the majority of colorectal cancers develop in patients with as yet no clearly identified genetic predisposition, and the factors responsible in these individuals have remained poorly defined. Hyperproliferation of the colonic epithelium is recognized as the most common alteration in high-risk conditions for colorectal cancer development and is believed to be the first step in a sequence of events leading to adenocarcinoma formation, growth, and malignant transformation (16). Several dietary and hormonal factors have been identified as possibly important in modulating the proliferative and tumorigenic potential in the colon (3, 31, 40). Recently, incompletely processed gastrins have emerged as a possible regulator of colonic growth and proliferation (6, 35, 38, 41).

Studies published in the early 1990s, from our laboratory and those of others, have indicated that glycine-extended gastrin exerts significant mitogenic effects in vitro on several cell types, including human and mouse colon cancer cells (6, 10, 35, 38, 41). Many primary colon cancers have been shown to express Gly-extended gastrin, but recent studies indicate that progastrin may represent the predominant form of gastrin produced by most human colon cancers (tumors reviewed in Ref. 2). Recent studies from our laboratory (39) and that of others (8) indicate a critical role of autocrine gastrins and their products in colon cancer development and is believed to be the first step in a sequence of events leading to adenocarcinoma formation, growth, and malignant transformation (16). Several dietary and hormonal factors have been identified as possibly important in modulating the proliferative and tumorigenic potential in the colon (3, 31, 40). Recently, incompletely processed gastrins have emerged as a possible regulator of colonic growth and proliferation (6, 35, 38, 41).

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Cocarcinogens are agents that do not initiate tumorigenesis but simulate cell proliferation and expand the pool of transformed cells. Thus gastrinlike peptides may function as cocarcinogens for transformed cells and accelerate tumorigenesis induced by other agents or genetic events. To investigate a possible cocarcinogenic role of nonamidated gastrins, we used a colon carcinogenesis model (18, 22, 51) and examined the effect of either elevated progastrin levels (hGAS transgenic mice) or elevated amidated gastrin levels (INS-GAS mice) on the formation of aberrant colonic crypt foci in response to AOM. The hGAS mice showed significantly higher numbers of aberrant crypt foci (140–200% increase) compared with that in wild-type (WT) and INS-GAS mice (P < 0.05) after AOM treatment. The bromodeoxyuridine-labeling index of colonic crypts also was significantly increased in hGAS mice vs. that in WT and INS-GAS mice. The results therefore provide evidence for a mitogenic and cocarcinogenic role of nonamidated gastrins (progastrin), which is apparently not shared by the amidated gastrins. Although nonamidated gastrins are now believed to mediate mitogenic effects via novel receptors, amidated gastrins mediate biological effects via different receptor subtypes, which may explain the difference in the cocarcinogenic potential of nonamidated vs. amidated gastrins. In conclusion, our results provide strong support for a cocarcinogenic role for nonamidated gastrins in colon carcinogenesis.

Mice overexpressing progastrin are predisposed for developing aberrant colonic crypt foci in response to AOM

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Recent studies show that nonamidated gastrins (Gly-gastrin and progastrin) stimulate colonic proliferation. However, the role of nonamidated vs. amidated gastrins in colon carcinogenesis has not been defined. We measured intermediate markers of carcinogenesis in transgenic mice overexpressing either progastrin (hGAS) or amidated gastrin (INS-GAS) in response to azoxymethane (AOM). The hGAS mice showed significantly higher numbers of aberrant crypt foci (140–200% increase) compared with that in wild-type (WT) and INS-GAS mice (P < 0.05) after AOM treatment. The bromodeoxyuridine-labeling index of colonic crypts also was significantly increased in hGAS mice vs. that in WT and INS-GAS mice. The results therefore provide evidence for a mitogenic and cocarcinogenic role of nonamidated gastrins (progastrin), which is apparently not shared by the amidated gastrins. Although nonamidated gastrins are now believed to mediate mitogenic effects via novel receptors, amidated gastrins mediate biological effects via different receptor subtypes, which may explain the difference in the cocarcinogenic potential of nonamidated vs. amidated gastrins. In conclusion, our results provide strong support for a cocarcinogenic role for nonamidated gastrins in colon carcinogenesis.

