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

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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.

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 GTPase-activating protein and the ERM family proteins (3–5), responsible for the genesis of familial adenomatous polyposis. However, further studies revealed that inactivation of another gene isolated from 5q21, APC, is responsible for the genesis of familial adenomatous polyposis (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 adherent 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 817–829. 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-BrdUrd¹ antibody was from Takara (Tokyo, Japan).

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 (Life Technologies, Inc.). cDNAs encoding the full-length human β-galactosidase were also cloned into the pME18S vector. A mutant MCC encoding Gin in place of Arg-506 was generated by site-directed mutagenesis (18) using the synthetic oligonucleotide 5′-CAT-GACTGCGCAAGAAGACGTG-3′. Another mutant encoding Thr and Ala in place of Lys-233 and Glu-234, respectively, was generated using the

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1 The abbreviations used are: BrdUrd, bromodeoxyuridine; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid.
synthetic oligonucleotide 5'-CGGGTGGGAGACGCGTGCTTGGA-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 h in DMEM containing 0.5% calf serum. Under these conditions, incubation with 50 μM BrdUrd for an additional 24 h resulted in labeling of less than 5% of the nuclei. For synchronized induction of the growth of fresh, medium-fed cells, 10% calf serum with 50 μM BrdUrd was added to serum-starved cells. During cell cycle experiments, the cell cycle was monitored by flow cytometric analysis with a FACScan/CellII/FIT DNA system (Becton Dickinson). SV40-transformed CV-1 (COS-7) cells were cultured in DMEM supplemented with 10% fetal calf serum.

Immunoprecipitation—Cells were labeled for 4 h in methionine-free DMEM containing [35S]methionine (100 μCi/ml, 1200 Ci/mmol, DuPont NEN). For 3P labeling, cells were labeled for 2 h in phosphate-free DMEM containing [32P]orthophosphate (200 μCi/ml, Amersham). The labeled cells were lysed in solubilization buffer containing 10 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, and 10 μg/ml 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). For ty,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 cells lysed were resolved on an 8% SDS-polyacrylamide gel and transferred to poly(vinylidene difluoride) membrane for immunodetection. 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 × 10^7) were suspended in 2 ml of KMP buffer (5 mM KCl, 1 mM MgCl₂, 20 mM PIPES, pH 7.0). The cells were then allowed to swell for 5 min at 4°C and stirred with 20 strokes in a Potter-Elvehjem homogenizer (Wheaton). The nuclei were pelleted by centrifugation at 1000 × 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 × 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 × 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-m cushion of KMP containing 50% sucrose and centrifuged at 15,000 × g for 40 min. The supernatant and interface were further centrifuged in a 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 these fractions was confirmed by staining with 4',6-diamidino-2-phenylindole (DAPI).

Immunofluorescence Analysis—Frozen sections of intestine and cerebral cortex of normal male 5-day 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 fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibodies (Amersham Corp.) diluted 1:100 in PBS at room temperature for 1 h. Subsequently, the sections were stained with uranyl acetate and lead citrate and analyzed with a J EOL 1200 EX transmission electron microscope. Control sections were incubated with the anti-MCC antibodies that had been preabsorbed with an excess of antigen used for immunization.

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 817–829 of the predicted protein. The antibodies recognized [35S]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 [35S]methionine- and [32P]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 [35S]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 [35S]methionine were prepared according to the method of Senda et al. (21). The Lowry KWM ultrathin sections were prepared according to the method of Senda et al. (21). The Lowry sections were incubated at 4°C overnight with anti-MCC antibodies diluted 1:50 with PBS, followed by 10-nm colloidal gold-labeled goat anti-rabbit IgG antibodies (Amersham Corp.) 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 J EOL 1200 EX transmission electron microscope. Control sections were incubated with the anti-MCC antibodies that had been preabsorbed with an excess of antigen used for immunization.

Microinjection—NIH3T3 cells, which were grown on coverslips (2 × 10^3 cells/dish), were cultured in DMEM containing 0.5% calf serum for 24 h and then microinjected with the normal or mutated MCC 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 BrdUrd, and then cells were incubated for another 18 h. Cells were fixed in 3% formaldehyde in PBS for 30 min, dehydrated with 100% methanol for 10 min, and then treated with 4% HCl for 10 min. MCC and β-galactosidase were detected with the affinity-purified antibodies, followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibodies. BrdUrd was detected with an anti-BrdUrd monoclonal antibody BU-4, followed by rhodamine B isothiocyanate-conjugated goat anti-mouse IgG antibodies. To arrest the cells at G0/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 BrdUrd was added after incubation for another 6 h.
The cytoplasm of the epithelial cells showed weak immunoreactivity mainly along lateral cell borders of the epithelial cells (Fig. 3A), indicating 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. By contrast, control sections stained with anti-MCC antibodies that had been preabsorbed with antigenic peptide 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 anti-MCC antibodies that had been preabsorbed with antigenic peptide 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 [32P]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. NIH3T3 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 BrdUrd 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 BrdUrd incorporation. As summarized in Fig. 5, incorporation of BrdUrd 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 G0/G1 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. The MCC cDNA was also microinjected into cells that had been arrested at G0/G1 by aphidicolin treatment.
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 whether 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% Nonidet P-40 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. Additionally, 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 G0/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-K237T/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 β proteins (reviewed in Ref. 43), a growth arrest-specific membrane protein Gas1 (44), prohibitin (45), and a Ras-related transformation suppressor gene product K-Ras-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 (29), 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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