Cell cycle and apoptosis regulatory protein-1: a novel regulator of apoptosis in the colonic mucosa during aging

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Submitted 18 July 2007; accepted in final form 5 October 2007

Majumdar AP, Du J, Yu Y, Xu H, Levi E, Patel BB, Rishi AK. Cell cycle and apoptosis regulatory protein-1: a novel regulator of apoptosis in the colonic mucosa during aging. Am J Physiol Gastrointest Liver Physiol 293: G1215–G1222, 2007. First published October 11, 2007; doi:10.1152/ajpgi.00324.2007.—Although the regulatory mechanisms for age-related rise in proliferation and reduction in apoptosis in the colonic mucosa are yet to be fully delineated, we have demonstrated that these events are associated with increased expression and activation of epithelial growth factor receptor (EGFR)/ErbB-1 and some of its receptor family members (EGFRs), indicating their involvement in these processes. However, the downstream signaling events of EGFR and/or its family members regulating age-related changes in mucosal proliferation and apoptosis remain to be delineated. Cell cycle and apoptosis regulatory protein-1 (CARP-1), a novel growth signaling regulator that we isolated, participates in EGFR-dependent signaling. In the current investigation, we examined the involvement of CARP-1 in colonic mucosal growth-related processes during aging. We report that the age-related reduction in apoptosis in the colonic mucosa is associated with increased expression and tyrosine phosphorylation of not only EGFR but also ErbB-2 and ErbB-3. In contrast, protein and mRNA levels of CARP-1 as well as tyrosine phosphorylation of CARP-1 are decreased. Additionally, we have observed that administration of wortmannin, an inhibitor of phosphatidylinositol 3-kinase activity that accelerates apoptosis in the colonic mucosa of aged rats, causes a marked increase in expression and tyrosine phosphorylation of CARP-1. The age-related decline in CARP-1 expression could partly be attributed to increased methylation of the CARP-1 promoter. Taken together, our data suggest that not only EGFR but also its other members are involved in regulating colonic mucosal growth during aging and that CARP-1 may play a crucial role in transducing EGFRs signals.

colonic mucosal growth; epidermal growth factor receptor; ErbB-2; ErbB-3; cell cycle and apoptosis regulatory protein-1 methylation

THE SIZE OF A continuously renewing mucosal cell population in the gastrointestinal tract is not only determined by the rate of cell proliferation but also by cell loss, processes that are essential for maintenance of mucosal homeostasis in healthy adults (5, 14). Aging appears to disrupt this process (16). Morphological and biochemical studies from this and other laboratories have demonstrated increased proliferation and decreased apoptosis in the gastric and colonic mucosa of Fischer-344 rats, events that are seen during the development and progression of carcinogenesis (10–12, 16, 19, 28). Morphological studies of the colonic mucosa of human volunteers have further revealed that, whereas cell proliferation in young is confined to the lower two-thirds of the crypt, with aging there is a major shift from the base to the middle and upper third of the gland (25), a pattern commonly observed in colorectal cancer. These and other relevant observations have led us to suggest that aging predisposes the gastrointestinal tract to carcinogenesis (1, 16).

Although the regulatory mechanisms for age-related changes in gastrointestinal mucosal proliferation and apoptosis are yet to be fully delineated, we have reported that these events are accompanied by increased expression and activation of the epithelial growth factor receptor (EGFR/ErbB-1) and some of its family members, particularly ErbB-2/HER-2, suggesting that EGFR/ErbB-1 and HER-2/ErbB-2 play critical roles in the regulation of mucosal growth during aging (17, 26, 27). Numerous studies have demonstrated that EGFR/ErbB-1 and/or its family members (ErbB-2/HER-2, ErbB-3/HER-3, and ErbB-3/HER-4, hereafter referred to as EGFRs) regulate many cellular events, such as proliferation, differentiation, and apoptosis (6). However, the downstream signaling events of EGFRs regulating age-related changes in mucosal growth-related processes remain to be delineated.