transgenic mice

COLORECTAL CANCER REPRESENTS one of the leading causes of cancer-related mortality in the United States (1). Studies by Vogelstein and associates (see Ref. 13) have defined the sequence of many of the genetic events involved in the multistep progression from normal colonic mucosa to adenomatous polyt to malignant tumor in humans. In addition, a number of familial syndromes have been defined in which colon cancer develops at a high incidence because of germline mutations in known tumor suppressor genes (12). However, the majority of colorectal cancers develop in patients with as yet no clearly identified genetic predisposition, and the factors responsible in these individuals have remained poorly defined. Hyperproliferation of the colonic epithelium is recognized as the most common alteration in high-risk conditions for colorectal cancer development and is believed to be the first step in a sequence of events leading to adenocarcinoma formation, growth, and malignant transformation (16). Several dietary and hormonal factors have been identified as possibly important in modulating the proliferative and tumorigenic potential in the colon (3, 31, 40). Recently, incompletely processed gastrins have emerged as a possible regulator of colonic growth and proliferation (6, 35, 38, 41).

Studies published in the early 1990s, from our laboratory and those of others, have indicated that glycine-extended gastrin exerts significant mitogenic effects in vitro on several cell types, including human and mouse colon cancer cells (6, 10, 35, 38, 41). Many primary colon cancers have been shown to express Gly-extended gastrin, but recent studies indicate that progastrin may represent the predominant form of gastrin produced by most human colon cancers (tumors reviewed in Ref. 2). Recent studies from our laboratory (39) and that of others (8) indicate a critical role of autocrine gastrins (largely nonamidated gastrins) in the proliferative potential of human colon cancer (39) and mouse colon (8) cells. Transgenic mice with increased plasma progastrin levels (hGAS) have been shown to exhibit markedly increased colonic proliferation, consistent with a mitogenic role for progastrin in the colon (49).
which form various DNA adducts including 0- to 6-methylquinone, resulting in G to A point mutations. This mutation can potentially activate specific oncogenes such as k-ras and inactivate tumor suppressor genes (52). AOM shows particular specificity for the colon (19), and treatment of mice with AOM has been shown to lead to development of single to multiple aggregates of atypical crypts known as aberrant crypt foci (ACFs), followed later by colonic adenocarcinomas (19, 30, 32). Mutated k-ras and APC genes are similarly detected in the ACFs and colonic adenomas of both humans (11, 26, 42) and AOM-treated mice (17, 26, 52). ACFs are therefore considered reliable intermediate markers for colon tumorigenesis and are likely precursors of colonic adenomas in human patients as well (45).

A number of previous studies have examined the effects of gastrin on carcinogen-induced tumor formation in rodents, but these earlier studies focused solely on amidated gastrins, and the results of these studies have been mixed. Some studies have shown that amidated gastrin enhances the growth of colon cancer induced by chemical carcinogens in rats (reviewed in Ref. 47), whereas other studies have shown that endogenous hypergastrinemia induced by acid-suppressing drugs does not enhance colon cancer induced by chemical carcinogens (29). However, the role of progastrin in enhancing colonic carcinogenesis has not been examined previously. The results of the present studies indicate that in contrast to amidated gastrin, elevated levels of progastrin significantly enhance the risk of developing preneoplastic lesions in the colon.

MATERIALS AND METHODS

Animals. Transgenic hGAS and INS-GAS (both in an FVB/N background) and control FVB/N mice, as described previously (49), were used in this study. Transgenic and WT mice were age and sex matched, had similar body weights, and were raised in a common facility at Massachusetts General Hospital (MGH). The hGAS transgenic mice contain a chimeric rat insulin-human gastrin gene, resulting in overexpression of gastrin in mouse hepatic tissue and markedly elevated (1–100 nM) serum levels of human progastrin (49). The INS-GAS mice contain a chimeric rat insulin-human gastrin transgene, resulting in overexpression of gastrin in pancreatic beta cells and elevated (~150 pM) serum levels of human amidated gastrin (49). Specific pathogen-free mice were bred in the MGH OLAR Facility. At 3–5 wk of age the mice were shipped to the Environmental Containment Facilities (ECL) at The University of Texas Medical Branch (UTMB; Galveston, TX). On arrival at UTMB, mice were quarantined and tested for pinworms twice, over a period of 2 wk. Only pathogen- and pinworm-free animals were used. Animals were housed in a microisolator in solid-bottom polycarbonate cages, fed a commercial diet (M/R rodent sterile diet 7012; Harlan Tech Labs; Indianapolis, IN), and given ad libitum water ad libitum. The animals were maintained on a 12:12-h light-dark cycle, and all experiments were conducted during the daytime. All experiments were approved by the Subcommittee on Research and Animal Care at MGH and the Committee on Animal Care at UTMB.

