Hunter, 1993; Xiong *et al.*, 1993; Noda *et al.*, 1994). Thus, it will be interesting to examine whether APC may also regulate the expression or activity of the proteins involved in controlling the function of cyclin–CDKs.

As mentioned above, p53 and pRB have been reported to function upstream and downstream of cyclin–CDK, respectively, and to block cell cycle progression from the G_0/G_1 to S phase. Our results imply that APC also blocks cell cycle progression in the G_1 phase. Additionally, we have recently found that the product of the Wilms tumour suppressor gene *WT1* blocks cell cycle progression from the G_0/G_1 to S phase and this activity is abrogated by the overexpression of cyclin–CDK (Kudoh *et al.*, 1995). Furthermore, in WT1-overexpressing cells both CDK2 and CDK4 activities are refractory to induction by serum stimulation. However, while p53, pRB and WT1 are nuclear proteins which exert their effects by transcriptional regulation, APC is a cytoplasmic protein. Accordingly, the mechanism of APC-mediated cell cycle block would involve a pathway not shared with p53, pRB or WT1. Elucidation of the APC pathway-mediated growth arrest may provide new insights into the understanding of the negative regulation of cellular proliferation. In addition, it should be noted that our study does not exclude the possibility that APC has the ability to block cell cycle progression at point(s) other than the G_1 phase. In this regard, it is interesting to note that pRB has recently been reported to have the potential to arrest cells in G_2 (Karantza *et al.*, 1993). p53 has been reported to be a component of a spindle checkpoint that ensures the maintenance of diploidy (Cross *et al.*, 1995). Thus it remains to be elucidated whether APC has an additional ability to block cell cycle progression at different point(s) such as the G_2 phase.

In conclusion, overexpression of APC was found to inhibit cell growth by blocking cell cycle progression from the G_0/G_1 to S phase. Furthermore, inhibition of the activity of CDK2 was suggested to be involved in mediating the APC-induced cell cycle block. These findings raise the possibility that APC may play a role in the transmission of a negative signal such as the contact inhibition signal into the nucleus and thus negatively regulate cell cycle progression.

Materials and methods

Construction of APC expression plasmids

The APC expression plasmid pUHDneoAPC was generated by inserting the full-length protein coding region (*Sal*I–*Bam*HI fragment) of the APC cDNA and the geneticin-resistance gene into the pUHD10-3 vector, which carries a tetracycline-responsive cytomegalovirus promoter (Gossen and Bujard, 1992). APC-302, -932 and -1338 were generated by site-directed mutagenesis with the synthetic oligonucleotides 5′-TGTCAGCCTT-CAAGGTGCAGA-3′, 5′-GTAAGTGTTTCAATGTGTATG-3′ and 5′-ACTAGAACCCTACAGTCTGCT-3′, respectively (Kunkel, 1985). APC-ΔN was constructed by digesting the APC cDNA with *Nde*I followed by blunting with T4 DNA polymerase and self-ligation.

Antibodies

Anti-APC-C-ter were prepared by immunizing rabbits with a synthetic peptide corresponding to amino acids 2830–2843 of APC and purified by affinity chromatography on a column to which the synthetic peptide had been covalently linked. Antibodies against the N-terminal region (anti-APC-N-ter) were prepared by immunizing rabbits with a bacterially synthesized APC fragment (amino acids 119–250) fused to glutathione *S*-transferase.

Immunoprecipitation

Human osteosarcoma HOS and mouse fibroblast NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and calf serum, respectively. Cells were labelled for 4 h in methionine-free DMEM containing 1 mCi of [^{35}S]methionine (100 μCi/ml, 1200 Ci/mmol, New England Nuclear) and lysed in solubilizing buffer [50 mM Tris, pH 7.2, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 50 mM β-glycerophosphate, 5 mM DTT, 1 mM sodium orthovanadate, 0.05 mM NaF, 0.1 mM (*p*-amidinophenyl)methanesulphonyl fluoride and leupeptin (5 μg/ml)] (Akiyama *et al.*, 1992). The lysates were incubated with antibodies for 1 h at 4°C. The immunocomplexes were adsorbed to protein A–Sepharose 4B and washed extensively with solubilizing buffer. Samples were analysed by SDS–PAGE followed by autoradiography.

Colony formation assay

CV-1 cells were transfected with 40 μg of the APC expression plasmid (pUHDneoAPC) along with 20 μg of the transactivator plasmid by electroporation using a Gene Pulser (Bio-Rad) (960 μF, 260 V). At 24 h after transfection, cells were trypsinized, diluted 1:10, and cultured in the presence of 600 μg/ml of geneticin for 3 weeks. The plates were fixed and stained with Giemsa.

Micro-injection

NIH 3T3 cells, which were grown on coverslips (5×10^5 cells/cm^2), were cultured in DMEM containing 0.4% calf serum for 24 h and then micro-injected with the normal or mutated APC expression plasmid or β-galactosidase expression plasmid (pUHDneoβ-galactosidase) (200 μg/ml DNA). After incubating for 24 h in the same conditions, medium was replaced with fresh DMEM containing 10% calf serum and 50 ng/ml BrdU, and the cells were then incubated for another 18 h. Cells were fixed in 3.7% formaldehyde in PBS for 30 min, treated with 100% methanol for 10 min and then treated with 2 N HCl for 10 min. The APC protein was detected with anti-APC-N-ter antibodies and FITC-conjugated goat anti-rabbit IgG (Cappel). BrdU was detected with an anti-BrdU monoclonal antibody BU-4 (Takara) followed by RITC-conjugated goat anti-mouse IgG (Cappel). For experiments performed in the presence of tetracycline (1 μg/ml) and those with APC-ΔN, the β-galactosidase cDNA (pBAβ-galactosidase) was co-micro-injected with the APC cDNA and micro-injected cells were identified by staining for β-galactosidase.

Flow cytometry

NIH 3T3 cells (3×10^6 cells) were co-transfected with 5 μg/0.8 ml of pMEDAF, a vector containing the human DAF cDNA, 20 μg/0.8 ml of the transactivator plasmid and 40 μg/0.8 ml of the APC expression plasmid by electroporation using a Gene Pulser (Bio-Rad) (960 μF, 260 V) as described (Inoue *et al.*, 1993). Cells were cultured in DMEM containing 10% calf serum for 5 h and then in DMEM containing 0.4% calf serum for 48 h. Subsequently, the medium was replaced with fresh DMEM containing 10% calf serum, and the cells were incubated for another 12 h. Cells were then stained with biotin-conjugated anti-DAF monoclonal antibody B-1A10 and phycoerythrin-conjugated Streptavidin, and immunostained cells were separated by flow cytometry using a FACS Vintage Machine (Becton Dickinson).

In vitro kinase assay and Western blotting analysis

DAF^+ NIH 3T3 cells separated by flow cytometry were lysed in solubilizing buffer [50 mM Tris, pH 7.2, 1% NP40, 0.15 M NaCl, 5 mM dithiothreitol, 0.1 mM (*p*-amidinophenyl)methanesulphonyl fluoride with leupeptin at 5 μg/ml] and subjected to immunoprecipitation with anti-CDK2 antibodies as described (Akiyama *et al.*, 1992). The immunocomplexes were incubated with 1 μg of histone H1 in 50 μl of kinase reaction buffer [50 mM Tris, pH 7.2, 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM [γ-^{32}P]ATP (5 μCi)] for 20 min at 30°C. CDK4-associated kinase

activity was assayed using 1 μg of bacterially produced GST–pRB as a substrate according to Matsushime *et al.* (1994). Samples were analysed by 12% SDS–PAGE followed by autoradiography.

For Western blotting analysis, lysates obtained as described above were resolved by 12% SDS–PAGE and transferred onto poly(vinylidene difluoride) membranes (Immobilon-P, Millipore). Blots were probed with antibodies to CDK2 or CDK4 and subsequently with alkaline phosphatase-conjugated second antibodies.

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Constitutive Overexpression of CDK2 Inhibits Neuronal Differentiation of Rat Pheochromocytoma PC12 Cells*

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Changes in the levels of cyclins A, D, and E, p21, and cyclin-dependent kinase 2 (CDK2) were examined in rat pheochromocytoma PC12 cells during neuronal differentiation induced by nerve growth factor (NGF). Expression of cyclin A decreased to an undetectable level after 5 days of exposure to NGF, while expression of CDK2 decreased gradually after day 3. In contrast, the levels of cyclins D1 and E increased gradually through day 10, yet the amount of cyclin E associated with CDK2 decreased concomitant with a decrease in the CDK2 protein level. p21 expression increased gradually after day 7, while the level of CDK2-associated p21 remained unchanged. When human cDNAs encoding cyclins and CDK2 were introduced into PC12 cells, only CDK2 overexpression inhibited NGF-induced differentiation. The cell lines overexpressing CDK2 showed stable and high levels of CDK2 kinase activity during differentiation, whereas parental and vector-transfected cell lines displayed a marked decline in CDK2 kinase activity 1 day after NGF treatment. In cell lines overexpressing cyclins A, D, and E, this reduction of the kinase activity was not apparent until day 3. These results suggest that down-regulation of CDK2 activity is a crucial event for the neuronal differentiation of PC12 cells.

The commitment to cellular differentiation is a highly controlled stochastic process consisting of successive steps that require specific signals for survival and simultaneous loss of proliferative potential. The determination as to whether cells continue to proliferate or differentiate appears to be executed during the G_1 phase of the cell cycle when several types of G_1 cyclins and cyclin-dependent kinases (CDKs)[1] interact in various combinations (1–7). The D-type cyclins assemble primarily with CDK4, whose activity is detectable at mid-G_1 and which increases as cells approach the G_1-S boundary (8–10). Cyclin E associates with CDK2 and induces maximal levels of kinase activity at the G_1-S transition (11–13). In addition, cyclin A expression peaks at the G_1-S boundary and accumulates in early S phase, activating both CDK2 and Cdc2 (14–16).

Cyclin-CDK complexes are believed to play an essential role in the G_1-S transition. Constitutive ectopic expression of cyclin D or E in normal fibroblasts has been reported to shorten G_1 and reduce the dependence of cells on growth factors (17–20). In addition, microinjection of antibodies against cyclin D1 during G_1 phase prevents cells from entering S phase (17). Inhibition of the function of CDK2 has also been reported to prevent the entry of cells into the S phase (21–23). The fact that cyclin-CDKs play a crucial role in the G_1 phase implies that the regulation of their functions is also critical for the commitment to cell differentiation. Indeed, ectopic expression of D-type cyclins and CDK4 was reported to inhibit granulocyte colony-stimulating factor-induced differentiation of murine myeloid cells and erythroleukemia cells, respectively (24, 25).

