Journal of Cellular Biochemistry 93:345–357 (2004)

Zinc Stabilizes Adenomatous Polyposis Coli (APC) Protein Levels and Induces Cell Cycle Arrest in Colon Cancer Cells

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Abstract In the present study, we investigated the mechanisms by which zinc causes growth arrest in colon cancer cells. The results suggest that zinc treatment stabilizes the levels of the wild-type adenomatous polyposis coli (APC) protein at the post-translational level since the APC mRNA levels and the promoter activity of the *APC* gene were decreased in HCT-116 cells (which express the wild-type *APC* gene) after treatment with $ZnCl_2$. Increased levels of wild-type but not truncated APC proteins were required for the $ZnCl_2$-mediated G_2/M phase arrest in different colon cancer cell lines. We further tested whether serum-stimulation, which induces cell cycle arrest in the S phase, can relieve $ZnCl_2$-induced G_2/M phase arrest of HCT-116 cells. Results showed that in the HCT-116 cells pretreated with $ZnCl_2$, the serum-stimulation neither changed the distribution of G_2/M phase arrested cells nor the increased levels of APC protein. The G_2/M phase arrest correlated with retarded growth of HCT-116 cells. To further establish that wild-type APC protein plays a role in $ZnCl_2$-induced G_2/M arrest, we treated SW480 colon cancer cells that express truncated APC protein. We found that $ZnCl_2$ treatment did not induce G_2/M phase arrest in SW480 cells; however, the cell growth was retarded due to the loss of E-cadherin and α-tubulin levels. These results suggest that $ZnCl_2$ inhibits the proliferation of colon cancer cells (which carry the wild-type *APC* gene) through stabilization of the APC protein and cell cycle arrest in the G_2/M phase. On the other hand, $ZnCl_2$ inhibits the proliferation of colon cancer cells (which carry the mutant *APC* gene) by disrupting cellular attachment and microtubule stability. J. Cell. Biochem. 93: 345–357, 2004.

Key words: colon cancer; zinc; APC; cell cycle; cellular attachment

Zinc is required for the normal functioning of a large number of enzymes, and is a structural component of many proteins, including hormones, neuropeptides, and receptors. Zinc deficiency has a profound adverse effect on cellular biochemistry and is recognized as a worldwide public health problem [Berg and Shi, 1996]. Zinc deficiency is often associated with the impairment of memory, growth retardation, male hypogonadism, neurosensory changes, frequent infections, delayed wound healing, depressed immune function, mental disturbances, and impaired cognitive functions [Shankar and Prasad, 1998]. Most of these effects can be reversed by the inclusion of zinc supplements in the diet [Prasad, 1998]. In animal studies, a zinc-deficient diet has shown to enhance *N*-nitrosomethylbenzylamine (NMBA)-induced esophageal cell proliferation and the incidence of esophageal tumors [Fong and Magee, 1999]. The above condition becomes normalized by the re-addition of zinc to the diet [Fong et al., 2001]. Furthermore, the addition of zinc to cell culture media has been reported to inhibit the growth of prostate [Liang et al., 1999; Uzzo et al., 2002], lymphoblastoid [Prasad et al., 2001], and colon [Park et al., 2002] cancer cells in vitro.

Abbreviations used: APC, adenomatous polyposis coli; CAT, chloramphenicol acetyltransferase; FACS, fluorescence activated cell sorting; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2yl-)-2,5-diphenyl tetrazolium bromide.

Grant sponsor: National Cancer Institute/National Institutes of Health; Grant numbers: RO1-CA77721, RO1-CA97031.

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Received 21 November 2003; Accepted 20 April 2004
DOI 10.1002/jcb.20156

Previous studies have indicated that zinc deficiency is one of the primary risk factors in the development of colorectal carcinogenesis [Martin Mateo and Martin, 1988; Song et al., 1993]. Chronic treatment of zinc-deficient mice with the colon carcinogen dimethylhydrazine (DMH) caused rapid formation of adenomatous polyps and invasive adenocarcinomas [Carter et al., 1997]. The formation of multiple adenomatous polyps or aberrant crypt foci (ACF) is a characteristic of the initial stages of the development of colon cancer and is often linked with mutations in the adenomatous polyposis coli (*APC*) gene [Fearnhead et al., 2001; van Es et al., 2001; Bienz, 2002]. A direct causal relationship has been established between the loss-of-function of the *APC* gene and the development of colorectal cancer in Apc(Min/+) mice. These mice carry a premature stop codon in one allele of the *Apc* tumor suppressor gene, develop multiple intestinal adenomas, and are a murine model of familial APC [Moser et al., 1990; Su et al., 1992]. It is well established that compared to wild-type C57BL/6J mice, Apc(Min/+) mice exhibit rapid growth of intestinal tumors and enhanced susceptibility to carcinogens such as azoxymethane (AOM), heterocyclic amines, and ethylnitrosourea (ENU). In addition to mutations, a reduction in the expression of APC has been found to be associated with susceptibility to AOM-induced colorectal carcinogenesis in rats [Shoemaker et al., 1995; Steffensen et al., 1997; Kishimoto et al., 2002; Suzui et al., 2002] and the induction of lung carcinogenesis by urethane, *N*-nitrosodiethylamine, or 3-methylcholanthrene in (A/J × 57BL/6) F1 or A/J mice [Oreffo et al., 1998]. From these studies, it is clear that APC is involved in the control of proliferation of both colonic and extra-colonic tissues.

The APC protein has several known functions. One is to control cell cycle progression, which has been reported to involve the modulation of the G_0/G_1-S phase components by the retinoblastoma (Rb)-pathway in serum-induced NIH-3T3 cells [Baeg et al., 1995] and controls entry into S-phase through its ability to regulate the cyclin D/RB pathway in colon cancer cells [Heinen et al., 2002]. It is also anticipated that APC may play a role in the G_2/M phase transition, especially when the APC protein is hyperphosphorylated during the M-phase and is a target of the M-phase kinase, p34Cdc2 [Dobashi et al., 1996; Trzepacz et al., 1997]. Furthermore, zinc has been found to control cell cycle progression in human malignant lymphoblastoid cell lines by functioning during the G_1 to S phase transition [Chesters et al., 1993; Prasad et al., 1996] while in prostate cancer cell lines it arrests the cells in the G_2/M phase [Liang et al., 1999]. Thus, it appears that zinc deficiency, mutations of the *APC* gene, and the reduced level expression of the wild-type *APC* gene are involved in cellular proliferation and tumor growth. It is not clear, however, whether there is a relationship between the zinc deficiency and the APC levels in colon cancer cells. Since the supplementation of zinc is found to reverse many of the effects of zinc deficiency [Prasad, 1998; Fong et al., 2001], we hypothesized that zinc supplementation increases the levels of the wild-type APC protein and causes cell cycle arrest to prevent abnormal proliferation. Our results suggest that $ZnCl_2$ inhibits the proliferation of colon cancer cells either by cell cycle arrest in G_2/M phase (cells express the wild-type APC protein) or by disrupting cell–cell communication and microtubule stability (cells express the truncated APC protein).

MATERIALS AND METHODS

Maintenance and Treatment of Cells

The human colon cancer cell lines HCT-116, SW48, LS174T (expressing wild-type APC), SW480, and LoVo (expressing mutant APC) were grown at 37°C under a humidified atmosphere of 5% CO_2 in McCoy's 5a medium, except LoVo cell line which was grown in Ham's F12 medium. In each case, the medium was supplemented with 10% fetal bovine serum (FBS; Cell Grow, Mediatech, VA), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. After cells reached 60% confluence, fresh medium containing 0.5% FBS and antibiotics were added to each plate and further incubated for an additional 20 h. The regimens for treatment with $ZnCl_2$ (Sigma Chem. Co., St. Louis, MO) are given in the figure legends.

Western Blot Analysis

Changes in APC and cell cycle-related protein levels subsequent to treatment of colon cancer cells with $ZnCl_2$ were determined by Western blot analysis using whole cell extracts, as described previously [Narayan and Jaiswal, 1997]. The antibodies used to detect the levels of the various proteins on the blots included mouse

monoclonal antibodies specific for: APC (Ab-1, Oncogene Research Products, Cambridge, MA); α-tubulin (Sigma Chem. Co.); β-catenin (H-1α) Cdc2-p34 (17), Cdk2 (H-298), cyclin B1 (GNS-1), cyclin A (BF-683) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and E-cadherin (BD Biosciences, Palo Alto, CA).