Recently, we identified a 130-kDa perinuclear protein, cell cycle and apoptosis regulatory protein (CARP-1), also known as CCAR-1, a novel growth regulator that participates in EGFR-dependent signaling (23, 24). CARP-1, which was isolated using an antisense-functionalized gene knockout approach (23), has been shown to be an important mediator of growth-related processes (23, 24). We have reported that CARP-1 regulates apoptosis by diverse agents, including chemotherapeutics, adriamycin etoposide, and EGFR inhibitor(s) (24). Treatment of human colon cancer cells with EGFRs inhibitors results in increased expression of CARP-1 and apoptosis (24). That CARP-1 is a critical regulator of EGFR-dependent apoptosis was underscored by the observation that antisense-dependent depletion of CARP-1 results in abrogation of apoptosis by inhibition of EGFRs by EGFR-related protein, a pan-erbB inhibitor that targets multiple members of the EGFR family (15, 22, 29). Increased expression of CARP-1, on the other hand, results in repression of various cell cycle- and proliferation-related genes such as a p21Rac1, c-Myc, cyclin B1, extracellular signal-regulated kinase (ERK) regulator mi- togen/extracellular signal-regulated kinase-2, and topoisomerase IIα while causing elevated levels of CDKI p21WAF1/CIP1 and activation of caspases-3 and -9 (23, 24). The current investigation was undertaken to examine the role of CARP-1 in

First published October 11, 2007; doi:10.1152/ajpgi.00324.2007.
transducing EGFR signals and in turn colonic mucosal growth during aging. Herein, we demonstrate that, whereas aging is associated with increased colonic mucosal proliferative activity, apoptosis in the colonic mucosa is decreased. These changes are associated with activation of not only EGFR but also the other members of its family and decreased expression and tyrosine phosphorylation of CARP-1. The age-related decrease in CARP-1 expression could partly be attributed to hypermethylation of the gene. Furthermore, inhibition of phosphatidylinositol 3-kinase (PI 3-kinase) signaling, which stimulates apoptosis in the colonic mucosa of aged rats, is associated with increased activation of CARP-1.

**METHODS**

*Reagents.* Polyclonal rabbit antibodies to CARP-1 were generated by Sigma-Genosys as described previously (23). Antibodies to caspase-9, ErbB-2, phosphotyrosine, Y254EFGF, Y446EFGF, Y1248ErbB-2, Y1221ErbB-2, and Y1298ErbB-3 were purchased from Cell Signaling (Beverley, MA). Anti-Y1173EGFR antibodies were from Upstate Biotech (Lake Placid, NY) while anti-ErbB-3, anti-proliferating cell nuclear antigen (PCNA), and anti-β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Wortmannin was obtained from Calbiochem (La Jolla, CA) and 5-aza-2′-deoxycytidine was from Sigma Chemical (St. Louis, MO). Goat anti-rabbit IgG conjugated with horseradish peroxidase and enhanced chemiluminescence (ECL) were obtained from Amersham (Arlington Heights, IL). Immobilon-P nylon membrane was from Millipore (Bedford, MA). Concentrated protein assay dye reagent was from Bio-Rad Laboratories (Hercules, CA). Molecular weight marker and DMEM were from GIBCO-BRL (Grand Island, NY). All other reagents were of molecular biology grade and were purchased from Sigma or Fisher Scientific.

*Animals and collection of tissues.* Male Fischer-344 rats aged 4–5 (young) and 21–24 (old) mo were used. The animals were purchased from the National Institute on Aging (Bethesda, MD) 2 mo before the experiment. During this period, they were housed two per cage and had access to Purina rat chow and water ad libitum. The reasons for using Fischer-344 rats for aging studies are because of purity of breeding, low susceptibility to spontaneous colorectal cancer, and their ability to maintain body weight. All animals were fasted overnight before being killed. The overnight-fasted animals were either used without any intervention or injected intraperitoneally with either wortmannin (0.1 mg/kg body wt in 15% dimethyl sulfoxide), 5-aza-2′-deoxycytidine (10 mg/kg body wt in 50% acetic acid), or the appropriate vehicle (controls) before being killed. All overnight-fasted animals, except those treated with wortmannin, were killed, 5-aza-2′-deoxycytidine, or the corresponding vehicle, were killed between 8:00 and 10:00 A.M. The animals injected with wortmannin or vehicle were killed in the afternoon, between 1:00 and 3:00 P.M. The animals injected with 5-aza-2′-deoxycytidine or vehicle were killed 16 h (overnight) later. From all experiments, the entire colon (% of total colon) was removed, cut open along the longitudinal median, and rinsed thoroughly in cold normal saline. The distal (~6 cm proximal to the rectum) and proximal (~6 cm distal to cecum) parts of the colon were cut out and used in this investigation. A small section (~0.5 cm) of the distal colon was fixed in 10% buffered formalin for immunohistochemical analysis. From rest of the region, mucosa was obtained by scraping with glass slides. Mucosal aliquots were either processed immediately or stored at −80°C.