In the present study, we investigated the roles of cyclins and CDK2 in the process of the neuronal differentiation of PC12 cells. In addition, we examined the expression of p21, a negative regulator of several cyclin-CDK complexes, including cyclin D-CDK4, cyclin E-CDK2, and cyclin A-CDK2 (26–28). Rat pheochromocytoma PC12 cells have proven to be a good model for neuronal differentiation due to their characteristic responsiveness to NGF. This response consists of partial growth arrest and acquisition of phenotypic properties typical of sympathetic ganglion, such as prominent neurite outgrowth (29). Herein, we demonstrated that suppression of CDK2 activity is a critical step in the NGF-induced differentiation of PC12 cells.

EXPERIMENTAL PROCEDURES

Cell Lines—PC12 cells were grown on plastic dishes without collagen coating in Dulbecco' modified Eagle's medium, containing 10% heat-inactivated horse serum and 5% fetal bovine serum. For NGF treatment, cells were cultured on collagen-coated plastic dishes in Dulbecco' modified Eagle's medium supplemented with 2% horse serum and 1% fetal bovine serum. NGF purified from mouse submaxillary glands was purchased from Wako Ltd. Cells that have one or more neurites with the length more than twice the diameter of the cell body were defined as "differentiated" according to the previously described criteria (30). Alternatively, cells that have neurites with a length of more than three times the diameter of the cell body were defined as "well differentiated." The number of such "differentiated" cells per 500 cells was counted and used to compare the extent of differentiation among different samples.

Plasmids and Transfection—cDNA of human cyclin A was subcloned into pMKITneo (gift from Dr. Maruyama, Tokyo Medical and Dental Universtity), and cDNAs of cyclins D1 and E, CDK2, and p21 were subcloned into the pME18 vector. All of these cDNAs are under the control of the SRα promotor and were used for transfection into PC12 cells. PC12 cells (6×10^5 cells) were transfected with 5 μg of cDNAs along with (cyclin D1, cyclin E, CDK2, and p21) or without (cyclin A) 0.5 μg of the neomycin-resistant gene, pSV2neo, by use of Lipofectamine (Life Technologies, Inc.) followed by selection for G418 (Life Technologies, Inc.) resistance (500 μg/ml). Three weeks after transfection, 50 clones were picked up for each cDNA transfectant and were maintained in medium containing 100 μg/ml G418. For each cDNA-transfectant, three clones showing the highest expression were selected by immunoblotting analysis and used for further experiments.

Antibodies—Polyclonal antibodies against cyclin A and cyclin E

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[1] The abbreviations used are: CDK, cyclin-dependent kinase; NGF, nerve growth factor; GST, glutathione *S*-transferase; RB, retinoblastoma; INK, inhibitor of CDK; KIP, CDK inhibitory protein.

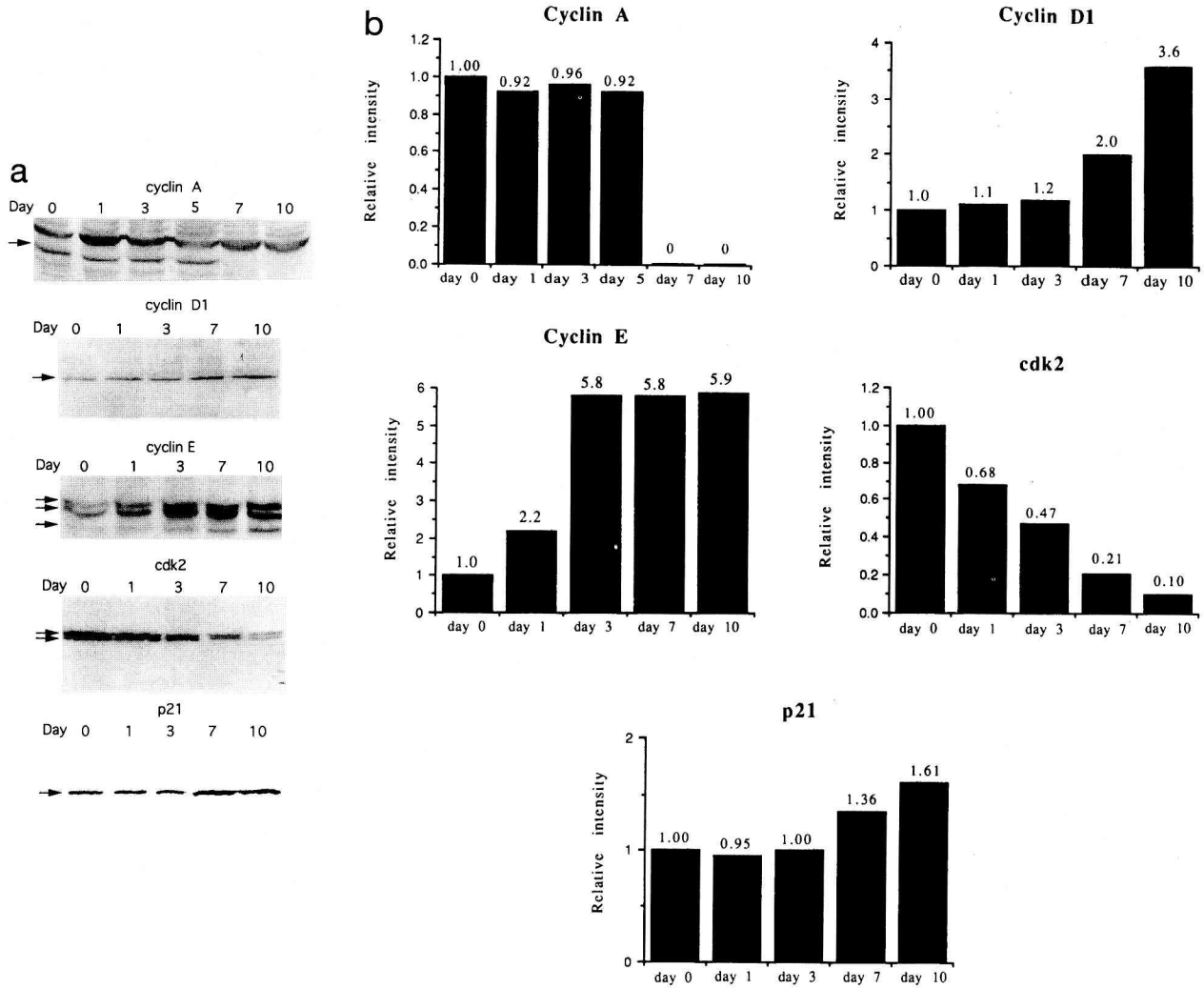

FIG. 1. **Protein levels of cyclins A, D1, and E, CDK2, and p21 during NGF-induced neuronal differentiation of PC12 cells.** *a*, lysates were prepared from cells that had been cultured in medium containing 20 ng/ml of NGF and harvested at the indicated times and were subjected to immunoblotting analysis to detect cyclins A, D1, and E, CDK2, and p21. *b*, histograms were generated by quantitating the intensity of the bands on nitrocellulose filters in panel *a* with a dual wavelength flying spot scanner. All values are indicated by ratios relative to those obtained on day 0. In the case of cyclin E and CDK2, the most slowly migrating bands were subjected to quantification.

were obtained from Upstate Biochemicals Incorporated. Polyclonal antibodies against cyclin D1, CDK2, and p21 were prepared by immunizing rabbits with synthetic peptides corresponding to the 15, 12, and 17 carboxyl-terminal amino acid residues, respectively, of these proteins.

Immunoblotting and Immunoprecipitation—For immunoblotting, cells were lysed in high salt lysis buffer (0.5% Nonidet P-40, 50 mM Tris-HCl (pH 8.0), 0.25 M NaCl, 5 mM EDTA, 50 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin). The protein concentration of each sample was determined using the Bio-Rad protein assay. 50 μg of protein was analyzed on an SDS-polyacrylamide gel electrophoresis gel (10 or 12.5% of acrylamide gel) and then transferred to a polyvinylidene difluoride membrane (Millipore Corp.). Following blocking in skim milk for 3 h, the membrane was incubated with primary antibodies to human cyclin A (1:200 dilution), cyclin D1 (1:100), cyclin E (1:150), CDK2 (1:100), or p21 (1:75). Each protein was detected by sequential binding of a specific primary antibody followed by alkaline phosphatase-conjugated secondary antibody (Promega, 1:6000 dilution). For immunocomplex kinase reactions, cells were lysed in solubilizing buffer (50 mM Tris (pH 7.2), 1% Nonidet P-40, 0.15 M NaCl, 50 mM β-glycerophosphate, 5 mM dithiothreitol, 1 mM Na_3VO_4, 0.05 mM NaF, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin) (31). Lysates (500 μg of protein) were incubated with anti-CDK2 antibody (diluted 1:150) for 1 h followed by an additional 1 h incubation with protein A-Sepharose beads at 4 °C. For immunoprecipitation followed by immunoblotting, cells were lysed in Nonidet P-40 lysis buffer (50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin) (32). Lysates (200 μg of protein) were incubated with anti-CDK2 antibody immobilized on Sepharose or with $p13^{suc1}$-Sepharose for 4 h at 4 °C. The precipitates were used for further immunoblotting analysis for cyclin A, cyclin E, or p21.

In Vitro Kinase Reactions—Lysates of each cell line (500 μg of protein) were prepared as described above and were subjected to immunoprecipitation with anti-CDK2 antibody diluted 1:150 in a total volume of 300 μl. A bacterially expressed fragment of the RB protein (amino acids 385–928) fused to glutathione *S*-transferase was purified by affinity chromatography on glutathione-Sepharose and was used as a substrate (0.5 μg of protein) in 50 μl of kinase reaction buffer containing 50 mM Tris-HCl (pH 7.2), 10 mM $MgCl_2$, 1 mM dithiothreitol, 20 mM [γ-^{32}P]ATP (5 μCi; 1 μCi = 37 kBq) (31). After incubation for 10 min at 25 °C, the sample was analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

Densitometric Analysis—Densitometric quantification of the data obtained by immunoblotting analysis (nitrocellulose filters) and *in vitro* kinase assay (x-ray films) was done with a Dual wavelength flying spot scanner (Shimadzu Ltd.). Since more than one band was detected in immunoblotting analysis of cyclin E and CDK2, the most slowly migrating band was subjected to quantification.

RESULTS

Expression of Endogenous Cyclins, CDK2, and p21 during Differentiation of PC12 Cells—PC12 cells were cultured in medium supplemented with 2% horse serum, 1% fetal bovine serum, and 20 ng/ml NGF for 10 days. At days 0, 1, 3, 7, and 10,

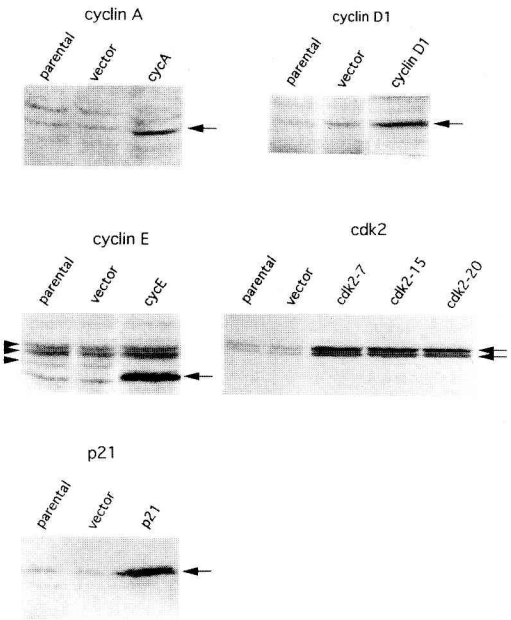

FIG. 2. **Forced expression of ectopic cyclins A, D1, and E, CDK2, and p21 in PC12 cells.** Representative clones and control clones (parental and vector-introduced cell lines) were lysed as described under "Experimental Procedures." Lysates (50 μg of protein) were subjected to immunoblotting analysis to detect cyclins A, D1, and E, CDK2, and p21. The positions of three different forms of endogenous rat cyclin E are indicated by *arrowheads*. Ectopically expressed human cyclins, CDK2, and p21 are indicated by *arrows*.