Northern Blot Analysis and Chloramphenicol Acetyltransferase (CAT)-Reporter Activity

The APC mRNA levels were determined by Northern blot analysis [Narayan and Jaiswal, 1997]. The total RNA loading in each sample and the transfer efficiency during blotting were normalized using an 18 S RNA probe. The promoter activity of the *APC* gene was determined by CAT-reporter assays using a cloned *APC* gene promoter, as described previously [Jaiswal and Narayan, 2001a,b].

FACScan Analysis

A detergent and proteolytic enzyme-based technique was used for nuclear isolation and analysis of DNA content of cells in different phases of the cell cycle. After treatment with $ZnCl_2$, the cells were harvested, processed, and nuclei stained with propidium iodide, as described previously [Jaiswal et al., 2002]. The cellular DNA content was analyzed using a Becton-Dickinson FACScan flow cytometer. At least 10,000 cells per sample were considered in the gated regions used for calculations. The ranges for G_0/G_1, S, G_2/M and sub-G_1 phase cells were established based upon the corresponding DNA contents as displayed in the histograms. Results were analyzed using the Modfit program and were expressed as the percentage of the total gated cells.

Histone H1 Kinase Assay

The immunocomplexes of the Cdk2/cyclin A and Cdc2/cyclin B1, which are specific for histone H1 phosphorylation, were used for the determination of kinase activity. The immunocomplexes were isolated with 150 μg of cell lysate using 2 μg of anti-Cdk2 and anti-Cdc2 antibodies as described earlier [Jaiswal et al., 2002]. The immunocomplexes were pre-incubated with a buffer containing 12.5 mM Hepes at pH 7.5, 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 2 mM EGTA, 0.5 mM orthovanadate, 0.5 mM NaF, and 0.5 mM DTT for 5 min at 30°C. The reaction was initiated with 10 μg histone H1 (Upstate Biotech, Inc., Lake Placid, NY), 20 μM ATP, and 1 μCi [γ^{32}P]ATP (3,000 Ci/mmol). After 20 min of incubation at 30°C, the reaction was stopped by the addition of SDS–PAGE sample buffer and boiled for 5 min. The ^{32}P-labeled histone H1 was separated on a 12% SDS–PAGE, and the signal was detected by autoradiography.

MTT Assay

The proliferation of HCT-116 and SW480 cell lines after treatment with $ZnCl_2$ for various time intervals was measured using an MTT cell proliferation assay kit from ATCC (Manassas, VA). In principle, the viable cell number is directly proportional to the purple formazan color of the reduced MTT dye, which can be quantitatively measured by spectrophotometry. Briefly, 500 cells were plated in triplicate in 96-well flat bottom tissue culture plates. Cells were allowed to grow for 20 h in 0.5% FBS containing medium, then treated with 100 μM of $ZnCl_2$ for different periods. After the treatment, 10 μl of MTT reagent was added to each well and incubated at 37μC for 4 h for the formation of purple color crystals of formazan. Then 100 μl of detergent solution was added to each well. The reaction mixture was incubated in the dark for 2–4 h (or sometimes overnight) at room temperature and the developed color density was measured spectrophotometrically at 570 nm using the micro-plate reader (Vmax Kinetic Microplate Reader, Molecular Device, Sunnyvale, CA).

Cell Morphology

The morphological changes in the control and $ZnCl_2$-treated cells were examined under an inverted microscope (Zeiss Axioplan-2 Imaging, Thornwood, NY) at 20-times magnification.

RESULTS

Treatment With $ZnCl_2$ Stabilizes the Levels of APC Protein and Induces G_2/M Phase Cell Cycle Arrest in HCT-116 Cells

Our present studies were directed toward understanding whether supplementation with extracellular zinc increases the amount of APC protein in colon cancer cells to a level that is sufficient to induce cell cycle arrest. For these studies, we utilized the human colon cancer cell line HCT-116, which expresses wild-type APC protein (310 kDa). The cells were grown in 0.5% FBS for 20 h prior to the addition of $ZnCl_2$. We

found a dose- and time-dependent increase in the APC protein level with the addition of $ZnCl_2$ (Fig. 1A and C). There was no change in the α-tubulin levels in $ZnCl_2$-treated HCT-116 cells, which served as a control for this experiment (Fig. 1B). The increase in the levels of APC protein in response to the addition of extracellular $ZnCl_2$ could be due to transcriptional up-regulation of *APC* gene expression or to stabilization of the APC protein. To address this issue, we first determined the levels of APC mRNA in HCT-116 cells. The cells were treated with different concentrations of $ZnCl_2$ for 30 h or 100 μM of $ZnCl_2$ for different periods, and the levels of APC mRNA were determined by Northern blot analysis. A dose- and time-dependent decrease in the levels of APC mRNA was observed (Fig. 1D). To further establish whether the decrease in the APC mRNA levels was due to decreased transcriptional activity of the *APC* gene, we determined the promoter activity of the *APC* gene in HCT-116 cells with and without $ZnCl_2$ treatment. We used cloned pAPCP promoter for these studies, which contains a genuine *APC* gene regulatory region as described in our previous studies [Jaiswal and Narayan, 2001a,b]. The pAPCP plasmid DNA was transfected into the HCT-116 cells and then treated with different concentrations of $ZnCl_2$ for 30 h. The CAT-reporter activity was measured to determine the effect of $ZnCl_2$ treatment on the pAPCP promoter activity. Results showed a decrease in the pAPCP promoter activity in HCT-116 cells after $ZnCl_2$ treatment (Fig. 1E), which was parallel to the decrease in APC mRNA level (Fig. 1D). This suggests that the increase in the levels of APC protein in HCT-116 cells after $ZnCl_2$ treatment does not arise due to increased *APC* gene expression and may therefore result from an increase in the stability of the APC protein. One of the mechanisms by which the $ZnCl_2$ treatment may cause stabilization of APC in HCT-116 cells may be due to reduced activity of caspase-3, which is known to induce the degradation of APC [Webb et al., 1999]. To test this hypothesis, we pre-treated HCT-116 cells with 100 μM $ZnCl_2$ for 1 h and then treated them with 500 μM methylmethane sulfonate (MMS), a DNA-alkylating agent, for an additional 30 h. MMS treatment is known to induce caspase-3 activity in cancer cells [Fishel et al., 2003]. The protein levels of APC and caspase-3 were determined in these cells. The results showed that $ZnCl_2$ pre-treatment blocked MMS-induced caspase-3 activity as well as the degradation of APC (Fig. 2, compare lane 3 with 4). Although the control cells showed an uninduced caspase-3 level, the APC level was increased after $ZnCl_2$ treatment (Fig. 2, compare lane 1 with 2). This may suggest that the low cellular level of caspase-3 without MMS treatment may be sufficient to regulate APC