**Immunoprecipitation and Western blot analysis.** Colonic mucosal aliquots from young and old rats were homogenized in lysis buffer (50 mM Tris·HCl, pH 7.4, 100 mM NaCl, 2.5 mM EDTA, 2.5 mM Na2VO4, 0.5% Triton X-100, 0.5% Nonidet P-40, and 5 μg/ml of aprotinin, pepstatin, and leupeptin) and subsequently subjected to centrifugation at 10,000 g for 10 min. The supernatant was used for immunoprecipitation and Western blot analysis according to our standard protocol (17, 27). In all Western blot analyses, protein concentration was standardized among the samples. Briefly, 100-μg proteins were separated on a 10% SDS-PAGE and subsequently electroblotted to Immobilon-P nylon membranes. The membranes were blocked overnight with 5% BSA or nonfat dried milk in buffer containing 20 mM Tris·HCl, pH 7.6, 100 mM NaCl, and 0.01% Tween 20 (TBS-T). The membranes were incubated for 3 h with respective anti-phosphotyrosine, phospho-ErbB3, or caspase-9 antibodies at the manufacturer-suggested dilutions in TBS-T buffer containing 5% BSA at room temperature. For determination of the levels of the tyrosine-phosphorylated form of CARP-1, aliquots of cell lysates containing 1.0 mg of proteins were first incubated with anti-CARP-1 (α2) antibodies and Sepharose G at 4°C for 3 h. The immunocomplexes were then subjected to Western blot analysis with anti-phospho-tyrosine antibodies as described above. All Western blots were performed at least three times using mucosal extracts from multiple rats.

All immunoblots were scanned by HP Precision Pro 3.13 (Hewlett-Packard, Packard, Palo Alto, CA). Densitometric measurements of the scanned bands were performed using the digitized scientific software program UN-SCNAT.

**Immunohistological and in situ hybridization.** For immunohistochemical staining, an immunoperoxidase method was used with a streptavidin biotinylated horseradish peroxidase complex (Dako, Carpinteria, CA). The rat colonic tissues were formalin fixed and paraffin embedded, and 5-μm serial sections were generated. The tissue sections were deparaffinized and microwaved for 15 min in citrate buffer (0.1 M citrate acid and 0.1 M sodium citrate, pH 6.0) for antigen retrieval. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide and subsequently incubated with 5% horse serum to block nonspecific binding. The slides were then incubated at room temperature for 2 h with polyclonal antibodies to CARP-1 (α1) or PCNA at 1:1,000 or 1:50 dilution, respectively. At least 10 well-oriented crypts on each slide and five slides from each sample were examined.

For apoptosis determination, the slides were subjected to terminal deoxynucleotidyltransferase dUTP nick-end labeling (TUNEL) staining using a cell-death detection kit (In situ cell death detection POD kit; Roche Applied Science). The avidin-biotin technique was performed with matched components (secondary biotinylated antibody and avidin-peroxidase complex) from the Dako-labeled streptavidin-biotin system according to the manufacturer’s suggested protocol. The slides were then reacted with amino ethyl carbazole, counterstained with Harris’ hematoxylin, and examined by a pathologist. At least 10 well-oriented crypts on each slide and five slides from each sample were examined.