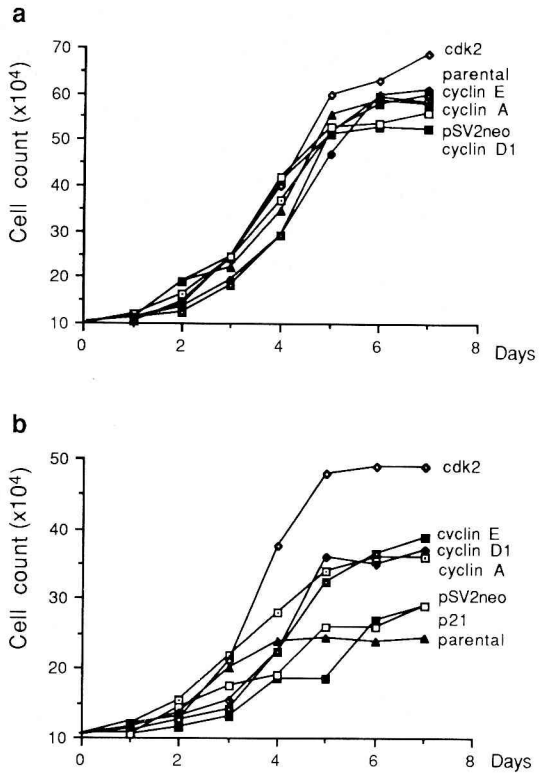

FIG. 3. **Growth rates of cell lines.** Growth rates of parental PC12 cells and derivative clones expressing cyclins A, D1, and E, CDK2, and p21 are shown in the absence (*a*) or presence (*b*) of NGF (20 ng/ml). For CDK2-expressing cell lines, only the growth curve of the representative clone K7 is shown because all 3 CDK2-overexpressing cells revealed quite similar growth rates. Cell numbers represent the mean values of triplicate experiments.

cells were lysed, and the expression of cyclins A, D1, and E, CDK2, and p21 was evaluated by immunoblotting analysis using specific antibodies. As shown in Fig. 1, expression of cyclin A was dramatically suppressed to an undetectable level after day 5. The expression of CDK2 showed a gradual but significant decrease to 21% of the initial level after NGF treatment for 7 days. In contrast, the expression of cyclins D1 and E increased gradually, reaching 4- and 6-fold higher levels, respectively, through the 10 days of observation. The level of p21 remained unchanged for at least 3 days and increased to 1.6-fold thereafter.

Introduction of the cDNAs Encoding Human Cyclins, CDK2, and p21—To evaluate the significance of the changes in the expression of cyclins, CDK2, and p21 described above, we asked whether forced expression of these molecules alters the sensitivity of PC12 cells to NGF-induced neuronal differentiation. Expression constructs containing the cDNAs of the human cyclins A, D1, and E as well as CDK2 and p21 were transfected separately into PC12 cells, and clonally derived cell lines were established. Immunoblotting analysis of the cloned cell lines showed that the bands corresponding to the ectopically overexpressed gene products of cyclins A and D, CDK2, and p21 were almost identical in size to the endogenous gene products (Fig. 2). The levels of the overexpressed proteins were approximately 5-, 5.5-, 12-, and 20-fold higher, respectively, than that of the endogenous proteins. As reported previously with Rat-1 cells (19, 20, 33), ectopically expressed human cyclin E could be detected as a protein of 50 kDa, while the endogenous rat cyclin E was detected as three more slowly migrating bands (Fig. 2), consistent with the fact that the human and rat cyclin E genes encode proteins of 395 and 491 amino acids, respectively (11, 34). The level of the exogenous 50-kDa cyclin E was 3-fold higher than that of the endogenous 55-kDa cyclin E. The 50-kDa human cyclin E species has been shown to be competent to regulate cell cycle progression as well as CDK2 kinase activity (19, 20).

Effects of NGF Stimulation on the Growth of PC12 Cells—NGF-induced differentiation of PC12 cells is known to be accompanied by a reduction in the growth rate (29). Thus, the effects of overexpressing cyclins A, D, and E, CDK2, and p21 on the growth of PC12 cells were examined by culturing these cell lines in medium supplemented with 10% horse serum and 5% fetal bovine serum, in the absence or presence of NGF (20 ng/ml). In the absence of NGF, all of the cell lines exhibited similar growth rates (Fig. 3*a*). After 3 days of culture in the presence of NGF, the growth of cell lines overexpressing cyclin A (clone A26), cyclin D1 (clone D3), cyclin E (clone E4), and p21 (clone P7) as well as the parental and vector-transfected cells declined significantly. However, in striking contrast to these cells, cell lines overexpressing CDK2 (clones K7, K15, and K20) did not show a significant reduction in cell growth for at least 5 days (Fig. 3*b*).

Morphological Changes Associated with NGF-induced Differentiation of PC12 Cells—All of the established cell lines showed morphologies very similar to the parental PC12 cells in the absence of NGF; cells were round or polygonal in shape and were loosely adherent on the dish (Fig. 4*a*). When the parental cells and vector-transfected cells were stimulated with NGF, approximately 40% of the cells displayed prominent neurite outgrowth at day 1 (Fig. 4*b*). Cyclin A-, D, and E-expressing cells began to show neurites from day 3 (Fig. 4*c*). By contrast, all of the three independent CDK2-expressing cell lines did not show significant neurite outgrowth through the 7 days of observation (Fig. 4*d*). The neurites developed in cyclin A-, D-, and E-expressing cells at day 3 were still shorter than those in parental cells and vector-transfected cells at day 1. For example, neurite outgrowth was observed in 34% of cyclin A-expressing cells at day 3 (Fig. 4*e*). Furthermore, only 12% of cyclin

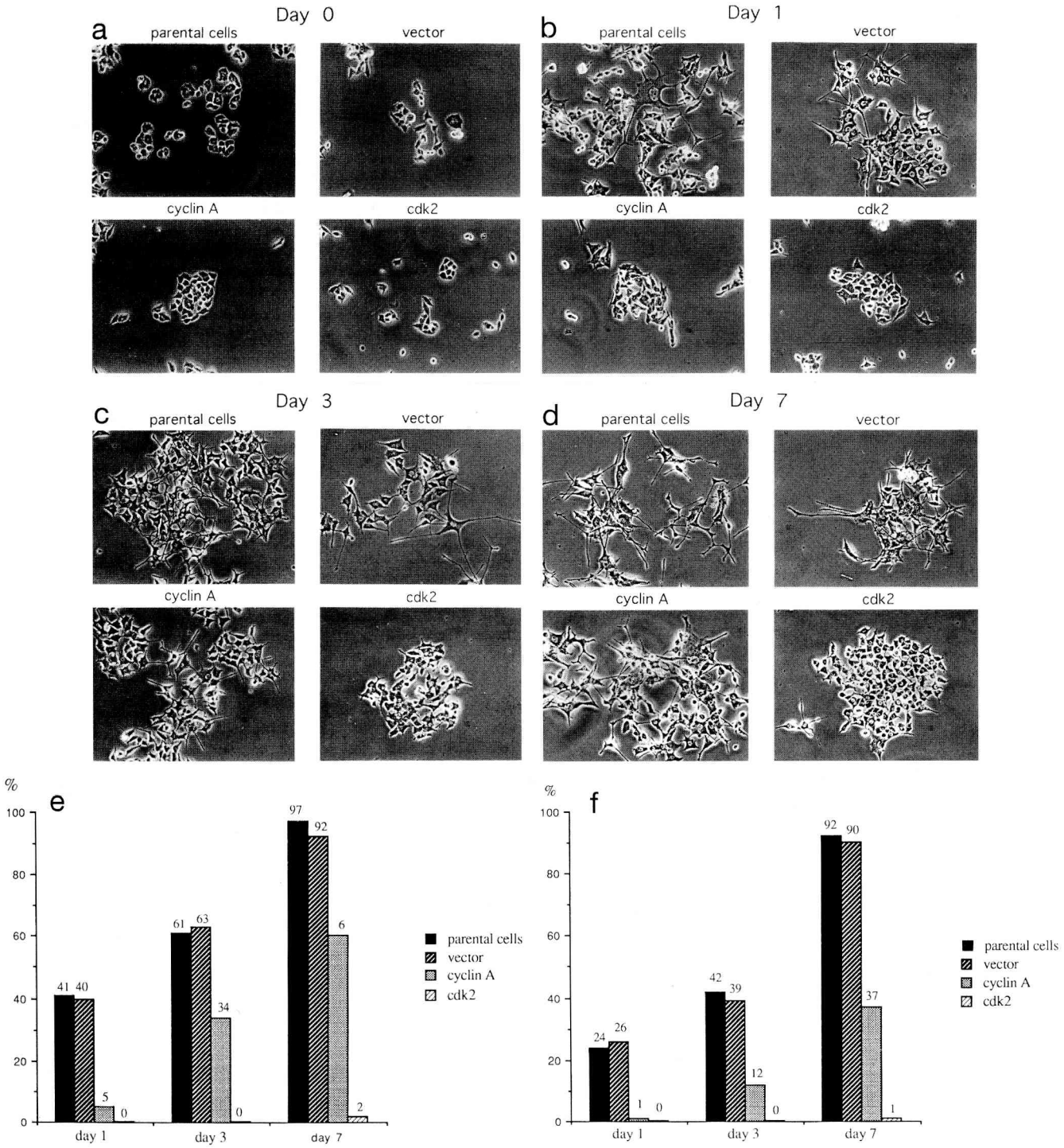

FIG. 4. **Morphological changes in cell lines.** Morphological changes in PC12 cells and derivative clones in the absence (*a*) or presence (*b*, *c*, *d*) of NGF (20 ng/ml) are shown. Since the changes in morphology of the cell lines overexpressing cyclins A, D1, and E and p21 were quite similar, only representative photomicrographs of cyclin A-expressing cells are shown. For CDK2-expressing cell lines, only the photographs of clone K7 are shown because all 3 CDK2-overexpressing clones exhibited similar morphological features. *e*, *f*, 500 cells from the parental, vector-transfected, and cyclin A-overexpressing cell cultures were counted at the indicated times, and the number of cells having neurites with length more than twice (*e*), or 3 times (*f*) the diameter of the cell body were calculated as a percentage of cells counted. Since the data obtained with cell lines overexpressing cyclins A, D1, and E and p21 were quite similar, only the data obtained with cyclin A-expressing cells are shown.