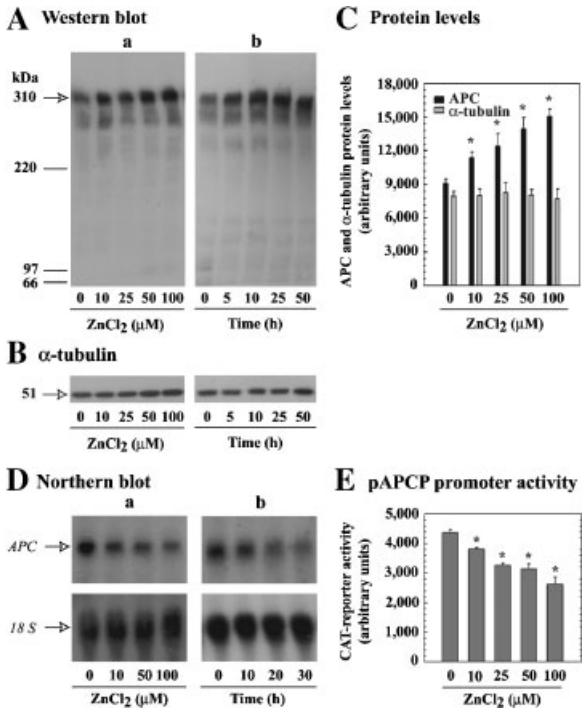

Fig. 1. Adenomatous polyposis coli (APC) protein and mRNA levels and *APC* gene's promoter activity in HCT-116 cells treated with $ZnCl_2$. Cells were treated with different concentrations of $ZnCl_2$ for different periods and processed for Western or Northern blot analysis. **Panel A**: Shows the results of Western blot analysis. Subset (**a**) represents a concentration curve in which cells were treated for 30 h and (**b**) represents a time course of 100 μM of $ZnCl_2$ treatment. **Panel B**: Shows the α-tubulin protein levels in each sample from untreated and $ZnCl_2$-treated cells. **Panel C**: Represents the quantitative analysis of APC and α-tubulin protein levels of HCT-116 cells treated with different concentrations of $ZnCl_2$ for 30 h. Data are mean ± SE of five and three independent experiments for APC and α-tubulin, respectively. **Panel D**: Is an autoradiogram of a Northern blot analysis. Subset (**a**) represents a concentration curve in which cells were treated for 30 h and (**b**) represents a time course of 100 μM of $ZnCl_2$ treatment. Arrows indicate the position of the APC protein, APC mRNA, and 18 S RNA. The autoradiograms shown are representatives of three independent experiments. **Panel E**: Depicts the cloned APC promoter (pAPCP) activity in the HCT-116 cells. Cells were transfected with pAPCP plasmid and then treated with different concentrations of $ZnCl_2$ for 30 h. Arbitrary units of the CAT-reporter activity are shown. Data are mean ± SE of three independent experiments. *Significantly different than untreated control (Student's *t*-test, $P < 0.05$).

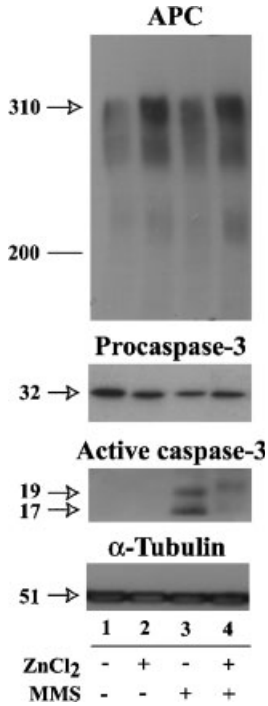

Fig. 2. $ZnCl_2$ stabilizes APC protein levels by checking caspase-3 activity in HCT-116 cells. Cells were pre-treated with 100 μM $ZnCl_2$ for 1 h and then treated with 500 μM MMS for 30 h. Cells were harvested and processed for Western blot analysis for APC, procaspase-3, caspase-3, and α-tubulin protein levels. Data are representative of two independent experiments.

protein level, which may be blocked after $ZnCl_2$ treatment in control cells. Alternatively, it is also possible that other mechanisms of $ZnCl_2$-induced stabilization of APC protein are functional in HCT-116 which is not yet clear.

We further determined the effect of $ZnCl_2$ on cell cycle checkpoints in HCT-116 cells. These cells were treated with different concentrations of $ZnCl_2$ for 30 h, and the cell cycle profile was determined by FACScan analysis. Results showed a dose-dependent decrease in the G_0/G_1 phase arrest and an increase in S-G_2/M phase arrest of HCT-116 cells; however, the extent of the arrest into the G_2/M phase at 100 μM of $ZnCl_2$ treatment was 3.8-fold greater than the S phase (1.8-fold) (Table I). The population of sub-G_1 cells representing the debris and dead cells was unchanged in HCT-116 cells after $ZnCl_2$ treatment for 30 h. Since approximately 1% of the cells were found in sub-G_1 phase, it indicated that the $ZnCl_2$ up to 100 μM concentration is not cytotoxic to the HCT-116 cells (Table I). These results suggest that after $ZnCl_2$ treatment, the HCT-116 cells are first arrested in the S phase and then moved into the G_2/M phase where they are arrested for a longer period. Thus, the increased level of APC protein paralleled the G_2/M phase arrest in HCT-116 cells in response to $ZnCl_2$ treatment.

Truncated APC Protein Levels do not Support $ZnCl_2$-Induced G_2/M Phase Arrest

If increased levels of APC protein are necessary for $ZnCl_2$-induced G_2/M phase arrest, it is likely that cells expressing truncated APC protein may lose the property of $ZnCl_2$-induced G_2/M phase arrest that occurs in HCT-116 cells. We directly tested this hypothesis using several colon cancer cell lines harboring either wild-type or mutant *APC* genes. The SW480 and LoVo cell lines carry mutations at codon 1338 and 1114, respectively, thus expressing truncated APC proteins. The SW48 and LS174T cell lines express wild-type APC proteins [Ilyas et al., 1997]. Although these cell lines are not isogenic, they are widely used in studies for comparison of the role of wild-type and truncated APC proteins. These cell lines were grown in McCoy's 5a medium containing 0.5% FBS for

TABLE I. Cell Cycle Profile of HCT-116 Colon Cancer Cell Line Treated With Different Concentrations of $ZnCl_2$

	DNA content (%)			
$ZnCl_2$ (μM)	G_0/G_1	S	G_2/M	Sub-G_1
0	76.72 ± 0.55 (1.0)	17.65 ± 0.67 (1.0)	5.63 ± 0.26 (1.0)	0.73 ± 0.04 (1.0)
10	72.26 ± 0.40 (0.9)	20.48 ± 0.75 (1.2)	7.27 ± 0.35 (1.3)	0.74 ± 0.04 (1.0)
25	68.25 ± 0.35 (0.9)	23.38 ± 0.35 (1.3)	8.37 ± 0.41 (1.5)	0.89 ± 0.09 (1.2)
50	61.52 ± 0.32 (0.8)	24.32 ± 0.46 (1.4)	14.16 ± 0.22 (2.5)	1.07 ± 0.07 (1.5)
100	45.92 ± 1.30 (0.6)	32.49 ± 1.49 (1.8)	21.58 ± 1.24 (3.8)	1.27 ± 0.04 (1.7)

Cells were grown in 0.5% FBS for 20 h and then treated with different concentrations of $ZnCl_2$ for 30 h. After treatment, cells were processed for cell cycle analysis by FACScan. The ranges for G_0/G_1, S, G_2/M, and sub-G_1 phase arrested cells were established on the basis of the corresponding DNA content of the histograms. At least 10,000 cells per sample were considered in the gated regions for calculations. Data are mean ± SE of percent distribution of DNA content of three different experiments. Numbers given in parentheses are the fold change with reference to untreated cells.

TABLE II. Cell Cycle Profile of Different Colon Cancer Cell Lines Treated With 100 μM $ZnCl_2$ for 30 h

	DNA content (%)			
	G_0/G_1	S	G_2/M	Sub-G_1
Cell lines with wild-type *APC* gene				
SW48				
Control	74.71 ± 0.23 (1.0)	14.18 ± 0.41 (1.0)	11.11 ± 0.29 (1.0)	2.01 ± 0.23 (1.0)
$ZnCl_2$	39.79 ± 1.07 (0.5)	36.48 ± 1.02 (2.6)	23.73 ± 0.27 (2.1)	4.26 ± 0.29 (2.1)
LS174T				
Control	77.48 ± 0.51 (1.0)	15.55 ± 0.39 (1.0)	6.99 ± 0.20 (1.0)	3.56 ± 0.27 (1.0)
$ZnCl_2$	65.70 ± 0.32 (0.9)	23.86 ± 0.25 (1.5)	10.44 ± 0.45 (1.5)	6.69 ± 0.32 (1.9)
Cell lines with mutant *APC* gene				
SW480				
Control	70.12 ± 0.94 (1.0)	20.99 ± 0.39 (1.0)	8.90 ± 0.49 (1.0)	1.29 ± 0.10 (1.0)
$ZnCl_2$	69.93 ± 0.25 (1.0)	20.12 ± 0.25 (1.0)	9.95 ± 0.22 (1.1)	0.92 ± 0.10 (0.7)
LoVo				
Control	67.97 ± 0.39 (1.0)	24.89 ± 0.53 (1.0)	7.60 ± 0.57 (1.0)	3.92 ± 0.57 (1.0)
$ZnCl_2$	70.83 ± 0.59 (1.0)	18.83 ± 0.62 (0.8)	10.00 ± 0.47 (1.3)	2.05 ± 0.13 (0.5)

Cells were grown in 0.5% FBS for 20 h and then treated with different concentrations of $ZnCl_2$ for 30 h. After treatment, cells were processed for cell cycle analysis by FACScan as described in Table I. Data are mean ± SE of three different experiments. Numbers given in parentheses are the fold change with reference to untreated cells.