Induction of ACFs in animals with chemical carcinogen AOM. Male and female mice, housed in separate cages, were placed in separate laminar flow hoods within the ECL facility. We first established optimal conditions for inducing ACFs in FVB/N mice in response to AOM. Male and female mice were injected with various doses of AOM (5–20 mg/kg body wt), and animals were monitored for the induction of an optimal number of ACFs per colon within 2 wk of the last injection. A dose of 15 mg/kg body wt and higher was determined to be toxic to >50% of the male mice but was less toxic to the female mice. More than 80% of the male and female mice (hGAS, INS-GAS, and WT) survived a dose of 12–13 mg/kg body wt per week. At lower doses all animals survived. Both male and female mice, in each group of animals (hGAS, INS-GAS, and WT), developed a significant number of ACFs per animal (>4 per colon) on treatment with 12 mg AOM/kg body wt per week ×2 within 2 wk of the last injection. At lower doses of 10 mg/kg body wt, only a few INS-GAS and WT mice developed significant number of ACFs per animal (<2 per animal). Based on these results, we chose 12 mg of AOM/kg body wt per week ×2, as an optimal dose for inducing ACFs in male and female mice of all animals groups, and animals were killed 2 wk after the last injection. In experiments 1 and 2, an equal number of animals per group were treated with AOM or with the vehicle control (saline). None of the control, saline-injected animals, from all groups of animals, developed aberrant crypts within the time frame of the studies. Therefore, in experiments 3–5 only two to four animals per group were used as control (non-AOM treated). In all cases, AOM (Sigma Chemical; St. Louis, MO) was purchased within a week of the start of the experiment and diluted to the required dose with sterile saline within 30 min of the injections. Animals were injected with either AOM or saline intraperitoneally in 0.1 ml saline/10 g body wt. Animals were weighed once a week and at the time of death. In experiments 3 and 4, each animal was injected with 10 mg of bromodeoxyuridine (BrdU; Boehringer Mannheim; Indianapolis, IN) per kg body wt in ~0.2 ml saline intraperitoneally 1 h before being killed. Animals were killed either by cervical dislocation or decapitation (for purposes of blood collection). Within 3 min after the animals were killed the full-length colon (from the anal to cecal end) was removed and flushed with saline. The colons were slightly distended by filling them with the saline solution under mild pressure, slit open by midline incision, and fixed flat in 70% ethanol on Whatman filter papers (with the mucosal side up), as described previously (51). Twenty-four hours after fixation, the colonic specimens were placed in 70% ethanol in tightly sealed scintillation vials until further analysis for intermediate end points.

Analysis of colons for intermediate end points. At the time of ACF analysis (within 1 wk of collection), colons were removed from 70% ethanol and briefly stained with methylene blue (0.3%) for enumeration of ACFs as described by McLellan and Bird (19). The total number of ACFs per colon, their location, and the number of aberrant crypts per foci were recorded using light microscopy (magnification ×10–12.5). ACFs that involved only one crypt were termed singlets and those that involved multiple crypts per lesion were termed doublets (2 crypts) or multiplets (triplets, quadruplets), depending on the number of crypts involved per foci. Representative ACFs from the hGAS mice are shown in Fig. 1A. In all experiments, the majority of the ACFs were singlets, whereas a significant number of doublets and triplets were also observed. Multiplets, involving more than three crypts per foci, were rare. Representative ACFs from each colon were marked with India ink, punched out with a skin biopsy punch (2.5-mm diam), embedded in paraffin blocks, and processed for tissue sectioning by published procedures (26, 51). Simultaneously, uninvolved crypts (UCs) that were at a distance from the aberrant crypts (ACs) were similarly
Fig. 1. Light microscopic view of gross and histological features of normal and aberrant crypt foci in azoxymethane (AOM)-treated FVB/N mice. A: micrographs a, b, and c illustrate surface appearance of aberrant crypt foci (ACFs) in mucosa of mouse colon. Single (S), double (D), triple (T), or multiple (M) aberrant crypts are present in these views of luminal surface. Crypts surrounding aberrant crypts represent uninvolved crypts that are normal in their staining pattern. Colon is stained with 0.3% methylene blue and photographed with dissecting microscope using transmitted light (magnification $\times$160). B: micrograph is representative hematoxylin and eosin (H&E) section of mucosa illustrating appearance of normal crypt from untreated animal. Cells form in single layer along side of crypt. Nuclei demonstrate rounded uniform appearance normally observed. Micrographs b and c are from representative H&E-stained sections that illustrate aberrant crypts (A) from AOM-treated animals. Cells are in multiple levels along crypt wall and are dysplastic in appearance. Note significant reduction in number of goblet cells and elongated nuclei (arrows) in aberrant crypts (b and c) compared with that observed in normal crypt (a). Crypts that are much less aberrant and more normal in appearance surround aberrant crypts. Dark layer along luminal surface is India ink used to locate crypts during tissue preparation. (Thickness 6 µm, magnification $\times$310)
punched out and processed for tissue sectioning. Control, normal crypts (NCs) from each group of nontreated animals were also processed for tissue sectioning.