A-expressing cells developed neurites having a length more than 3 times the diameter of the cell body, which we defined as "well differentiated" (Fig. 4*f*). By contrast, parental cells and vector-transfected cells developed neurites in about 60% of the entire population at day 3, and 40% were well differentiated (Fig. 4, *e* and *f*).

Changes in CDK2 Kinase Activity—We next examined the changes in CDK2 kinase activity following immunoprecipitation from the lysates of the cell lines during NGF-induced differentiation. CDK2 immunoprecipitates were assayed for their ability to phosphorylate a fragment of bacterially produced pRB in the presence of [γ-^{32}P]ATP. As shown in Fig. 5, a rapid decline in kinase activity to about 55% of starting levels at day 1 and to 2% at day 3 was detected in parental cells and a vector-transfected cell line (clone neo5). In cell lines overexpressing cyclins A, D1, and E and p21, an apparent decrease in

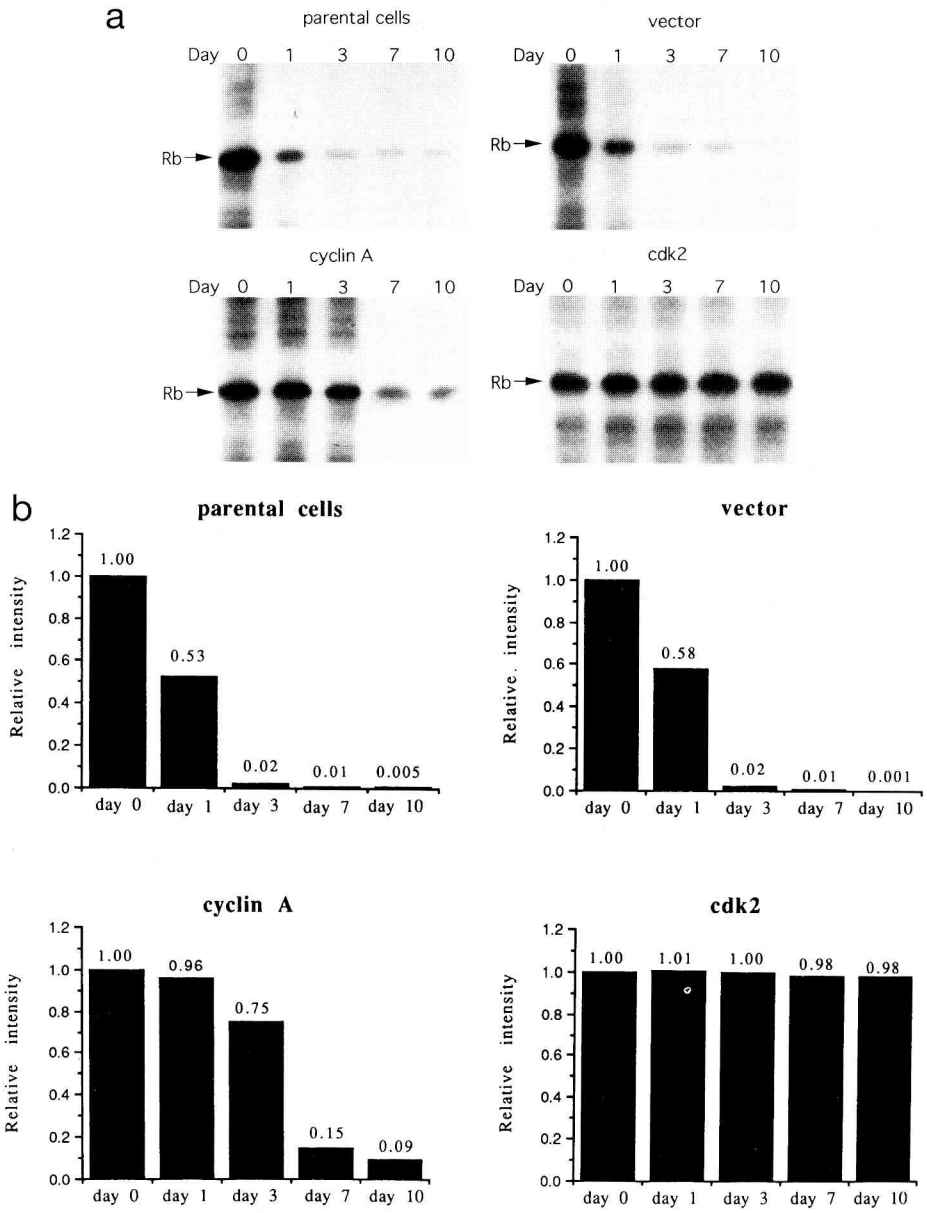

FIG. 5. **Kinase activities associated with CDK2.** *a*, CDK2-associated kinase activity in parental PC12 cells and derived cell lines expressing cyclins A, D1, and E, CDK2, and p21 during NGF treatment (20 ng/ml) are shown. Lysates prepared at the indicated times were immunoprecipitated with anti-CDK2 antibody. The immunocomplex was assayed for kinase activity using GST-RB fusion protein as a substrate. Since the changes in kinase activity of the cell lines overexpressing cyclins A, D1, and E and p21 were quite similar, only the results of a representative cyclin A-expressing cell line are shown. *b*, The histograms were generated by quantitating the intensity of radioactive signals on the x-ray films in panel *a* with a dual wavelength flying spot scanner. All values are indicated by ratios relative to those obtained on day 0.

the activity of CDK2 to 75% and 15% of the initial levels was detected at day 3 and day 7, respectively (Fig. 5*b*; only the data obtained with cyclin A-expressing cells are shown). However, CDK2 kinase activity in all of the cell lines overexpressing CDK2 (clones K7, K15, K20) remained high throughout the 10 days of observation.

Immunoblotting Analysis of CDK-associated Cyclin A, Cyclin E, and p21—To evaluate the levels of cyclin A, cyclin E, and p21 associated with CDK, we subjected p13^{suc1} precipitates and CDK2 immunoprecipitates prepared from NGF-stimulated cells to immunoblotting analysis using antibodies against cyclins A and E and p21, respectively. As shown in Fig. 6, the amount of cyclin A associated with CDK that bound to p13^{suc1}-Sepharose decreased after day 3, in parallel with the previously observed levels of cyclin A expression (Fig. 1). However, the levels of CDK-associated cyclin E gradually decreased to 60% of the initial level at day 7, despite the fact that the level of cyclin E expression gradually increased during the time course (Fig. 6; compare to Fig. 1). The level of p21 associated with CDK2 was minimally affected by the presence of NGF.

DISCUSSION

In the present study, we examined alterations in the expression levels of cyclins, CDK2, and p21 during the NGF-induced neuronal differentiation of PC12 cells. Furthermore, we examined the effects of overexpression of these cell cycle regulators on the differentiation of PC12 cells. The results obtained from these experiments suggest that CDK2 is a key regulator of neuronal differentiation. The activity of CDK2 dramatically decreased following the addition of NGF, and constitutive overexpression of CDK2, but not of any cyclins tested, significantly blocked differentiation of PC12 cells. Ectopically expressed CDK2 probably exerted its effect by forming complexes with endogenous cyclins E and A and possibly with cyclins D2 and D3, *i.e.* those cyclins whose expression may be high enough to interact with CDK2 (2, 8, 32, 35). On the other hand, overexpression of cyclins A, D1, and E had a weaker effect. These findings are different from those previously reported using myeloid and erythroleukemia cell lines. Ectopic overexpression of cyclins D2 and D3 was reported to have inhibited granulocyte colony-stimulating factor-induced differentiation of murine myeloid cells. The mechanism of these results was attrib-

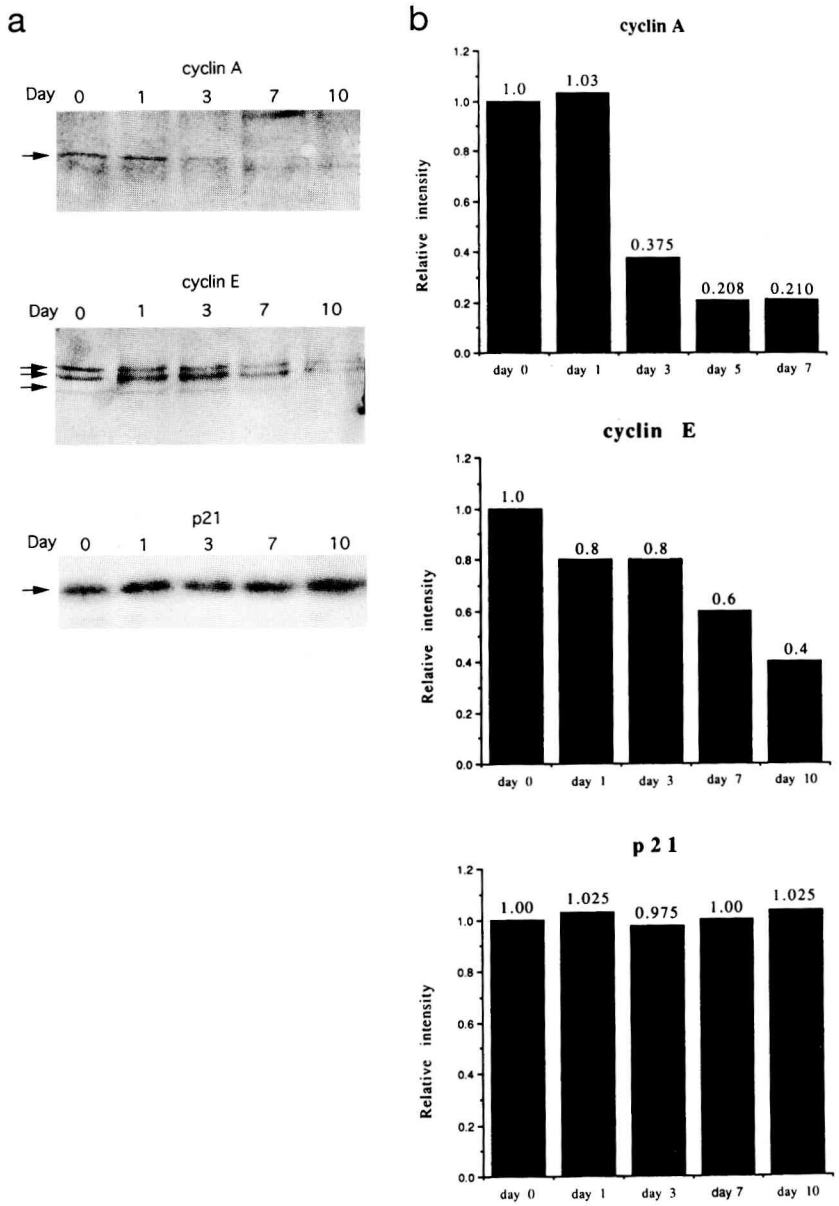

FIG. 6. **Levels of CDK-associated cyclin A, cyclin E, and p21 during differentiation.** *a*, lysates prepared from PC12 cells treated with NGF for the indicated time periods were precipitated with p13^{suc1} (for cyclin A and cyclin E) or immunoprecipitated with anti-CDK2 antibody (for p21). The immunocomplex was subjected to immunoblotting analysis with antibodies against cyclin A, cyclin E, and p21. *b*, the histograms were generated by quantitating the intensity of the bands on nitrocellulose filters in *panel a* with a dual wavelength flying spot scanner. All values are indicated by ratios relative to those obtained on day 0. In the case of cyclin E, the most slowly migrating bands were subjected to quantification.

uted to the interaction of ectopically expressed cyclin D2 or D3 with CDK2, which was persistently expressed in that cell line (24). Kiyokawa *et al.* (25) determined that the suppression of CDK4 expression is a critical event in the pathway of terminal differentiation of the erythroleukemia cell line MEL. In addition, Jahn *et al.* (36) reported that CDK2 activity does not change during differentiation of the mouse skeletal myogenic cell line C2C12. These differences may be due to cell type-specific molecular mechanisms of cell cycle modulation and differentiation.