20 h, and were then treated with 100 μM of $ZnCl_2$ for 30 h. The cell cycle profile of these cell lines with and without $ZnCl_2$ treatment was determined by FACScan analysis and is given in Table II. After treatment with $ZnCl_2$, there was no significant G_2/M phase arrest in SW480 and LoVo cells versus 1.5- and 2.1-fold G_2/M phase arrest in SW48 and LS174T cells, respectively (Table II). These results provide further support that the wild-type and not the truncated APC protein is necessary for $ZnCl_2$-induced G_2/M phase arrest in colon cancer cells. In addition to APC, the SW480 cell line also harbors a mutation in the *p53* gene. Whether p53 plays a role in $ZnCl_2$-induced G_2/M phase arrest is not clear. However, it does not seem likely that p53 plays a major role in $ZnCl_2$-induced S-G_2/M phase arrest, since p53 is wild-type in HCT-116, SW48, LS174T, and LoVo cell lines but the S-G_2/M phase arrest is found only in HCT-116, SW48, LS174T cells but not in LoVo cells (Table II).

Serum-Stimulation of $ZnCl_2$-Pretreated HCT-116 Cells Failed to Abolish G_2/M Phase Arrest

To examine whether G_2/M phase arrest of HCT-116 cells after $ZnCl_2$ treatment in serum-withdrawal growth conditions were reversible by serum-stimulation conditions and to determine whether the APC protein levels correlated with this change, FACScan and Western blot analysis were performed on untreated and $ZnCl_2$-treated cells. The HCT-116 cells were grown in duplicate in low-serum (0.5%, v/v)-containing (indicated as serum-withdrawal) medium for 20 h and were then treated with 100 μM of $ZnCl_2$ for different periods. To examine whether serum-stimulation can overcome $ZnCl_2$-induced G_2/M phase arrest of HCT-116 cells and allow them to return into the S phase, 10% FBS (v/v) was supplemented to the medium. These cells were grown for 10, 20, and 30 h in high-serum conditions and then processed for FACScan analysis (Table III, protocol c). After serum-stimulation, approximately 55% of the $ZnCl_2$-untreated (or control) HCT-116 cells regrouped into the S phase in 20 h. Only 35 and 12% of the cells remained in the G_0/G_1 and G_2/M phases, respectively (Table III, compare protocol c with a). These results are consistent with previous findings [Corbeil and Branton, 1997; Wells et al., 1997]. On the other hand, the pre-treatment of cells with $ZnCl_2$ caused a 2- to 2.7-fold increase in G_2/M phase arrest after serum-stimulation for 10, 20, or 30 h. However, the serum-stimulated cells in the absence of $ZnCl_2$ progressed from S to G_2/M to G_0/G_1 phase in 30 h (Table III, compare protocol d with b). Thus, $ZnCl_2$-induced G_2/M phase arrest is independent of serum-stimulation. Since these experiments were limited to 30 h, it is not clear whether the $ZnCl_2$-induced G_2/M phase arrest is reversible. It is possible that $ZnCl_2$ treatment may slow the transition of cells from G_2/M to G_0/G_1 phase or the entry from S to G_2/M phase.

We further tested whether the cell cycle profile of HCT-116 cells after $ZnCl_2$ pretreatment

TABLE III. $ZnCl_2$-Induced G_2/M Phase Arrest of HCT-116 Cells was Unaffected by Serum-Stimulation

Protocols
(a) Cells → serum w/d for 20 h ——————————————————→ harvest
(b) Cells → serum w/d for 20 h → 100 μM $ZnCl_2$ for different periods ——————→ harvest
(c) Cells → serum w/d for 20 h → 10% FBS for different periods ——————→ harvest
(d) Cells → serum w/d for 20 h → 100 μM $ZnCl_2$ for 10 h → 10% serum for different periods → harvest

Results

	DNA content (%)			
	G_0/G_1	S	G_2/M	Sub-G_1
Serum-stimulation for 10 h				
(a)	64.63 ± 0.41 (1.0)	20.87 ± 0.45 (1.0)	14.51 ± 0.10 (1.0)	0.54 ± 0.07 (1.0)
(b)	26.58 ± 0.12 (0.4)	38.02 ± 2.07 (1.8)	35.40 ± 1.90 (2.4)	0.95 ± 0.12 (1.8)
(c)	44.32 ± 0.14 (0.7)	41.38 ± 0.65 (2.0)	14.30 ± 0.61 (1.0)	0.59 ± 0.17 (1.1)
(d)	30.96 ± 1.33 (0.5)	29.43 ± 0.70 (1.4)	39.60 ± 0.63 (2.7)	1.32 ± 0.01 (2.4)
Serum-stimulation for 20 h				
(a)	72.69 ± 0.10 (1.0)	17.20 ± 0.54 (1.0)	9.83 ± 0.38 (1.0)	0.74 ± 0.13 (1.0)
(b)	51.57 ± 0.45 (0.7)	27.24 ± 0.11 (1.6)	21.19 ± 0.56 (2.2)	0.93 ± 0.12 (1.3)
(c)	34.67 ± 0.14 (0.5)	55.46 ± 2.37 (3.2)	12.42 ± 0.71 (1.3)	1.08 ± 0.26 (1.5)
(d)	27.10 ± 0.69 (0.4)	51.47 ± 2.90 (3.0)	18.95 ± 1.07 (1.9)	1.09 ± 0.30 (1.5)
Serum-stimulation for 30 h				
(a)	74.46 ± 0.54 (1.0)	17.52 ± 0.10 (1.0)	8.03 ± 0.56 (1.0)	0.73 ± 0.09 (1.0)
(b)	43.94 ± 0.16 (0.6)	41.87 ± 1.10 (2.4)	15.52 ± 2.25 (1.9)	0.95 ± 0.05 (1.2)
(c)	64.80 ± 0.14 (0.9)	18.85 ± 1.04 (1.1)	16.35 ± 1.86 (2.0)	0.55 ± 0.08 (0.8)
(d)	48.48 ± 0.35 (0.7)	31.00 ± 0.88 (1.8)	20.53 ± 0.94 (2.6)	1.56 ± 0.03 (2.1)

The protocol for these experiments is given in parentheses—(a–d). Cell cycle analysis was determined by FACScan. The ranges for G_0/G_1, S, and G_2/M phase arrested cells were established on the basis of the corresponding DNA content of the histograms. At least 10,000 cells per sample were considered in the gated regions for calculations. Data are mean ± SE of percent distribution of DNA contents of three different experiments. Numbers given in parentheses are the fold change with reference to untreated cells (protocol a).

or serum-stimulation was linked with the levels of S or G_2/M phase arrest-associated cyclin-dependent kinases. In $ZnCl_2$-treated HCT-116 cells, growing in serum-withdrawal growth medium or after serum-stimulation, the levels of S phase arrest-associated Cdk2 and cyclin A protein levels and Cdk2/cyclin A kinase activity (measured with the phosphorylation of histone H1) were not significantly different than untreated (control) cells (Fig. 3A, compare lane 1 with 2 and 3 with 4, respectively). The effect of $ZnCl_2$-pretreatment on the G_2/M