Labeling index. Serial sections of longitudinal ACs and UCs from AOM-treated animals and NCs from control animals were prepared, and representative sections were stained with hematoxylin and eosin. The histological data obtained from the hematoxylin and eosin-stained crypts were used to confirm the aberrant-nonaberrant nature of the crypts as in the definition of McEllean and Bird (18, 19), as described recently by Olivo and Wargovich (26). In all cases, lesions or foci, identified by us as containing ACs, were determined to display typical features of dysplasia (Fig. 1B). A representative NC from untreated WT animals is shown in Fig. 1B for comparison. Sections of ACs, UCs, and NCs were immunocytochemically stained to determine the presence of BrdU-labeled cells, as a function of cells in the S phase of the cell cycle, used as a marker of the proliferative index of epithelial cells within a crypt. Cells positive for the presence of BrdU were stained using the In Situ Cell Proliferation Kit (Boehringer Mannheim) as published previously (37). Briefly, sections were deparaffinized, digested with trypsin, treated with 4 N HCl, exposed to the monoclonal antibody to BrdU conjugated to alkaline phosphatase, followed by a red chromogen, and lightly counterstained with hematoxylin. The immunostained sections were examined by bright-field microscopy, and the labeling index (LI) of individual ACs, UCs, and NCs was determined. To determine the LI, images were digitally captured, nuclei were counted, and the percentage of nuclei stained for the presence of BrdU in the crypts was calculated using Image Tool for Windows (version 1.28) image analysis software (University of Texas Health Science Center; San Antonio, TX). The angular transformation (arsin %) was performed, and ANOVA with Newman-Keuls analysis of groups was used to determine statistical significance (P < 0.05).

Results
Effects of AOM treatment on number of ACFs in hGAS, INS-GAS, and WT mice AOM was given at a dose of 12 mg/kg body wt in a series of five sets of experiments 1-5, involving hGAS and control mice, as described in MATERIALS AND METHODS. Control mice received saline injections alone. In experiments 4 and 5,
AOM was additionally given to INS-GAS mice, at the same dose and schedule. In experiments 1 and 4 female mice were used, and in experiments 2, 3, and 5 male mice were used. The administration of AOM had no significant effect on the body weight of the animals during the observation period. The body weight of male and female mice for all three groups of animals (hGAS, INS-GAS, and WT) increased similarly by 10–20% during the 4-wk experimental period (data not shown).

In all groups examined, ACFs (Fig. 1) were more prevalent in the distal one-third of the colon, with fewer numbers present in the middle and proximal thirds of the colon. ACFs were found in all groups of AOM-treated mice but were absent in all groups of saline-treated mice. In all five experiments involving the hGAS mice, the total number of ACFs per colon was greater in the hGAS mice compared with that in the WT mice, and this difference was significant in almost all cases (P < 0.05, Table 1).

In contrast, the total number of ACFs per colon was similar in the INS-GAS mice compared with that in the WT mice, in the experiments 4 and 5 in which INS-GAS mice were used (Table 1). On an average, the percent increase in total number of ACFs per colon compared with that in the corresponding WT mice (irrespective of the sex of the animal) was similar in all experiments (Table 1). We therefore combined the data from all experiments, and the percent change in the total number of ACFs per colon in the transgenic (hGAS, INS-GAS) vs. that measured in the WT mice is presented in Fig. 2. As can be seen from Fig. 2, on average the total number of ACFs per animal was significantly increased.