Although the levels of CDK2 and cyclin A were found to be suppressed during NGF-induced differentiation, neurite outgrowth apparently preceded this decrease. Hence, suppression of the expression levels of CDK2 or cyclin A may not be of primary importance in the induction of differentiation.

In contrast to CDK2 and cyclin A, the levels of cyclins D1 and E were found to increase gradually during differentiation of PC12 cells. Although overexpression of G_1 cyclins has been reported to accelerate G_1 phase in several cultured cell lines (17–20), the increase in cyclin D1 and E expression observed at the later stages of differentiation of PC12 cells may not be involved in the acceleration of the G_1/S transition; indeed, our experiments using p13^{suc1} precipitates showed a gradual decrease in the amount of cyclin E associated with CDK. Similar up-regulation of cyclins has been reported to occur during the differentiation of various cell lineages. For example, the expression of cyclin D1 has been reported to increase in neurons at the onset of rat brain maturation as well as during differentiation of PC12 h cells (37, 38). During 12-*O*-tetradecanoylphorbol-13-acetate-induced differentiation of human promyelocytic leukemia HL60 cells into macrophage-like cells, cyclin D1 expression is also up-regulated, although its expression is down-regulated in Me_2SO-induced granulocytic cells (39). The expression of cyclin D3 is also known to increase during differentiation of mouse erythroleukemia MEL cells (25) and rat myoblast L6 cells (40). In the latter case, the kinase activity of the cyclin D3 complex, which presumably includes CDK2 and CDK4, has been shown to be markedly suppressed in the differentiated myotubes. A similar phenomenon was also observed in senescent human diploid fibroblasts (41). Cyclin E-associated CDK2 activity is very low in senescent cells, although the amounts of cyclins D1 and E are 10–15-fold higher than observed in quiescent early passage cells. Taken together, these findings raise the possibility that G_1 cyclins

may play some roles other than G_1/S acceleration during cellular differentiation. For example, it has been observed that the G_1 cyclins can form complexes with pRB or p107, although the significance of these complexes is still unclear (2, 42–44).

Since the changes in the expression levels of CDK2 and cyclin E do not seem to be of primary importance in the down-regulation of CDK2 activity during differentiation of PC12 cells, an alternative mechanism may involve CDK inhibitor proteins (6, 7). However, expression of p21 was not significantly changed during differentiation of PC12 cells. Furthermore, overexpression of p21 neither induced differentiation nor growth retardation. Consistent with these results, no significant suppression of CDK2 kinase activity was observed in p21-overexpressing cells. One possible reason may be that the levels of ectopically expressed p21 in these established cell lines was not high enough to block the function of CDK2, although there was an approximately 20-fold increase in p21 level as compared with the parental cells. Presumably, cell lines overexpressing p21 at higher levels were not established due to its cell proliferation-inhibitory activity. In addition to p21, a growing number of CDK inhibitors, such as $p16^{INK4}$, $p15^{INK4B}$, $p27^{KIP1}$ and $p57^{KIP2}$, have recently been reported (6, 7, 45, 46). Interestingly, the TGF-β-mediated cell cycle block has been shown to involve $p15^{INK4B}$ and $p27^{KIP1}$ (6, 7, 47). Additionally, it has been reported that a protein, SNT, that appears as a doublet of 78–90 kDa in SDS-polyacrylamide gel electrophoresis gels and which coprecipitates with $p13^{suc1}$-agarose is rapidly phosphorylated on tyrosine in neurons and PC12 cells treated with differentiation factors but not in those treated with mitogens (48). Since $p13^{suc1}$ associates with cyclin-CDK, this finding raises the possibility that SNT may link the differentiation signal mediated by receptor tyrosine kinases to the cell cycle regulator CDKs; *i.e.* SNT may act as a negative regulator of CDKs. Thus, the contribution of CDK inhibitors as well as of SNT to the NGF-induced inhibition of CDK2 activity remains to be elucidated in future studies.

Cyclin-CDK is believed to phosphorylate cellular proteins important for cell cycle regulation (6, 7). One of the main targets of cyclin-CDK is pRB, which negatively regulates cell cycle progression through the G_1 phase (31, 43). pRB also plays a crucial role in early neuronal and hematopoietic development as demonstrated by the analysis of mice carrying a targeted mutation in the RB gene (49–51). The amount of underphosphorylated pRB, which is believed to be the active form of pRB, is increased by extracellular signals, which induce cell cycle arrest and differentiation (52, 53). The results obtained in this study suggest that the NGF-induced reduction in CDK2 activity may be responsible for the accumulation of the underphosphorylated form of pRB during differentiation of PC12 cells. Accordingly, constitutive overexpression of CDK2 may block differentiation by driving the phosphorylation of pRB. Consistent with this notion, ectopic overexpression of adenovirus E1A, which associates with and inactivates pRB as well as p107, has been demonstrated to inhibit NGF-induced neuronal differentiation of PC12 cells (54–56). Further detailed analysis of the function of pRB, as well as the identification of other CDK2 substrates, may prove important for our understanding of the precise mechanism by which PC12 cells differentiate.

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Subcellular localization of the APC protein: Immunoelectron microscopic study of the association of the APC protein with catenin

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Mutations in the APC gene are linked to the development of sporadic colorectal tumors as well as to familial adenomatous polyposis. Recently, the APC protein was reported to associate with catenins, proteins that bind to the cell adhesion molecule E-cadherin. In the present study, we examined the distribution and localization of the APC protein and α -catenin in the normal mouse intestine by light and immunoelectron microscopy using specific antibodies. The APC protein was found to be localized in microvilli and in the apical and lateral cytoplasm of the epithelial cells, whereas α-catenin was detected only in the lateral cytoplasm. Double-labeling immunoelectron microscopy showed colocalization of the APC protein with α-catenin in the lateral cytoplasm, especially along the lateral plasma membrane, although a certain portion of the APC protein in this region was distributed independently of α-catenin. These results suggest that a portion of the APC protein localized in the lateral cytoplasm of intestinal epithelial cells functions in cooperation with catenins, whereas the APC protein in microvilli and in the apical cytoplasm has other functions independent of catenins.

Keywords: tumor suppressor gene; APC; FAP; catenin; colorectal tumor

Introduction

Mutations in the adenomatous polyposis coli (APC) gene contribute to the progression of colorectal tumorigenesis. Germline mutations in APC result in an autosomal dominantly inherited disease called familial adenomatous polyposis (FAP), which is characterized by the development of multiple colorectal adenomatous polyps that are prone to develop carcinomas later. Somatic mutations are linked to the development of sporadic colorectal tumors including small benign adenoma (Groden *et al.*, 1991; Nishisho *et al.*, 1991; Miyoshi *et al.*, 1992a,b; Powell *et al.*, 1992, Vogelstein and Kinzler, 1993). APC mutations are also associated with extracolonic disorders; for example Gardner's syndrome includes benign soft tissue and bony tumors, dental abnormalities, desmoid tumor and polyps in the upper gastrointestine (Gardner and Richards, 1953). Additionally, mice with germline mutations of the murine homolog of the APC gene (Min, for multiple intestinal neoplasia) have been reported to manifest a phenotype similar to that of FAP (Moser *et al.*, 1990; Su *et al.*, 1992).

The APC gene is localized to the human chromosome 5q21-22 (Herrera *et al.*, 1986; Bodmer *et al.*, 1987; Leppert *et al.*, 1987), consists of 16 exons, one non-coding and 15 coding (Groden *et al.*, 1991), and is expressed in all tissues that have been tested. The product of the APC gene is a 300 kDa protein composed of 2843 amino acids (Groden *et al.*, 1991; Kinzler *et al.*, 1991; Smith *et al.*, 1993). Both germline and somatic mutations are almost all either nonsense or frame-shift mutations that result in a COOH-terminal truncation of the APC gene product (Groden *et al.*, 1991; Nishisho *et al.*, 1991; Miyoshi *et al.*, 1992a,b; Powell *et al.*, 1992; Su *et al.*, 1993a). Cell fractionation experiments and immunohistochemical analyses suggested that APC is present as insoluble aggregates in the cytoplasm (Smith *et al.*, 1993). Furthermore, it has recently been reported that the APC protein is associated with the cell adhesion molecule E-cadherin-associated proteins α-, β-, and γ-catenin, suggesting that APC is involved in cell adhesion (Nagafuchi *et al.*, 1991; Takeichi, 1991; Shimoyama *et al.*, 1992; Oda *et al.*, 1993; Rubinfeld *et al.*, 1993; Su *et al.*, 1993b; Shibata *et al.*, 1994). However, little has been investigated regarding the distribution and localization of the APC protein and catenins in normal tissues. Thus, in the present study, we have developed polyclonal antibodies to human APC and examined the subcellular localization of the APC protein by immunohistochemical and immunoelectron microscopic analysis. Furthermore, double-labeling immunoelectron microscopy was performed to see whether the APC protein and α-catenin are colocalized at the subcellular level in normal intestinal epithelial cells.

Results

Polyclonal antibodies against APC protein

To study the subcellular localization of the APC protein, we generated anti-human APC polyclonal

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antibodies, anti-APC-C-ter, by immunizing rabbits with a synthetic peptide corresponding to the COOH-terminal 14 amino acids of APC. The antibodies were affinity purified and were tested for their ability to immunoprecipitate the APC protein from the human osteosarcoma cell line HOS. HOS cells were metabolically labeled with [^{35}S]methionine, lysed and subjected to immunoprecipitation with the anti-APC-C-ter antibodies. Similar to the result of Smith *et al.* (1993), anti-APC-C-ter immunoprecipitated a large amount of the 300 kDa APC protein from HOS cells and immunoprecipitation was blocked by preincubating the antibodies with the antigenic peptide (Figure 1). From mouse cerebellum lysate, the anti-APC-C-ter antibodies also immunoprecipitated the 300 kDa APC protein, which was detectable by subsequent Western blotting analysis. As reported previously (Rubinfeld *et al.*, 1993; Su *et al.*, 1993b), α- and β-catenin were detected in the immunoprecipitates prepared withanti-APC-C-ter antibodies (data not shown). These results suggest that anti-APC-C-ter antibodies recognize the APC protein.