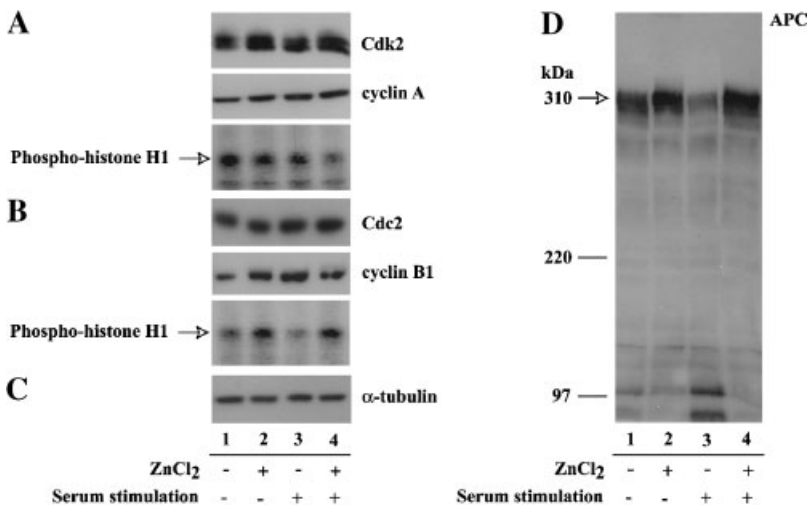

Fig. 3. Protein levels of Cdk2/cyclin A, Cdc2/cyclin B1, and APC in HCT-116 cells after serum-stimulation. The protocols for treatment of these cells were the same as given in Table III. After treatment, cell lysates were processed for Western blot analysis and kinase activity. **Panel A**: Shows the Cdk2 and cyclin A protein levels and phosphorylated histone H1 levels for Cdk2/cyclin A kinase activity. **Panel B**: Depicts the Cdc2 and cyclin B1 protein levels and phosphorylated histone H1 levels for Cdc2/cyclin B1 kinase activity. **Panel C**: Shows the α-tubulin protein level that was used as a control for protein loading in each lane on the gel or any effect of $ZnCl_2$ treatment on cytoskeletal organization of these cells. **Panel D**: Shows the Western blot analysis of the APC protein level which was unchanged after serum-stimulation in $ZnCl_2$-untreated but remained increased in $ZnCl_2$-pretreated HCT-116 cells. Data are representative of three independent experiments.

phase arrest-associated Cdc2 protein level was unchanged, while the cyclin B1 protein level was increased in HCT-116 cells growing in either serum-withdrawal growth medium or after serum-stimulation (Fig. 3B, compare lane 1 with 2 and 3 with 4, respectively). Subsequently, we examined whether Cdc2/cyclin B1 kinase activity changed under serum-withdrawal growth conditions and serum-stimulation conditions in control and $ZnCl_2$-pretreated HCT-116 cells. We found an increased Cdc2/cyclin B1 kinase activity (measured with the phosphorylation of histone H1) in both serum-withdrawal and serum-stimulated conditions in response to $ZnCl_2$-pretreatment (Fig. 3B, compare lane 1 with 2 and 3 with 4, respectively). These results indicate that $ZnCl_2$ treatment induces G_2/M phase arrest, which is correlated with the increased Cdc2/cyclin B1 kinase activity that is independent of serum-induced signaling mechanisms in HCT-116 cells. Although the Cdc2/cyclin B1 protein levels were abundant after serum-stimulation, the Cdc2/cyclin B1 kinase activity was lower in these cells. We think that this is due to activation levels, not the protein level of the Cdc2/cyclin B1 complex, which is necessary for its kinase activity. The Cdc2/cyclin B1 kinase activity is regulated by the phosphorylation/dehosphorylation of Cdc2. Upon phosphorylation of Cdc2 at Thr14 and Tyr15 (within the ATP-binding pocket of the Cdc2 catalytic subunit), the delay of mitotic entry of cells is controlled [Fattaey and Booher, 1997]. Dephosphorylation of both Thr14 and Tyr15 is required for activation of Cdc2 at the G_2/M transition. Thus, it is likely that although the protein level of Cdc2 remains same in $ZnCl_2$ treatment and serum-stimulation conditions without $ZnCl_2$ treatment, it is its phosphorylation that delays cell cycle arrest in G_2/M phase. The α-tubulin levels were unchanged in control and $ZnCl_2$-treated cells, indicating that $ZnCl_2$ treatment did not alter microtubule stability and cytoskeletal integrity in HCT-116 cells (Fig. 3C).

We then tested whether the APC protein level was correlative to the $ZnCl_2$-induced G_2/M phase arrest after serum-stimulation in HCT-116 cells. The APC protein level was significantly increased in HCT-116 cells growing in low-serum-containing medium after $ZnCl_2$ treatment (Fig. 3D, compare lane 1 with 2). However, the APC protein level was unchanged (or decreased) after serum-stimulation in $ZnCl_2$-untreated cells, but remained increased in $ZnCl_2$-pretreated HCT-116 cells (Fig. 3D, compare lane 3 with 4). Thus, the increased levels of APC protein and Cdc2/cyclin B1 kinase activity are linked with G_2/M phase arrest after $ZnCl_2$ treatment, which are independent of serum-stimulation.

Growth Arrest of HCT-116 and SW480 Cell Lines in Response to $ZnCl_2$ Treatment are Through Different Mechanisms

Since the cell lines with wild-type APC showed G_2/M phase arrest (Tables I and II) while cell lines with truncated APC did not show a significant arrest in any phase of the cell cycle (Table II), we expected that after $ZnCl_2$ treatment the growth of the former cell lines would be much slower than the latter. To test our hypothesis, we measured the growth of HCT-116 (carrying wild-type APC protein) and SW480 (carrying truncated APC protein) cell lines by MTT assay with and without $ZnCl_2$ treatment. Cells were grown in 0.5% FBS for 20 h and then treated with 100 μM of $ZnCl_2$ for different periods and processed for an MTT assay. Results showed a time-dependent decrease in the growth of both the cell lines (Fig. 4). These results suggest that after $ZnCl_2$ treatment, the decrease in the growth of HCT-116 cells was attributed to the cell cycle arrest in G_2/M phase. However, the decrease in growth of the SW480

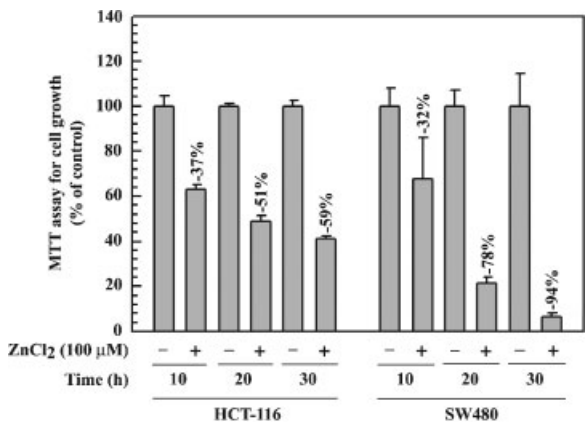

Fig. 4. Growth characteristics of HCT-116 and SW480 cells treated with $ZnCl_2$. Cells were grown in 0.5% FBS-containing medium for 20 h and then treated with 100 μM of $ZnCl_2$ for different periods. The cell growth was measured by MTT assay as described in ''Materials and Methods.'' Data are presented as percent change of untreated versus $ZnCl_2$-treated cells and are mean $\pm$ SE of four different determinations. The decreased percentage growth is shown by negative numbers on the top of the graphs.

cells might be attributed to an alternative mechanism(s).

To determine a possible role of $ZnCl_2$ treatment in the growth retardation of SW480 cells, we first examined phenotypic changes in these cells to gain some direction for further studies. We found that after $ZnCl_2$ treatment, most of the HCT-116 cells remained flat and attached to petri dishes, except a few cells that had started rounding at 30 h treatment (Fig. 5A). On the other hand, most of the SW480 cells showed a rounding phenotype even at 10 h treatment period (Fig. 5B). These cells were viable and attached to the petri dishes. The rounding of these cells suggested that they might be losing their attachment with the petri dishes, which might be one of the reasons for their growth retardation.