Fig. 3. Bromodeoxyuridine (BrdU) labeling of crypts from WT and hGAS mice. Micrographs of sections illustrating normal (N) crypts (taken from control, non-AOM-treated, WT and hGAS mice) and uninvolved (U) and aberrant (A) crypts (taken from AOM-treated WT and hGAS mice from experiments 3-5) are immunostained for presence of BrdU and counterstained with hematoxylin. Uninvolved crypts from AOM-treated mice that were used for BrdU-labeling index (LI) determination were taken from regions free of aberrant crypts. Representative sections of N, U, and A crypts from WT and hGAS (HG) mice are shown in micrographs a–f. BrdU-labeled cells contain darkly stained nuclei. %BrdU-labeled cells per crypt was determined from similar sections, as described in Materials and Methods, and data obtained are presented in Fig. 4. Dark stain on luminal surface of aberrant crypts is India ink used to localize crypts during preparation. As can be seen, normal crypts from hGAS mice (d) demonstrated significantly higher number of BrdU-labeled cells compared with that in normal crypts from WT mice (a). Similarly uninvolved crypts from hGAS, AOM-treated mice (e) presented higher proportion of BrdU-labeled cells compared with that in uninvolved crypts from WT mice (b). Labeling of aberrant crypts (A) of hGAS mice (f) was similar to that of aberrant crypts (A) of WT mice (c). Labeled cells were present in both upper and lower compartments in hGAS mice (f), whereas BrdU-labeled cells were mostly confined to lower compartment of aberrant crypts in WT mice (c). Thickness 6 µm, magnification x310.
by $-61 \pm 12.6\%$ in the hGAS vs. WT mice. The percent change in the total number of ACFs per animal in the INS-GAS vs. WT mice was insignificant (Fig. 2).

The multiplicity of the crypts per foci (i.e., singlets vs. doublets vs. multiplets) was analyzed for each group of mice. The results are presented in Table 1. The majority of the foci contained single ACs (singlets). A significant number of ACFs also contained two (doublets) or more than two (multiplets) ACs per foci. In all experiments other than experiment 3, the total number of singlets was significantly higher in the hGAS vs. that in the WT mice (Table 1). The total number of doublets and multiplets per colon was on average higher in the hGAS vs. WT mice, achieving statistical significance in experiments 1, 3, and 5. On the other hand, the multiplicity of crypts per foci in the colons of INS-GAS mice was similar to that in the WT mice (Table 1).

Effect of AOM on BrdU-labeling index of colonic crypts in hGAS, INS-GAS, and WT mice. BrdU staining was carried out in both AOM-treated and -untreated mice, and the LI was calculated as the percent cells labeled within a whole crypt. In the AOM-treated animals, UC and AC foci were analyzed separately for BrdU staining and calculation of the LI. BrdU labeling in the NCs of untreated WT mice was generally present at very low levels in the lower one-third of the crypts (Fig. 3a). On AOM treatment, the BrdU labeling increased markedly both in the UCs and ACs of the WT mice (Fig. 3, b and c). The UCs in AOM-treated WT mice were labeled more heavily than the NCs of the WT mice, but the labeling was confined to the lower one-third of the crypts (Fig. 3b) and was significantly lower than the BrdU labeling of ACs in the WT mice (Fig. 3c). The BrdU labeling of NCs from saline-injected hGAS mice on the other hand was already high, being markedly higher than the labeling of NCs of WT animals (Fig. 3, a and d). The NCs of hGAS mice were labeled for BrdU mostly in the lower one-third of the crypts (Fig. 3d). In comparison, the ACs of the AOM-treated hGAS mice were labeled for BrdU in the lower as well as the upper compartments of the crypts (Fig. 3f). The labeling of UCs and ACs from AOM-treated hGAS mice could not be distinguished and appeared to be similar in its pattern of labeling within the lower two-thirds of the crypts (Fig. 3, e and f). Once again, the cases of INS-GAS mice, the BrdU labeling of NCs, UCs, and ACs was very similar to that of the corresponding crypts from the WT mice (data not shown).