Immunohistochemical analysis of the APC protein

To define the distribution and localization of the APC protein in the intestine, immunohistochemical studies were performed on frozen sections of normal mouse duodenum, proximal and distal colons using anti-APC-C-ter antibodies.

Epithelia of the duodenum, proximal and distal colons were immunoreactive with anti-APC-C-ter antibodies (Figure 2a–f). In each of these three regions of intestine, the staining intensity was uneven throughout the epithelium and varied depending on the level of intestinal villus and crypt. In the duodenum sample, APC immunofluorescence was strongest at the top of the villus and became fainter toward the crypt

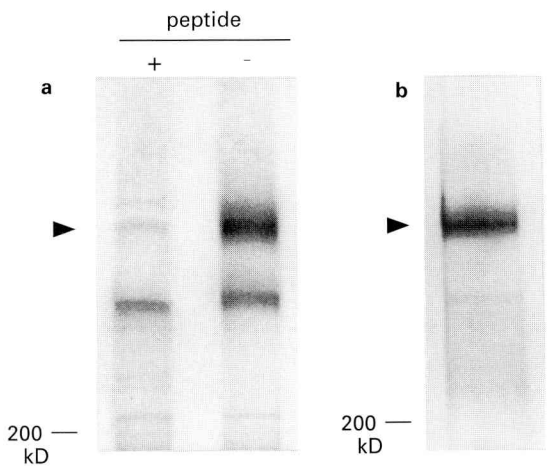

Figure 1 Detection of the APC protein by immunoprecipitation and Western blotting analysis using anti-APC polyclonal antibodies. (**a**) HOS cells were labeled with [^{35}S]methionine for 4 h. The APC protein was immunoprecipitated with anti-APC-C-ter antibodies or those preabsorbed with an excess of peptide antigen. The immunoprecipitates were analysed by SDS/PAGE gel electrophoresis followed by autoradiography. (**b**) Normal mouse cerebellum lysate immunoprecipitated with anti-APC-C-ter antibodies were separated by SDS/PAGE gel and transferred to a nylon membrane. The APC protein was detected with anti-APC-C-ter antibodies. The APC protein is indicated by an arrowhead

where only weak immunoreactivity was detected (Figure 2a). In the proximal and distal colons, the APC protein was most abundantly expressed in the surface epithelial cells. In crypts, the staining of the APC protein was higher in the upper portions and decreased toward the basal portions (Figure 2b and c).

In the epithelial cells of the duodenal villi, immunoreactivity toward the APC protein was prominent at both the apical and lateral cell borders, whereas staining was faint in the inner cytoplasm and negative in the nucleus and basal cell borders (Figure 2d). A similar immunostaining pattern was detected in the surface epithelial cells of the proximal colon (Figure 2e). In distal colon epithelial cells, the lateral cell borders showed the most marked immunoreactivity, while the apical cell borders were less intensely immunostained (Figure 2f). In a certain number of the distal colon epithelial cells, the basal cytoplasm exhibited a more intense staining than the apical ones. Mucus in goblet cells was negative for immunoreactivity. Self-fluorescence of mast cells in connective tissue under the epithelium was detected in both experimental and control sections. No immunoreactivity was detected in controls sections (data not shown).

We next examined expression of APC in intestinal neoplasia in the Min mice that carry a nonsense mutation at nucleotide 2549 of the murine homolog of the APC gene (Moser *et al.*, 1990; Su *et al.*, 1992). While anti-APC-C-ter antibodies detected expression of APC in the normal epithelial cells, no immunoreactivity was detected in the adenomas and adenocarcinomas developed in the intestinal tract (Figure 3).

Immunohistochemical analysis of α-catenin

Immunohistochemical localization of α-catenin in the duodenum, proximal and distal colons of normal mice were examined using monoclonal rat anti-mouse α-catenin antibody. The immunofluorescence corresponding to α-catenin was present only at the lateral cell borders of the epithelial cells in duodenum, proximal and distal colons (Figure 2g–i) and the staining intensity was even along the entire length of each immunofluorescent line. In contrast to APC, staining of α-catenin was not detected in the apical and basal cell borders. The cytoplasm was slightly immunopositive, whereas the nucleus exhibited no immunoreactivity. Interestingly, similar to the expression pattern observed for APC, the immunostaining of α-catenin was prominent at the top of the villus in the duodenum and in the surface epithelium of proximal and distal colons, but was weak in the base of the villus and crypts.

These results suggest that the APC protein is associated with α-catenin near the lateral plasma membrane, but is not associated with α-catenin in the apical region.

Immunoelectron microscopy of the APC protein and α-catenin

To further clarify the intracellular localization of the APC protein in normal mouse intestinal epithelial cells, we performed electron microscopic post-embedding immunocytochemistry using Lowicryl thin sections, anti-APC-C-ter antibodies and colloidal gold-conju-

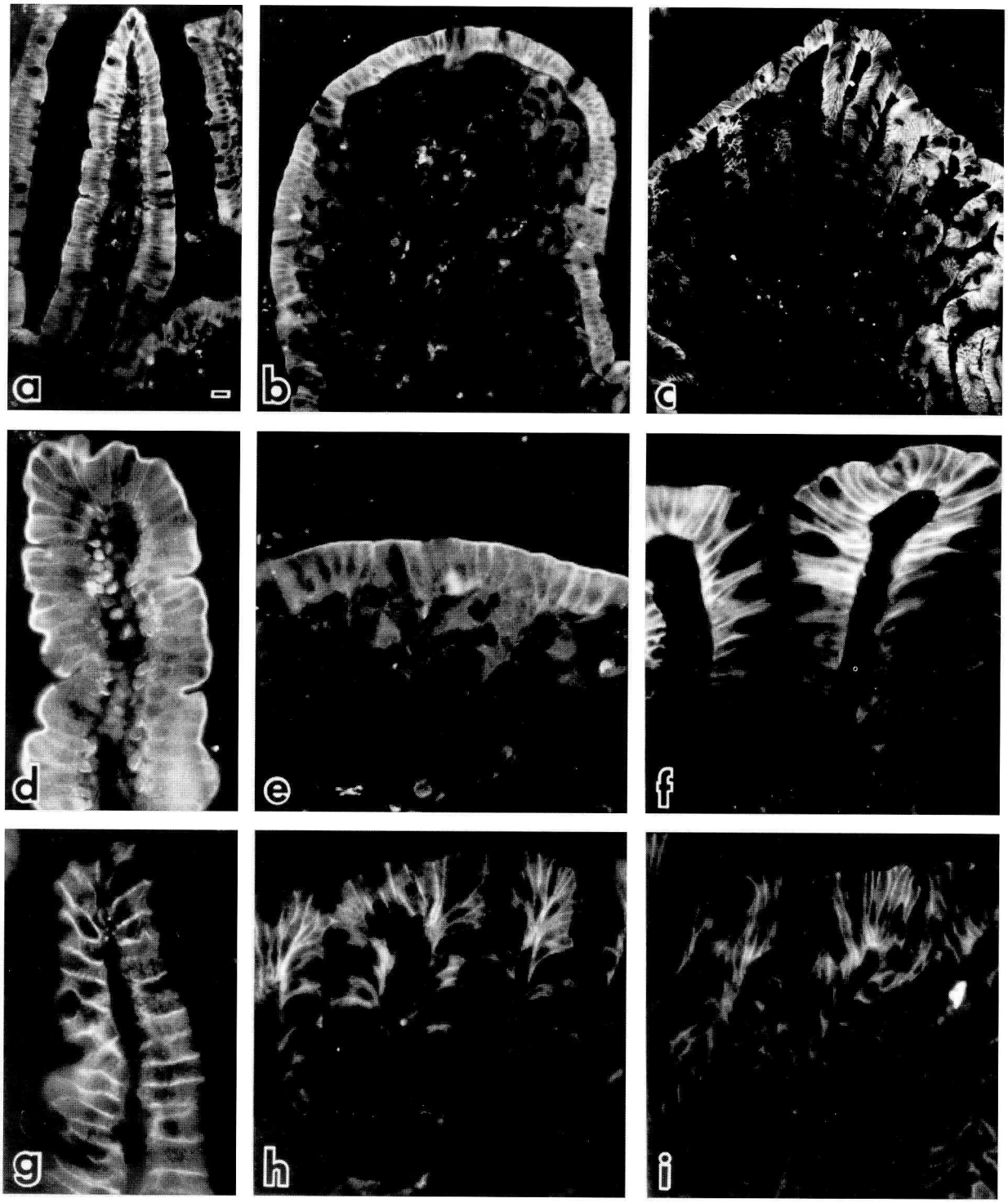

Figure 2 Immunohistochemical localization of the APC protein and α-catenin in mouse duodenum, proximal and distal colons. Frozen sections of duodenum (**a, d** and **g**), proximal (**b, e** and **h**) and distal (**c, f** and **i**) colons were stained with anti-APC-C-ter antibodies (**a–f**) or anti-α-catenin antibody (**g–i**). The expression of the APC protein was prominent at the top of a villus (**a**) and in the surface epithelium (**b** and **c**) and decreased gradually toward the base of villus and crypt (**a–c**). The APC protein was localized mainly at the apical and lateral cell borders of the epithelial cells (**d–f**). The localization of α-catenin was confined to the lateral cell borders (**g–i**) (Bar = 10 μm for **a–c** and 5 μm for **d–i**)

gated second antibody. A number of 10 nm gold particles which represent the localization of the APC protein were seen in microvilli, in the apical cytoplasm, and in the cytoplasm along the lateral plasma membrane (Figure 4). A few gold particles were scattered throughout the inner cytoplasm (Figure 4) and no significant labeling was detected in the nucleus (data not shown).