To determine whether cellular attachment is affected in SW480 cells as compared to HCT-116 cells in response to $ZnCl_2$ treatment, we measured the protein levels of E-cadherin and β-catenin. Results showed no changes in β-catenin levels in HCT-116 and SW480 cell lines after treatment with 100 μM of $ZnCl_2$ for 30 h (Fig. 6A). The E-cadherin level was also unchanged in HCT-116 cells; however, it was drastically decreased in a time-dependent manner in SW480 cells (Fig. 6B). Apart from E-cadherin, the level of the microtubular protein α-tubulin was decreased in a time-dependent manner in SW480 but not in HCT-116 cells after $ZnCl_2$ treatment (Fig. 6C). These results suggest that the morphological changes may be attributed to the loss of cellular attachment protein E-cadherin and microtubular protein α-tubulin, which might be a consequence of cell rounding and growth retardation of the SW480 cell line after $ZnCl_2$ treatment. Thus, in the HCT-116 cell line (with wild-type APC protein), the growth retardation after $ZnCl_2$ treatment is

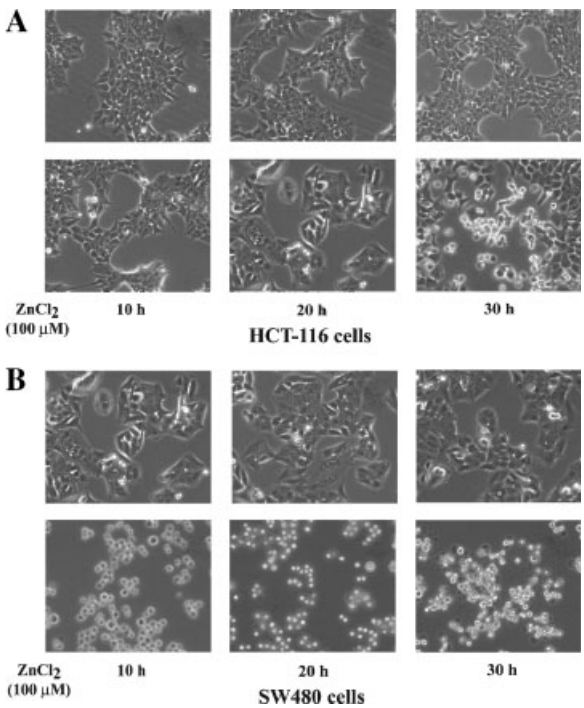

Fig. 5. Effect of $ZnCl_2$ treatment on the morphological changes in HCT-116 and SW480 cells. Cells were grown in 0.5% FBS-containing medium for 20 h and then treated with 100 μM of $ZnCl_2$ for different periods. After treatment, the cell morphology was examined by an inverted microscope. Photographs are the representatives of three different experiments.

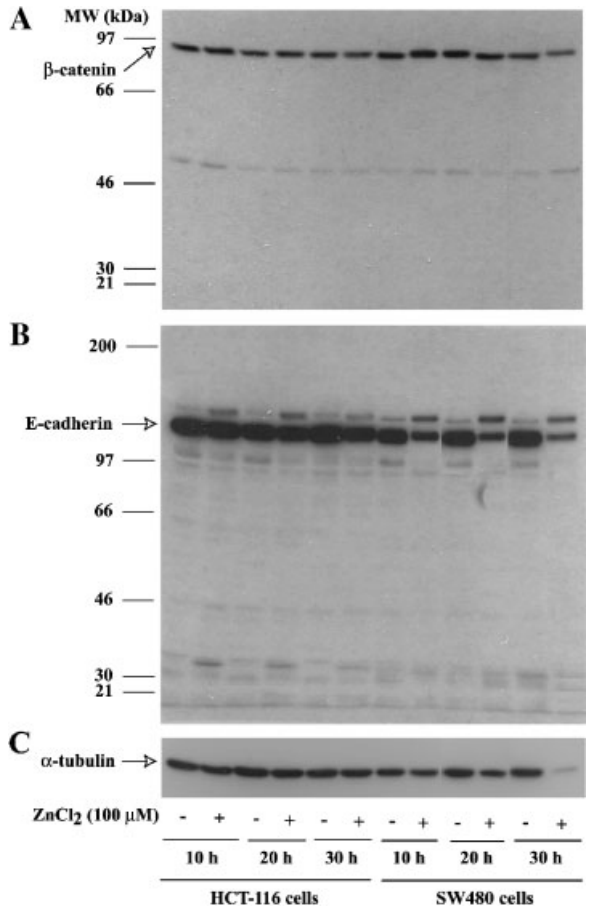

Fig. 6. Protein levels of E-cadherin and β-catenin in HCT-116 and SW480 cell lines after treatment with $ZnCl_2$. Cells were grown in 0.5% FBS-containing medium for 20 h and then treated with 100 μM of $ZnCl_2$ for different periods. After treatment, cell lysates were processed for Western blot analysis for β-catenin, E-cadherin, and α-tubulin protein levels as shown in **Panels A**, **B**, and **C**, respectively. Arrows indicate the molecular weight of the indicated proteins. Data are representative of three independent experiments.

due to increased G_2/M arrest, while in the SW480 cell line (with the truncated APC protein), is due to decreased cellular attachment and microtubule instability.

DISCUSSION

A number of research reports indicate clearly that zinc is involved in the regulation of growth in colorectal cancers [Martin Mateo and Martin, 1988; Song et al., 1993; Park et al., 2002; Schlegel-Zawadzka et al., 2002]; however, the mechanisms by which zinc might inhibit these effects are not well understood. It has been reported that zinc supplementation of the diet decreases AOM-induced adenomatous polyps as well as invasive adenocarcinomas in mice [Carter et al., 1997]. Since low level expression and the mutation of the *APC* gene have been associated directly with adenomatous polyposis in humans and animals [Moser et al., 1990; Fearnhead et al., 2001; van Es et al., 2001; Bienz, 2002; Kishimoto et al., 2002], while an increase in expression of APC has been found to arrest the cell cycle in the G_0/G_1-S phase [Chesters et al., 1993; Baeg et al., 1995; Heinen et al., 2002] and G_2/M phase [Dobashi et al., 1996; Liang et al., 1999], we formed the hypothesis that $ZnCl_2$-induced cell cycle arrest is mediated through increased levels of APC. A corollary to this hypothesis would be that mutations of the *APC* gene together with zinc deficiency would heighten the risk of colon cancer development by disturbing the regulation of the cell cycle and inducing cellular proliferation. Our results suggest that the addition of exogenous $ZnCl_2$ to the culture medium induces cell cycle arrest in the G_2/M phase. This may explain the protective role of $ZnCl_2$ against the growth of colon cancer.

The mechanisms by which $ZnCl_2$ treatment increased the APC protein level is not fully understood yet; however, it appears that it is a post-translational rather than a transcriptional phenomenon. In our studies we found that $ZnCl_2$ treatment decreased the APC mRNA level as a result of decreased *APC* gene expression, which was determined by the *APC* gene's promoter activity in HCT-116 cells. The HCT-116 cells express wild-type *APC* gene and protein. Now the question is how the APC protein levels increased under similar experimental conditions, if the *APC* gene expression is decreased after $ZnCl_2$ treatment. To address this question, we examined the role of caspases in $ZnCl_2$-induced stabilization of the APC protein. Since the APC protein is a known target for caspase-3-mediated degradation [Webb et al., 1999], we determined caspase-3 activity in HCT-116 cells with and without $ZnCl_2$ treatment to asses its role in the stabilization of the APC protein. We found a low level of caspase-3 activity in HCT-116 cells treated with or without $ZnCl_2$, thus it was not clear whether $ZnCl_2$-induced levels of APC were due to decreased caspase-3 activity. To test the possibility as to whether $ZnCl_2$ treatment inhibits caspase-3 activity, we treated HCT-116 cells with MMS in the presence or absence of $ZnCl_2$. The MMS treatment caused activation in caspase-3 and degradation of APC protein level in HCT-116 cells. In $ZnCl_2$ pre-treated cells, the caspase-3 activity decreased while APC protein level increased. These results suggest that $ZnCl_2$ treatment inhibits caspase-3 activity and stabilizes APC protein level in normal cells as well as MMS-treated cells. In previous studies, a zinc-mediated decrease in caspase-3 activity has been reported in human bronchial cells [Fanzo et al., 2001], which is consistent with our observations with colon cancer cells.