For in situ hybridization, the tissue sections were deparaffinized in xylene (Fisher) for two treatments of 5 min each and subsequently washed two times in 100, 80, and 70% ethanol for 5 min each wash, followed by a 5-min wash in diethyl pyrocarbonate (DEPC)-treated water. The slides were incubated in proteinate K (50 μg/ml in PBS buffer) at room temperature for 8 min. Excess biotin was blocked using the avidin blocking kit (Vector) essentially following the manufacturer’s guidelines. The slides were washed in DEPC-PBS at room temperature for 5 min and incubated in 20 μl of hybridization solution (Sigma-Aldrich) at 37°C for 3–4 h. Hybridization reaction was then carried out in fresh hybridization buffer containing 1.5 μg/ml of...
CARP-1 sense (5'-CTGCAGGAGACCACAGGGGCGT-3') or antisense (5'-ACGCCCCTTGTTGCCTCCTCAGG-3') single-stranded oligos (positions 1754–1777 of human CARP-1 cDNA accession no. AY249140) that had a biotin moiety positioned at their 5' ends (Integrated DNA Technologies, Coralville, IA). After hybridization for 14 h at 37°C, the buffer was removed, and the slides were washed in 0.5× saline-sodium citrate, followed by washing in double-distilled water. The slides were immersed in 3% H2O2 in methanol for 10 min and washed in PBS, followed by incubation in 10% FBS for 30 min. The tissues were then hybridized with antibiotic mouse monoclonal antibody (Roche) overnight at 4°C, washed in PBS, reacted with horseradish peroxidase-goat antimouse antibody (Chemicon) for 1 h, followed by incubation with substrate-chromagen solution diaminobenzidine (Vector), and counterstained with hematoxylin.

The staining score was determined by a pathologist to reflect the quantitative and qualitative intensity. Staining intensity was graded as negative, low, moderate, and high (grades 0, 1, 2, and 3, respectively). The percentage staining was multiplied with the intensity score to obtain a single numerical staining score.

Methylation-specific PCR analysis of rat CARP-1 promoter. Genomic DNA was extracted from colonic mucosa of rats of varying ages ranging from 4 to 23 mo essentially as described previously (21). Bisulfite modification of DNA to convert all unmethylated cytosines to uracil and then to thymidine during the subsequent PCR step while leaving the methylated cytosines unaffected was performed as described by Herman et al. (9). Modified DNA was purified using the Wizard DNA purification resin according to the manufacturer's instructions (Promega) and eluted in 50 μl of water. Modification was completed by the addition of NaOH (0.3 M final concentration) for 5 min at room temperature followed by ethanol precipitation. DNA was resuspended in water and used immediately or stored at −20°C. The bisulfite-treated DNA was used for PCR amplification of a putative rat CARP-1 promoter fragment that is located within a predicted methylation island (www.uscnorris.com/cpgislands2/cpg). Primer sequences for amplification of a 175-bp product (accession no. NW_047601, positions 10931556 to 10931730) for methylated promoter fragment were 5'-TATGATTTTATAGGTACGTTTCGG-3' (forward primer) and 5'-CTAACCTCCACTACCCCCACGTA-3' (reverse primer). The primers for amplification of unmethylated DNA fragment were 5'-TATGATTATATTAGGTATGTTTG-3' (forward primer) and 5'-CTAACCTCCACTACCCCCACATA-3' (reverse primer). For PCR amplification, the annealing temperature was 66°C. PCR amplification was performed by using ~300 ng of treated DNA as the template in a final volume of 50 μl. Reactions were hot started at 95°C for 5 min before amplification in a Eppendorf thermal cycler for 40 cycles (20 s at 95°C, 20 s at 66°C, and 20 s at 70°C) followed by a final 4-min extension at 72°C. Controls without DNA were performed for each set of PCRs. Each PCR product (15 μl) was directly electrophoresed and visualized following ethidium bromide staining of 2% agarose gels.

Statistical analysis. Where applicable, results were analyzed using ANOVA followed by Fischer’s protected least-significant differences or Scheffé’s test. A P value of <0.05 was designated as the level of significance.