Subsequently, we performed double-labeling immunoelectron microscopy to analyse the precise relationship between the APC protein and α-catenin. Five nm gold particles marking the localization of α-catenin were distributed mainly underneath the lateral plasma membrane and in the cytoplasm nearby (Figure 5a and b). A close association of the 5 nm and 10 nm gold particles was frequently observed along the lateral

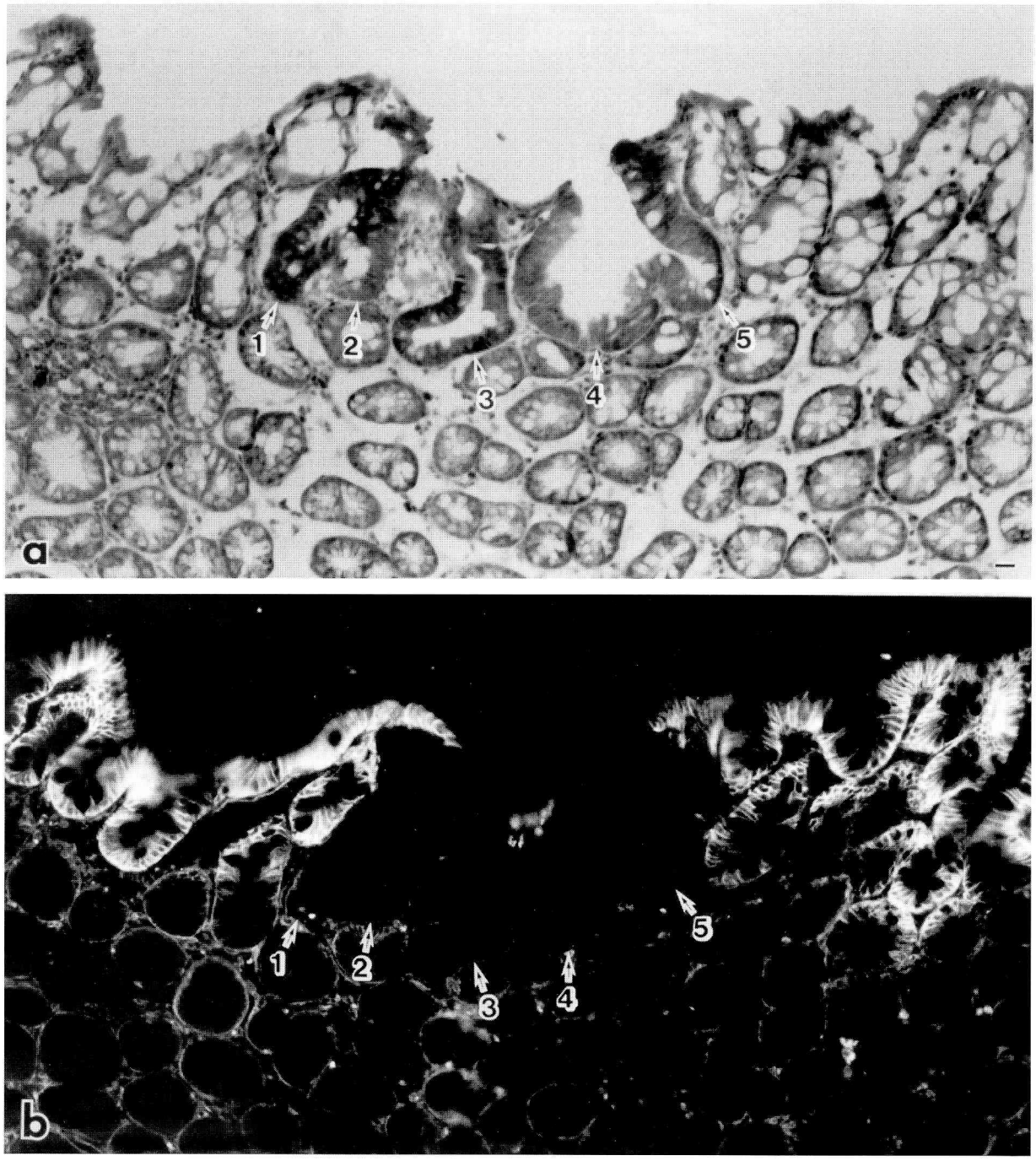

Figure 3 Immunohistochemical staining of the APC protein in the Min mouse distal colon including adenoma. Frozen sections were stained with hematoxylin-eosin (**a**) and the next serial ones were immunostained with anti-APC-C-ter antibodies (**b**). All of the crypts in adenoma (1–5 in **a**) showed no immunoreactivity for the APC protein (1–5 in **b**). The epithelium excluding adenoma exhibited the same immunostaining pattern as in the normal mouse colon (Bar = 20 μm)

plasma membrane from the apical to basal ends and in the cytoplasm nearby (Figure 5a and b). On the other hand, a certain number of 5 nm and 10 nm particles in the same region were localized independent of one another. The colocalization of the APC protein and α-catenin was rarely detected in other regions of the cell; few 5 nm gold particles were seen in the apical cytoplasm and on microvilli where the APC protein was abundantly expressed (Figure 5c). Neither particle was more than weakly detected in the inner cytoplasm and nucleus (data not shown). Very few 5 nm or 10 nm gold particles were seen in any regions of the epithelial cells in the control sections (Figure 5d).

Discussion

The predicted amino acid sequence of the APC protein has little sequence similarity to other proteins, providing few clues to its mechanism of action (Groden *et al.*, 1991; Kinzler *et al.*, 1991). However, the APC protein has recently been reported to form a cytosolic complex with catenins, suggesting that the APC protein is involved in cell adhesion (Nagafuchi *et al.*, 1991; Takeichi, 1991; Shimoyama *et al.*, 1992; Oda *et al.*, 1993; Rubinfeld *et al.*, 1993; Su *et al.*, 1993b; Shibata *et al.*, 1994). In addition, cell fractionation experiments and immunohistochemical

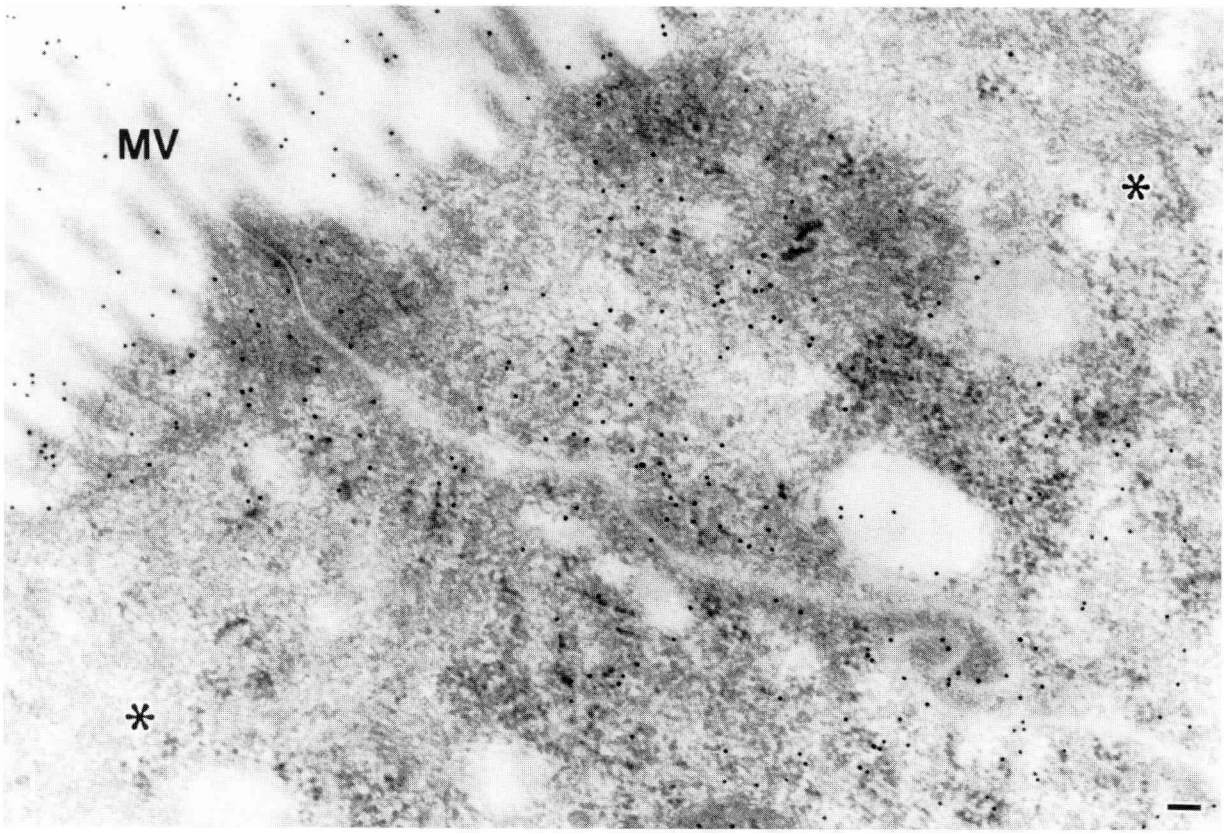

Figure 4 Intracellular localization of the APC protein in mouse duodenal epithelial cells detected by immunoelectron microscopy. Ultrathin sections were stained with anti-APC-C-ter antibodies and 10 nm colloidal gold-conjugated second antibody. A number of 10 nm particles marking the APC protein were seen on microvilli (MV), in apical cytoplasm and along the lateral plasma membranes. The APC protein was barely detected in the inner cytoplasm (*) (Bar = 100 nm)

studies suggested that the APC protein is present as insoluble aggregates in the cytoplasm (Smith *et al.*, 1993). In the present study, we analyzed whether the APC protein and α-catenin are colocalized in the normal mouse intestine by light and immunoelectron microscopy. Our immunohistochemical analysis demonstrated that both the APC protein and α-catenin are localized in the lateral cytoplasm of intestinal epithelial cells. Furthermore, double-labeling immunoelectron microscopy confirmed that both proteins are indeed closely colocalized in the lateral cytoplasm, in particular along the lateral plasma membrane. Hence the APC protein may function in a complex with catenins in the lateral cytoplasm of the intestinal epithelial cells.

The formation of a complex between the APC protein and catenins was reported to be independent of cadherin (Rubinfeld *et al.*, 1993; Su *et al.*, 1993b). Consistent with this notion, detailed analysis of the dynamics of cadherin-catenin complex formation recently showed that as much as 50% of catenins are not directly associated with E-cadherin (Hinck *et al.*, 1994; Näthke *et al.*, 1994). Thus, the APC α-catenin complex detected in this study is most likely to be independent of cadherin. On the contrary, β-catenin is presumably contained in the complex, since α-catenin is believed to bind indirectly to the APC protein through its association with β-catenin, the latter which directly binds to the APC protein (Rubinfeld *et al.*, 1993; Su *et al.*, 1993b). However, we were not able to examine the localization of β-catenin, because anti-β-catenin antibodies suitable for immunostaining and immunoelectron microscopy were not available.

It has recently been reported that the wild-type but not mutant APC protein associates with microtubules *in vivo* via its COOH-terminal region, a region which is typically deleted in colorectal cancers (Munemitsu *et al.*, 1994; Smith *et al.*, 1994). In addition, the APC protein was found to dramatically promote microtubule assembly *in vitro* (Munemitsu *et al.*, 1994). Nevertheless, we did not observe the filamentous staining pattern which is characteristic of microtubules. One reason for this discrepancy may be a difference in the level of the APC protein expressed in the cells examined; the staining patterns reported by Smith *et al.* and Munemitsu *et al.* were obtained with cells transiently overexpressing the transfected APC gene, whereas in our experiments the endogenous APC protein was stained. Overexpression of the APC protein may induce microtubule assembly in the living cells as observed *in vitro*.