To examine whether $ZnCl_2$-induced G_2/M phase arrest of HCT-116 cells was explicit and caused by increased levels of APC and correlated with increased activity of G_2/M phase-specific Cdc2/cyclin B1 kinase activity, we performed serum starvation-stimulation experiments. It is well established that cells grown in serum-free or low-serum conditions are predominantly arrested into the G_0/G_1 phase. After stimulation with high-serum conditions, the G_0/G_1 phase arrested cells move and accumulate into the S phase [Corbeil and Branton, 1997; Wells et al., 1997]. We reasoned that if $ZnCl_2$-induced G_2/M phase arrest is serum-dependent, then after serum-stimulation $ZnCl_2$-induced cell cycle arrest will consequently be relieved and the accumulation of cells will resume in S phase. Furthermore, these experiments also tested whether the level of APC changed after serum-stimulation. We found that the APC protein levels increased and remained unaffected after serum-stimulation in the $ZnCl_2$-pretreated HCT-116 cells. After serum-stimulation, S phase arrest associated Cdk2/cyclin A protein levels and their kinase activity were induced in $ZnCl_2$-untreated cells (data not shown). However, once these cells were pretreated with $ZnCl_2$, the shift

from $ZnCl_2$-induced G_2/M to S phase arrest was not facilitated after serum-stimulation. Furthermore, there was a clear association between G_2/M phase arrest associated Cdc2/cyclin B1 protein levels and their kinase activity with $ZnCl_2$ treatment, which was unaffected after serum-stimulation in the $ZnCl_2$-pretreated cells. These results indicate that $ZnCl_2$ treatment stabilizes APC protein levels, which may increase Cdc2/cyclin B1 kinase activity and induce G_2/M phase arrest in HCT-116 cells. In earlier studies, the role of APC has been suggested in G_2/M phase arrest of colon cancer cells [Dobashi et al., 1996; Trzepacz et al., 1997; Homma et al., 2002]. In a recent study using MCA3D (mouse immortalized epidermal keratinocytes) and HaCa4 (mouse squamous cell carcinoma) cell lines, the role of wild-type β-catenin and APC has been shown in cell cycle arrest and apoptosis [Olmeda et al., 2003]. These studies showed an increased cytoplasmic and nuclear localization of β-catenin and APC during S and G_2/M phase arrest of the cell cycle. To further prove our hypothesis that the increased levels of wild-type, not the truncated, APC protein plays a role in $ZnCl_2$-induced G_2/M phase arrest, we treated SW480 and LoVo colon cancer cell lines with $ZnCl_2$ under similar experimental conditions. The SW480 and LoVo cells lack wild-type but express truncated APC protein, thus they lose the functions of the wild-type APC. As expected, we found no G_2/M phase arrest in SW480 and LoVo cells versus a significant increase in the G_2/M phase arrest in $ZnCl_2$-treated HCT-116, SW48, and LS174T cells, which express wild-type APC protein. These results suggest a role of increased levels of wild-type APC in the G_2/M phase arrest, which are in agreement with previous studies performed with overexpression of APC in colon cancer cell lines [Homma et al., 2002].

Based on our results and previous reports, we expected that mutations in the *APC* gene may abolish $ZnCl_2$-induced cell cycle arrest and cause cellular proliferation. However, these predictions turned out to be different. In fact, the growth arrest occurred in both HCT-116 and SW480 cell lines carrying wild-type and truncated APC proteins, respectively. We further explored the mechanisms by which $ZnCl_2$ treatment might induce growth arrest in the SW480 cell line. SW480 cells became rounded after $ZnCl_2$ treatment, and this rounding phenomenon may be associated with the loss of cellular attachment protein E-cadherin and the microtubular protein α-tubulin. Interaction of APC with β-catenin and the members of the cadherin family of proteins have been implicated in cellular attachment [Gumbiner, 2000]. The C-terminal domain of E-cadherin interacts with β- and γ-catenin, which associate with α-catenin and form an E-cadherin complex with actin cytoskeleton. This complex maintains stable cellular attachment [Gumbiner, 2000]. APC becomes a part of the cellular attachment and cell–cell communication complex when it is linked with E-cadherin through the complex of β-catenin, γ-catenin, and actin filament [Ben-Ze'ev and Geiger, 1998]. Thus, the loss in wild-type activity or expression levels of APC, E-cadherin, and β-catenin can impair cellular attachment properties and result in growth arrest and apoptosis.

Microtubule stability and actin cytoskeletal integrity is necessary to maintain the shape, adherence junction, cell–cell communication, and cell migration. The role of APC in microtubule stability and actin cytoskeletal maintenance is predicted through its interaction with microtubule end-binding protein (EB1) [Berrueta et al., 1998] and β-catenin [Dikovskaya et al., 2001], respectively. Thus, the functional loss of one or more of these molecules such as APC, β-catenin, α-tubulin, or actin filament will produce disturbance in cell morphology. Since SW480 cells inherently express truncated APC protein, they are more susceptible for cellular disorganization. Thus, the decreased level of α-tubulin along with truncated APC protein may appear to be one of the mechanisms for rounding of SW480 cells after $ZnCl_2$ treatment. Furthermore, the loss of cellular attachment function due to a loss of E-cadherin expression provides additional burden on these cells to seize proliferation in response to $ZnCl_2$ treatment. Since changes in the E-cadherin and α-tubulin levels are not significant in HCT-116 cells, our results implicate that somehow the wild-type APC protein protects the loss of cellular levels of these proteins.

In summary, our studies provide a basis for further studies to examine the beneficial effect of zinc supplementation in the treatment of colorectal cancers that arise independently or dependently from mutations in the *APC* gene. Moreover, they provide the basis for further studies to examine the possibility that dietary zinc supplementation may prevent colon cancer

growth in high-risk populations by maintaining high cellular levels of wild-type APC protein, thereby maintaining an appropriate balance between cell growth and cell death. Furthermore, dietary zinc supplementation can be beneficial to those patients who may have colon cancer due to mutations in the *APC* gene. The growth of colon cancer cells can be reduced after dietary zinc supplementation by cell cycle arrest in the G_2/M phase in those cases where the wild-type *APC* gene is expressed. On the other hand, the dietary zinc supplementation can also reduce colon cancer cell growth by interfering at cellular attachment and microtubule stability by inducing the loss of E-cadherin and α-tubulin levels. Thus, dietary zinc supplementation can be a useful agent for the prevention of colorectal cancer growth in high-risk populations.

ACKNOWLEDGMENTS

We thank Dr. William A. Dunn, Dr. Fiona Hunter, Ms. Nirupama Gupta, Ms. Mary Wall, and Ms. Diana Kang for critically reading the article and Dr. Virendra Kumar for technical assistance.

REFERENCES

Baeg GH, Matsumine A, Kuroda T, Bhattacharjee RN, Miyashiro I, Toyoshima K, Akiyama T. 1995. The tumour suppressor gene product APC blocks cell cycle progression from G0/G1 to S phase. EMBO J 14:5618–5625.

Ben-Ze'ev A, Geiger B. 1998. Differential molecular interactions of beta-catenin and plakoglobin in adhesion, signaling and cancer. Curr Opin Cell Biol 10:629–639.

Berg JM, Shi Y. 1996. The galvanization of biology: A growing appreciation for the roles of zinc. Science 271: 1081–1085.

Berrueta L, Kraeft SK, Tirnauer JS, Schuyler SC, Chen LB, Hill DE, Pellman D, Bierer BE. 1998. The adenomatous polyposis coli-binding protein EB1 is associated with cytoplasmic and spindle microtubules. Proc Natl Acad Sci USA 95:10596–10601.

Bienz M. 2002. The subcellular destinations of APC proteins. Nat Rev Mol Cell Biol 3:328–338.

Carter JW, Lancaster H, Hardman WE, Cameron IL. 1997. Zinc deprivation promotes progression of 1,2-dimethylhydrazine-induced colon tumors but reduces malignant invasion in mice. Nutr Cancer 27:217–221.

Chesters JK, Petrie L, Lipson KE. 1993. Two zinc-dependent steps during G1 to S phase transition. J Cell Physiol 155:445–451.

Corbeil HB, Branton PE. 1997. Characterization of an E2F–p130 complex formed during growth arrest. Oncogene 15:657–668.