RESULTS

Aging is associated with increased proliferation and decreased apoptosis in the colonic mucosa. Previous studies from our laboratory have demonstrated that the colonic mucosa of aged (22- to 24-mo-old) Fisher-344 rats displays increased proliferation and decreased apoptosis when compared with their younger (4-mo-old) counterparts that results in overall enhanced cell survival (16–18). To further establish the consistency of this observation, we examined the proliferative

Fig. 1. Age-related changes in proliferative activity (determined by proliferating cell nuclear antigen immunoreactivity), and apoptosis (assessed by the terminal deoxynucleotidyltransferase dUTP nick-end labeling assay) in the distal colonic mucosa of Fischer-344 rats. Histogram depicts the mean ± SE of data from 4–5 animals in each group. *P < 0.01 (top) and *P < 0.025 (bottom) compared with the corresponding 4-mo-old controls.
activity and apoptosis in the colonic mucosa of young (4-mo-old) and aged (21-mo-old) Fischer-344 rats. We observed that the colonic mucosal proliferative activity (as determined by PCNA immunoreactivity) in 21-mo-old rats was ∼40% higher ($P < 0.01$) and apoptosis (as determined by TUNEL assay) 50% lower when compared with the corresponding values from 4-mo-old rats (Fig. 1). Furthermore, examination of distribution of apoptosis in the colonic crypts in young and aged rats revealed that apoptotic cells were present throughout the entire length of colonic crypts in both age groups. However, whereas in young rats the majority of apoptotic cells were present in the upper half (24 ± 4/focus in upper vs. 15 ± 3/focus in lower crypts) of the colonic crypt, in old animals they were mostly found in the lower half (6 ± 2/focus in upper vs. 18 ± 3/focus in lower crypts) of the crypt. The results suggest that aging is associated with changes in distribution of apoptosis in the colonic crypt.

Although the regulatory mechanisms for the age-related increase in proliferation and decrease in apoptosis remain to be fully elucidated, it is our working hypothesis that EGFR and/or other EGFR family member(s) play key roles in the regulation of these processes. Activation of EGFR and its family members through tyrosine phosphorylation is one of the earliest steps in induction of the signal transduction pathways (13). Moreover, activation of different members of the EGFR family is known to cause diversification of the signal transduction pathways (2, 3). To evaluate the role of EGFR and member(s) of its family (EGFRs) in regulating GI mucosal growth during aging, we examined the levels of total and tyrosine phosphorylated (activated) forms of EGFR and its family members in the colonic mucosa of overnight-fasted young (5-mo-old) and aged (23-mo-old) male Fischer-344 rats. Western immunoblot analysis using various antibodies that detect the phosphorylation and expression of EGFRs revealed increased expression and activation of EGFR, ErbB-2, and ErbB-3 in the colonic mucosa of aged than in young rats (Fig. 2). We also observed that the extent of phosphorylation of EGFR tyrosine residues at 845, 1068, and 1173 and of ErbB-2 tyrosine residue at 1248 and 1221 was higher in colonic mucosa of aged than in young rats (Fig. 2B), suggesting induction of multiple signaling cascades in the colonic mucosa during aging.

Carp-1 expression is downregulated in colonic mucosa during aging. Recently, we identified a 130-kDa perinuclear protein, Carp-1, that participates in EGFR-dependent signaling (23, 24). Attenuation of EGFR activation inhibits growth and induces expression of Carp-1, whereas depletion of Carp-1 inhibits EGFR-dependent apoptosis (24). Moreover, Carp-1 Tyr192 phosphorylation regulates EGFRs signaling and subsequent cell growth (24). Because increased proliferative activity and decreased apoptosis seen in the colonic mucosa with aging is accompanied by enhanced expression and activation of EGFRs (Fig. 2), we postulate that Carp-1 plays a critical role in regulating the growth-related processes in the GI mucosa during aging. To test this possibility, we examined the levels of Carp-1 in the proximal and distal colonic mucosa of young (4-mo-old) and aged (21-mo-old) rats by Western blot analysis. These results revealed a 30–60% reduction in Carp-1 levels in the proximal and distal colonic mucosa of 24-mo-old Fischer-344 rats compared with their 4-mo-old counterparts (Fig. 3).