One of the most intriguing findings in this study is that the APC protein is localized not only in the lateral cytoplasm but also on microvilli and in the apical cytoplasm of the intestinal epithelial cells where α-catenin is not present. Thus, the APC protein is likely to have additional functions independent of catenins, and also may associate with proteins other than catenins. Since microvilli is abundant in microfilaments but devoid of microtubules and intermediate filaments, it is interesting to

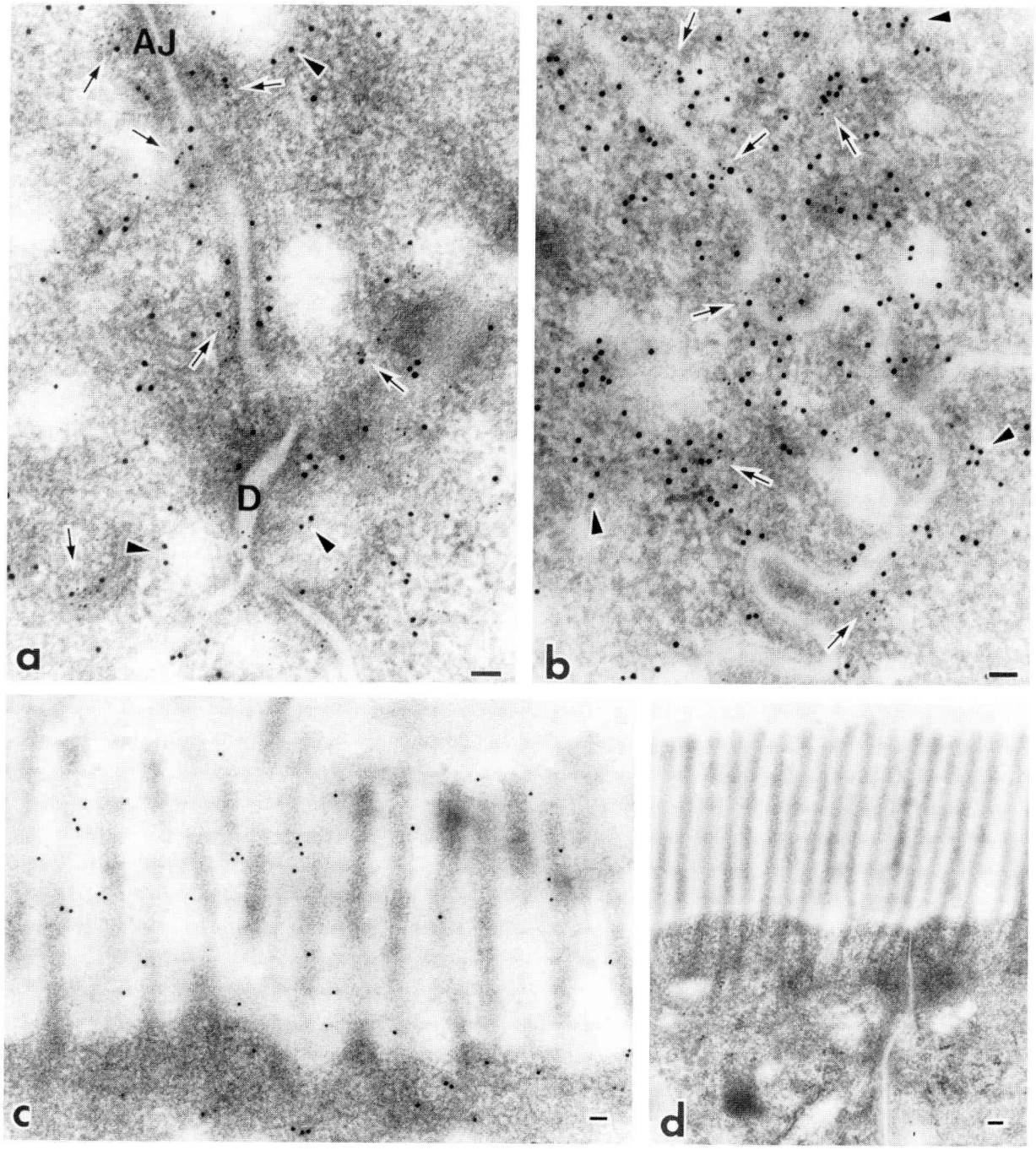

Figure 5 Double labeling-immunoelectron microscopic demonstration of the APC protein and α-catenin in mouse duodenal epithelial cells. Ultrathin sections were stained with anti-APC-C-ter antibodies, anti-α-catenin antibody, 10 nm colloidal gold-conjugated anti-rabbit IgG and 5 nm colloidal gold-conjugated anti-rat IgG. (**a**), (**b**) Colocalization of 10 nm (the APC protein) and 5 nm (α-catenin) particles was observed along the lateral plasma membranes and in the cytoplasm nearby (arrows). Independent localization of the APC protein was also detected in the same region (arrowheads). Colocalization of both proteins was very rare in the inner cytoplasm. AJ, adherence junction; D, desmosome. a, apical part of the epithelial cells; b, basal part of them. (**c**) The APC protein was present on microvilli and in the apical cytoplasm where α-catenin was barely detected. (**d**) No particles were detected when the control rabbit serum and control rat serum were used for staining in place of anti-APC-C-ter antibodies and anti-α-catenin antibody α-18 (Bars = 50 nm in **a**–**c** and 100 nm in **d**)

speculate that the APC protein is associated with microfilaments or microfilament-binding proteins in microvilli.

Similar to the finding of Smith *et al.* (1993), our immunohistochemical analysis showed that the staining of the APC protein gradually increases from the base of the crypt to the luminal surface, suggesting a relationship between the expression of the APC proteins and differentiation of intestinal epithelial cells. In this regard, it is interesting to note that APC shows significantly high expression in the brain (Bhat *et al.*, 1994; our unpublished observation). In particular, during the development of the cortex, cerebellum and retina, APC and mRNA expression was prominent in layers containing newly formed postmitotic neurons, with lower levels observed in the proliferative zones where neurogenesis occurs.

The anti-APC-C-ter antibodies did not stain the

adenomas and adenocarcinomas developed in the intestinal tract of the Min mice, while the antibodies specifically stained the normal epithelial cells. Since the protein product generated from the mutant APC gene of Min mice could be truncated polypeptide with 850 amino acids that is not recognized by the anti-APC-C-ter antibodies, APC detected in the normal epithelial cells of Min mice would be derived from the normal allele of the APC gene. Lack of the immunoreactivity of the adenomas and adenocarcinomas, therefore, implies that the normal allele of the APC gene is inactivated in the tumor cells, being consistent with the two-hit theory (Knudson, 1971).

In conclusion, a certain portion of the APC protein was found to colocalize with α-catenin in the intestinal epithelial cells but there also exists APC protein that is not colocalized with α-catenin. Thus, the APC protein seems to have both catenin-dependent and -independent functions. Identification of novel APC associated protein is currently under way in our laboratories.

Materials and methods

Cell culture

Human osteosarcoma HOS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum.

Antibodies

Anti-APC-C-ter antibodies used for immunoprecipitation and immunostaining were prepared by immunizing rabbits with a synthetic peptide corresponding to the COOH-terminal 14 amino acids of the human APC protein and purified by affinity chromatography on a column to which the synthetic peptide had been covalently linked. The sequence of the COOH-terminal 14 amino acids of the human APC protein was identical to that of the mouse APC protein. Anti-α-catenin monoclonal antibody α-18 was prepared as described elsewhere (Nagafuchi and Tsukita 1994).

Immunoprecipitation

Cells were labeled for 4 h in methionine-free DMEM containing 1 mCi of [^{35}S]methionine (100 mCi ml^{-1}, 1200 Ci mmol^{-1}, New England Nuclear) and lysed in solubilizing buffer [50 mM Tris (pH 7.2), 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 50 mM β-glycerophosphate, 5 mM DTT, 1 mM sodium orthovanadate, 0.05 mM NaF, 0.1 mM (p-amidinophenyl) methanesulfonyl fluoride and leupeptid (5 mg ml^{-1})]. The lysates were incubated with antibodies for 1 h at 4°C. The immunocomplexes were absorbed to protein A-Sepharose 4B and washed extensively with solubilizing buffer. Samples were analysed by 5% SDS-polyacrylamide gel electrophoresis followed by autoradiography. Tissues were also lysed, immunoprecipitated, and size fractionated in a 5% SDS-polyacrylamide gel and transferred to a nylon membrane, and the APC protein was detected by sequential binding of a specific first antibody followed by alkaline phosphatase-conjugated second antibodies.

Immunohistochemical analaysis

Duodenum, proximal and distal colons of normal male ddY mice and Min mice, which were obtained from The Jackson Laboratory, were used for immunohistochemical analysis. Frozen sections of the normal mouse organs were prepared as described previously (Senda *et al.*, 1991). The sections were incubated at 4°C overnight with anti-APC-C-ter antibodies diluted 1:50 with PBS or with anti-α-catenin antibody (α-18) diluted 1:1 with PBS. After washing in PBS, the sections were incubated at room temperature for 1 h with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG antibody (Seikagaku Kogyo) diluted 1:100 with PBS for anti-APC-C-ter antibodies, or in FITC-labeled goat anti-rat IgG antibody (Seikagaku Kogyo) diluted 1:100 for anti-α-catenin antibody. The sections washed in PBS were examined with a Fluophoto fluorescence microscope (Nikon). Control sections were incubated with normal rabbit or rat serum instead of anti-APC-C-ter or anti-α-catenin antibodies, respectively. Serial frozen sections of the Min mouse colon were prepared as they include both tumor and normal tissues. They, alternatively, were immunostained with anti-APC-C-ter antibodies as described above, and stained with hematoxylin-eosin.

Immunoelectron microscopy

Duodenum of normal male ddY mice aged 8 weeks were used for immunoelectron microscopic analysis. Lowicryl K4M ultrathin sections were prepared according to the method of Senda *et al.* (1991). The sections were incubated at 4°C overnight with anti-APC-C-ter antibodies diluted 1:50 with PBS. The sections were incubated with 10 nm-colloidal gold-conjugated goat anti-rabbit IgG antibody (Amersham) diluted 1:30 with PBS at room temperature for 2 h. The sections were washed, stained with uranyl acetate and lead citrate and examined with a H-700 transmission electron microscope (Hitachi). Control sections were incubated with normal rabbit serum. For double-labeling immunoelectron microscopy, the sections were incubated with anti-α-catenin antibody and anti-APC-C-ter antibodies diluted 1:50 at 4°C overnight. After washing, the sections were incubated at room temperature for 2 h with a mixture of 10 nm-colloidal gold-conjugated goat anti-rabbit IgG antibody diluted 1:30 and 5 nm-colloidal gold-conjugated goat anti-rat IgG antibody (Amersham) diluted 1:30. The sections were stained with uranyl acetate and lead citrate and examined with the electron microscope. Control sections were incubated with a mixture of normal rabbit serum and normal rat serum.

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