Dikovskaya D, Zumbrunn J, Penman GA, Nathke IS. 2001. The adenomatous polyposis coli protein: In the limelight out at the edge. Trends Cell Biol 11:378–384.

Dobashi Y, Bhattacharjee RN, Toyoshima K, Akiyama T. 1996. Upregulation of the *APC* gene product during neuronal differentiation of rat pheochromocytoma PC12 cells. Biochem Biophys Res Commun 224:479–483.

Fanzo JC, Reaves SK, Cui L, Zhu L, Wu JY, Wang YR, Lei KY. 2001. Zinc status affects p53, gadd45, and c-fos expression and caspase-3 activity in human bronchial epithelial cells. Am J Physiol Cell Physiol 281:C751–C757.

Fattaey A, Booher RN. 1997. Myt1: A Wee1-type kinase that phosphorylates Cdc2 on residue Thr14. Prog Cell Cycle Res 3:233–240.

Fearnhead NS, Britton MP, Bodmer WF. 2001. The ABC of APC. Hum Mol Genet 10:721–733.

Fishel ML, Seo YR, Smith ML, Kelley MR. 2003. Imbalancing the DNA base excision repair pathway in the mitochondria; targeting and overexpressing *N*-methylpurine DNA glycosylase in mitochondria leads to enhanced cell killing. Cancer Res 63:608–615.

Fong LY, Magee PN. 1999. Dietary zinc deficiency enhances esophageal cell proliferation and *N*-nitrosomethylbenzylamine (NMBA)-induced esophageal tumor incidence in C57BL/6 mouse. Cancer Lett 143:63–69.

Fong LY, Nguyen VT, Farber JL. 2001. Esophageal cancer prevention in zinc-deficient rats: Rapid induction of apoptosis by replenishing zinc. J Natl Cancer Inst 93: 1525–1533.

Gumbiner BM. 2000. Regulation of cadherin adhesive activity. J Cell Biol 148:399–404.

Heinen CD, Goss KH, Cornelius JR, Babcock GF, Knudsen ES, Kowalik T, Groden J. 2002. The APC tumor suppressor controls entry into S-phase through its ability to regulate the cyclin D/RB pathway. Gastroenterology 123:751–763.

Homma MK, Edwin GK, Yuasa Y, Homma Y. 2002. Association and regulation of casein kinase 2 activity by adenomatous polyposis coli protein. Proc Natl Acad Sci USA 99:5959–5964.

Ilyas M, Tomlinson IP, Rowan A, Pignatelli M, Bodmer WF. 1997. β-catenin mutations in cell lines established from human colorectal cancers. Proc Natl Acad Sci USA 94:10330–10334.

Jaiswal AS, Narayan S. 2001a. Upstream stimulating factor-1 (USF1) and USF2 bind to and activate the promoter of the adenomatous polyposis coli (*APC*) tumor suppressor gene. J Cell Biochem 81:262–277.

Jaiswal AS, Narayan S. 2001b. p53-dependent transcriptional regulation of the APC promoter in colon cancer cells treated with DNA alkylating agents. J Biol Chem 276:18193–18199.

Jaiswal AS, Marlow BP, Gupta N, Narayan S. 2002. Beta-catenin-mediated transactivation and cell–cell adhesion pathways are important in curcumin (diferuylmethane)-induced growth arrest and apoptosis in colon cancer cells. Oncogene 21:8414–8427.

Kishimoto Y, Morisawa T, Hosoda A, Shiota G, Kawasaki H, Hasegawa J. 2002. Molecular changes in the early stage of colon carcinogenesis in rats treated with azoxymethane. J Exp Clin Cancer Res 21:203–211.

Liang JY, Liu YY, Zou J, Franklin RB, Costello LC, Feng P. 1999. Inhibitory effect of zinc on human prostatic carcinoma cell growth. Prostate 40:200–207.

Martin Mateo MC, Martin G. 1988. Influence of metallic carcinogenesis in lung and colorectal neoplasia. Metals in neoplastic processes. Clin Physiol Biochem 6:321–326.

Moser AR, Pitot HC, Dove WF. 1990. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. Science 247:322–324.

Narayan S, Jaiswal AS. 1997. Activation of adenomatous polyposis coli (*APC*) gene expression by the DNA-alkylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine requires p53. J Biol Chem 272:30619–30622.

Olmeda D, Castel S, Vilaro S, Cano A. 2003. Beta-catenin regulation during the cell cycle: Implications in G2/M and apoptosis. Mol Biol Cell 14:2844–2860.

Oreffo VI, Robinson S, You M, Wu MC, Malkinson AM. 1998. Decreased expression of the adenomatous polyposis coli (Apc) and mutated in colorectal cancer (Mcc) genes in mouse lung neoplasia. Mol Carcinog 21:37–49.

Park KS, Ahn Y, Kim JA, Yun MS, Seong BL, Choi KY. 2002. Extracellular zinc stimulates ERK-dependent activation of p21(Cip/WAF1) and inhibits proliferation of colorectal cancer cells. Br J Pharmacol 137:597–607.

Prasad AS. 1998. Zinc deficiency in humans: A neglected problem. J Am Coll Nutr 17:542–543.

Prasad AS, Beck FW, Endre L, Handschu W, Kukuruga M, Kumar G. 1996. Zinc deficiency affects cell cycle and deoxythymidine kinase gene expression in HUT-78 cells. J Lab Clin Med 128:51–60.

Prasad AS, Bao B, Beck FW, Sarkar FH. 2001. Zinc activates NF-kappaB in HUT-78 cells. J Lab Clin Med 138:250–256.

Schlegel-Zawadzka M, Kopanski Z, Piekoszewski W, Tabak J, Puto T, Engel B. 2002. The disturbances of the zinc concentration in the blood in selected neoplasms. Biol Trace Elem Res 89:139–144.

Shankar AH, Prasad AS. 1998. Zinc and immune function: the biological basis of altered resistance to infection. Am J Clin Nutr 68(2 Suppl):447S–463S.

Shoemaker AR, Moser AR, Dove WF. 1995. *N*-ethyl-*N*-nitrosourea treatment of multiple intestinal neoplasia (Min) mice: Age-related effects on the formation of intestinal adenomas, cystic crypts, and epidermoid cysts. Cancer Res 55:4479–4485.

Song MK, Heng MC, Rolandelli R, Ament ME, Heng MK. 1993. Possible link between zinc intake and colon cancer. J Natl Cancer Inst 85:667–669.

Steffensen IL, Paulsen JE, Eide TJ, Alexander J. 1997. 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine increases the numbers of tumors, cystic crypts and aberrant crypt foci in multiple intestinal neoplasia mice. Carcinogenesis 18:1049–1054.

Su LK, Kinzler KW, Vogelstein B, Preisinger AC, Moser AR, Luongo C, Gould KA, Dove WF. 1992. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the *APC* gene. Science 256:668–670.

Suzui M, Okuno M, Tanaka T, Nakagama H, Moriwaki H. 2002. Enhanced colon carcinogenesis induced by azoxymethane in min mice occurs via a mechanism independent of beta-catenin mutation. Cancer Lett 183: 31–41.

Trzepacz C, Lowy AM, Kordich JJ, Groden J. 1997. Phosphorylation of the tumor suppressor adenomatous polyposis coli (APC) by the cyclin-dependent kinase p34. J Biol Chem 272:21681–21684.

Uzzo RG, Leavis P, Hatch W, Gabai VL, Dulin N, Zvartau N, Kolenko VM. 2002. Zinc inhibits nuclear factor-kappaB activation and sensitizes prostate cancer cells to cytotoxic agents. Clin Cancer Res 8:3579–3583.

van Es JH, Giles RH, Clevers HC. 2001. The many faces of the tumor suppressor gene *APC*. Exp Cell Res 264:126–134.

Webb SJ, Nicholson D, Bubb VJ, Wyllie AH. 1999. Caspase-mediated cleavage of APC results in an amino-terminal fragment with an intact armadillo repeat domain. FASEB J 13:339–346.

Wells JM, Illenye S, Magae J, Wu CL, Heintz NH. 1997. Accumulation of E2F-4.DP-1 DNA binding complexes correlates with induction of *dhfr* gene expression during the G1 to S phase transition. J Biol Chem 272:4483–4492.