To further determine whether the age-related decrease in Carp-1 protein in the colonic mucosa could be the result of decreased gene expression, the relative abundance and cellular distribution of Carp-1 mRNA were examined by in situ hybridization. Parallel immunohistological studies were carried out to examine the relative abundance and cellular distribution of Carp-1 protein. Carp-1 mRNA and its protein product was predominantly found in surface mucosal cells of the colon (Fig. 4). The relative abundance of Carp-1 mRNA and Carp-1 protein was decreased by 79 and 54%, respectively, in the colonic mucosa of aged (22-mo-old) rats compared with the corresponding values in young (4-mo-old) rats (Fig. 4). Taken together, the results show that the age-related decrease in apoptosis in the colonic mucosa is associated with reduced expression of Carp-1 and that this could partly be attributable to decreased gene expression. Moreover, the presence of Carp-1 primarily in the surface mucosal cells together with the fact that the majority of apoptotic cells are present in the upper part of the colonic crypt suggest an involvement of Carp-1 in regulating apoptosis in the colonic mucosa. On the other hand, since proliferative cells are primarily located at the bottom of the crypt, the presence of Carp-1 in surface cells suggests that Carp-1 may not play a significant role in modulating proliferative processes in the colonic mucosa.

Carp-1 is regulated by EGFR signaling. Activation of EGFRs leads to induction of several downstream signaling pathways, including PI 3-kinase (17, 27). We recently demonstrated that inhibition of PI 3-kinase activity by wortmannin leads to a marked increase in apoptosis in the colonic mucosa.
of older rats (17). If CARP-1 is considered to be a critical mediator of EGFR-dependent cell proliferation and apoptosis signaling, inhibition of PI 3-kinase by wortmannin, which stimulates apoptosis in the colonic mucosa of older animals, should lead to an increase in CARP-1 expression. To test this possibility, we examined the levels of the total and phosphor-
ylated forms of CARP-1 in the proximal and distal colonic mucosa of young (4-mo-old) and aged (21-mo-old) rats 6 h after a single injection of wortmannin, an experimental proto-
col that has earlier shown to significantly stimulate apoptosis in the colonic mucosa of aged but not in young rats (17). As expected, 6 h after a single injection of wortmannin, the levels of total CARP-1 in the proximal and distal colonic mucosa were increased significantly by 40–50% in 21-mo-old (aged) but not in 4-mo-old (young) rats compared with the corre-
sponding vehicle-treated controls (Fig. 5A). This was accom-
ppanied by a concomitant 50% increase in tyrosine phosphory-
lation of CARP-1 (Fig. 5B).

Age-related reduction in CARP-1 expression in the colonic mucosa is due, in part, to increased methylation. Although aging is found to be associated with decreased expression of CARP-1 in the colonic mucosa, the regulatory mechanisms for this are poorly understood. We hypothesize that this could partly be due to hypermethylation of the CARP-1 promoter. Despite the fact that the full-length promoter for CARP-1 has not been cloned, the database search [www.uscnorris.com/
cpgislands2/cpg.aspx] revealed the presence of a potential methylation island (CpG sequences) in the putative promoter region of the rat CARP-1 gene that is located on chromosome 20 (accession no. NW 047601, data not shown). To test our hypothesis, we conducted methylation-specific PCR (MS-PCR) amplification of bisulfite-treated genomic DNAs derived from the colonic mucosa of 4- to 23-mo-old Fischer-344 rats. A 175-bp CARP-1 promoter subfragment, contained within the predicted methylation island, was amplified using primers for methylated DNA. As shown in Fig. 6A, the MS-PCR amplifi-
cation of the methylated fragment of the putative rat CARP-1 promoter was higher in the colonic mucosa from rats >9 mo of age than those aged between 4 and 6 mo. The results suggest that hypermethylation of CARP-1 gene is partly responsible for

![Fig. 3. Representative Western blot showing decreased cell cycle and apopto-
sis regulatory protein-1 (CARP-1) expression in the proximal and distal colonic mucosa of 21-mo-old (aged) Fischer-344 rats. β-Actin served as an
internal control. Histogram depicts mean ± SE of 4–5 observations of the relative increase in CARP-1 over the corresponding β-actin. *P < 0.01 compared with 4-mo-old rats.](image)

![Fig. 4. Photomicrographs from immunohistological (A) and in situ (B) hybridization experiments showing the relative abundance and localization of CARP-1 protein and mRNA in the distal colonic mucosa of 4- and 22-mo-old Fischer-344 rats. Histogram depicts average ± SE of mean score from 4–5 observations. *P < 0.01 compared with 4-mo-old rats.](image)
the age-related decrease in CARP-1 expression in the colonic mucosa.

If hypermethylation is thought to be partly responsible for the age-related decrease in CARP-1 expression, then inhibition of methylation should reverse the situation. To test this postulation, 4- and 21-mo-old rats were injected with 5-aza-2'-deoxycytidine, a specific inhibitor of DNA methyltransferase activity (8), and the colonic mucosa was assayed for CARP-1 expression. PCR analysis of colonic mucosal DNA from rats 16 h after a single injection of 5-aza-2'-deoxycytidine or vehicle (controls) revealed that the relative concentration of the methylated fragment of the rat CARP-1 promoter was greatly decreased, whereas the unmethylated fragment was increased when compared with the corresponding levels in vehicle-treated controls. The results show the effectiveness of the current experimental protocol in inhibiting methylation of CARP-1 promoter (Fig. 6A). Western blot analysis revealed that inhibition of methylation by 5-aza-2'-deoxycytidine resulted in an approximately twofold increase in CARP-1 levels in the colonic mucosa of both 4- and 21-mo-old rats when compared with the corresponding vehicle-treated controls (Fig. 6C).

DISCUSSION

Homeostasis of the mucosa of the gastrointestinal tract is maintained by sustained proliferation of precursor cells and exfoliation of surface cells (5, 14). Earlier studies from this and other laboratories have demonstrated that aging is associated with increased proliferation and decreased apoptosis in the gastric and colonic mucosa, thereby disrupting mucosal homeostasis (10–12, 16, 19, 28). Although the regulatory mechanisms for the age-related changes in gastrointestinal mucosal proliferation and apoptosis remain to be fully delineated, our current data together with those reported earlier suggest a role for EGFR/ErbB-1 and/or its family member(s) in regulating these processes. Earlier, we demonstrated that aging is associated with increased expression and activation of EGFR and ErbB-2 in the gastric mucosa (26). Our current data further demonstrate that the age-related increase in proliferation and decrease in apoptosis is associated with increased expression and activation of not only EGFR and ErbB-2 but also ErbB-3, suggesting a role for all three members of the EGFR family.

Phosphorylation of tyrosine residues at different sites of the cytoplasmic domain of EGFRs is known to initiate different signaling events. For example, whereas phosphorylation of Tyr845 in the kinase domain of EGFR is thought to stabilize the activation loop by maintaining the enzyme in an active state to regulate epithelial growth factor-induced DNA synthesis, phosphorylation of Tyr1068 creates the binding site for Grb2, leading to activation of Ras and subsequently initiating the mitogen-activated protein kinase (MAPK)/ERK signaling cas-

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**Fig. 5.** Representative Western blot showing changes in total CARP-1 levels in proximal and distal colonic mucosa of 4- and 21-mo-old Fischer-344 rats (A) and the levels of tyrosine-phosphorylated forms of CARP-1 in the distal colonic mucosa 6 h after single injection of wortmannin (0.1 mg/kg) or vehicle (B). Histogram depicts mean ± SE of 4 experiments of the relative increase in total and tyrosine-phosphorylated form of CARP-1 over the corresponding controls. β-Actin served as internal control. *P < 0.01 compared with vehicle-treated animals.

**Fig. 6.** A: methylation-specific PCR amplification of putative CARP-1 promoter in the distal colonic mucosa of Fischer-344 rats of varying ages. B: changes in methylated and unmethylated levels of PCR-amplified putative CARP-1 promoter in the distal colonic mucosa of 22-mo-old rats 16 h after a single injection (ip) of 5-aza-2'-deoxycytidine (Aza). C: Western blot analysis showing changes in the levels of CARP-1 protein in the distal colonic mucosa of 4- and 21-mo-old rats 16 h after a single injection (ip) of 5-aza-2'-deoxycytidine. For PCR amplification studies, the DNA fragment (~175 bp) was amplified from colonic mucosal genomic DNA from 4- to 23-mo-old rats using the primer sequences described METHODS.
colonic mucosa of Fischer-344 rats could partly be attributed to gene, we conducted MS-PCR to determine whether the age-revealed the presence of a potential methylation island (CpG sequence of gene silencing (4). Because the database search of transcription factor binding and/or by formation of an enhancers generally suppresses their function. Methylation-within the regulatory regions of the gene such as promoters and be control of gene regulation. This is because methylation the major roles of DNA methylation in mammals is thought to decrease in CARP-1 expression in the colonic mucosa have not dependent mucosal cell growth.

EGFR-dependent regulation of colonic mucosal growth likely involves distinct and overlapping pathways. Recently, we identified a 130-kDa perinuclear phosphoprotein, CARP-1, a novel growth regulator that also participates in EGFR-dependent signaling (23, 24). Attenuation of EGFR activation inhibits growth and induces expression and tyrosine phosphorylation of CARP-1 (24). Elevated levels of CARP-1 in turn induce apoptosis that involves activation of p38 MAPK and caspsases-3 and -9, whereas depletion of CARP-1 inhibits EGFR-dependent apoptosis (24). Our current observation that the age-related rise in expression and activation of EGFR in the colonic mucosa is associated with decreased expression and tyrosine phosphorylation of CARP-1 suggests a role for CARP-1 in EGFR signal transduction pathways. Induction of CARP-1 has been shown to stimulate apoptosis (24). Our current observation that the age-related reduction in CARP-1 in the colonic mucosa is associated with a decrease in apoptosis shows a relationship between the two events and also suggests a role for CARP-1 in regulating colonic mucosal growth during aging. In support of the latter, we have observed that inhibition of PI 3-kinase activity by wortmannin, which stimulates apoptosis, is associated with increased expression and tyrosine phosphorylation of CARP-1. This, together with our earlier observation that inhibition of EGFR that enhances apoptosis is accompanied by increased expression and tyrosine phosphorylation of CARP-1, suggests that CARP-1 may be key regulator of EGFRs/PI 3-kinase-dependent mucosal cell growth.

Although the regulatory mechanisms for the age-related decrease in CARP-1 expression in the colonic mucosa have not been delineated, we have suggested that this could partly be because of increased methylation of the CARP-1 gene. One of the major roles of DNA methylation in mammals is thought to be control of gene regulation. This is because methylation within the regulatory regions of the gene such as promoters and enhancers generally suppresses their function. Methylation-induced suppression is thought to occur either by the blocking of transcription factor binding and/or by formation of an inactive chromatin state. However, it is still unclear whether methylation directly elicits gene inactivation or is a consequence of gene silencing (4). Because the database search revealed the presence of a potential methylation island (CpG sequences) in the putative promoter region of the rat CARP-1 gene, we conducted MS-PCR to determine whether the age-related decrease in CARP-1 protein and mRNA levels in the colonic mucosa of Fischer-344 rats could partly be attributed to hypermethylation of CARP-1 promoter. Our observation of a higher methylation of CARP-1 promoter in DNA from the colonic mucosa of Fischer-344 rats aged between 9 and 23 mo than those between 4 and 6 mo supports our contention that increased methylation of the CARP-1 promoter could partly be responsible for the age-related decrease in CARP-1 expression in the colonic mucosa. Further support for this contention comes from the observation that inhibition of methylation by 5-aza-2'-deoxycytidine, a specific inhibitor of DNA mehyltransferase activity, leads to increased expression of CARP-1.

In summary, our current data together with those reported earlier demonstrate that aging is associated with increased proliferation and decreased apoptosis in the colonic mucosa of Fischer-344 rats. These changes may in part be regulated by EGFR and its family members. Our current data, for the first time, also demonstrate a role for CARP-1 in regulating mucosal apoptosis during aging, as evidenced by 1) an age-related decrease in mucosal apoptosis in association with a concomitant reduction in CARP-1 expression and tyrosine phosphorylation and 2) the fact that inhibition of mucosal PI 3-kinase activity by wortmannin, which stimulates apoptosis, is associated with increased expression and tyrosine phosphorylation of CARP-1. The age-related decrease in expression of CARP-1 in the colonic mucosa could partly be the result of decreased transcription resulting from increased methylation of CARP-1 promoter, and inhibition of methylation reverses the situation.

GRANTS

This work was supported by National Institute on Aging Grant 2R01-AG-014343 (A. P. N. Majumdar) and by the Department of Veterans Affairs (A. P. N. Majumdar and A. K. Rishi).

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