To evaluate the BrdU LI, we initially counted representative crypts (NCs, UCs, and ACs) from all the WT, hGAS, and INS-GAS mice from experiments 3 and 4 in which we had injected the animals with BrdU (as described in MATERIALS AND METHODS). The data from WT and hGAS mice is presented in Fig. 4. The LI of NCs from hGAS mice was almost twice that of NCs from WT mice (Fig. 4) and confirms our previous findings (49). The LI of UCs and ACs from AOM-treated WT mice increased linearly and significantly compared with the LI of NCs from the WT control mice (Fig. 4). In the case of hGAS mice, however, because the LI of NCs in untreated mice was already quite high, it was increased by only $-40\%$ in UCs and by $-50\%$ in ACs on AOM treatment (Fig. 4). The LI of ACs in hGAS vs. that in WT mice was therefore not significantly different and may represent a maximizing-plateauing response of colonic mucosa to AOM. As determined with all other parameters, the LI of NCs, UCs, and ACs from INS-GAS mice was similar to that measured for the corresponding crypts in the WT mice (data not shown).

To confirm whether the LI measured for representative crypts from each animal was in fact a true reflection of the LI for the specific crypts from a particular group of animals, we further analyzed $-10–20$ crypts per animal (NCs and UCs) from all animals from the representative experiment 4. All available midline sections of aberrant crypts from this experiment were counted for this purpose, and the data thus obtained from a much larger sample number are presented in Table 2. The data in Table 2 are almost identical to the data presented in Fig. 4, further confirming that the LI of NCs and UCs from hGAS mice were indeed significantly higher than those from the corresponding crypts from WT and INS-GAS mice. Once again the LI of ACs was similar for all three groups of animals (Table 2 and Fig. 4).
Amidated gastrins, G17 or G34, was always collected during daytime (10 AM–12 noon). Food-stimulated gastrin levels in rodents are known to be at peak during nighttime and INS-GAS animals and aberrant crypts from WT, hGAS, and INS-GAS animals were counted from all animals in experiment 4. Values for aberrant crypts are means ± SE from 60–80 crypts per animal group, wherein >10–20 crypts per animal were counted from all animals in experiment 4. Values for aberrant crypts are means ± SE from 15–19 crypts per group of animals, wherein at least 2–3 aberrant crypts per animal were counted. *P < 0.05 vs. corresponding WT and INS-GAS values.

A significant increase in the total number of ACFs in AOM-treated hGAS mice vs. that in the WT mice (Table 1) likely reflects the higher LI activity of NCs and UCs in hGAS mice (that may have translated into an increased conversion of NCs to ACs on AOM treatment).

Correlation between ACF formation and levels of progastrin and gastrin. To examine a possible relationship between gastrin levels and ACF formation, plasma was obtained from AOM-treated animals when they were killed and analyzed for the concentration of progastrin and amidated gastrins. The results are shown in Table 3. In the case of WT mice, amidated gastrin levels were ∼40–80 pM, whereas progastrin levels were undetected (assay not sensitive at <40 pM). The levels of progastrin were variable but markedly elevated in the hGAS mice (∼1,000–8,000 pM), whereas the levels of amidated gastrin were similar to those of WT mice. In contrast, the INS-GAS mice showed approximately threefold elevations in serum levels of amidated gastrin (∼150 pM) but only slight increases in progastrin levels. The peak level of gastrins in INS-GAS mice, in response to food stimulation at night, however, is not known.

In animals in which gastrin determinations were obtained, attempts were made to correlate the total number of ACFs per animal with the concentration of progastrin and amidated gastrins. With respect to progastrin, no correlation could be discerned in the WT mice (no measurable progastrin) or the hGAS mice (where progastrin levels were >1,000 pM). However, in the INS-GAS mice, where progastrin levels ranged between 0 and 100 pM, there was a clear, positive correlation between progastrin levels and the number of ACFs per mouse (Fig. 5). In contrast, there appeared to be a negative correlation between the level of amidated gastrins and the number of ACFs per hGAS mouse (Fig. 5).

### DISCUSSION

The possibility that processing intermediates of gastrin (mainly Gly-gastrin and progastrin) may function as autocrine-endocrine growth factors has evolved as a novel concept in the last few years (2, 6, 10, 35, 38, 39, 41, 49). Our study for the first time suggests a role for incompletely processed progastrins in determining the risk of colon carcinogenesis, possibly through effects on stimulation of colon proliferation. Previous studies from our group showed that progastrin-expressing hGAS mice had a twofold increase in colonic proliferation at 10–12 mo of age (49). The present study demonstrates that this twofold increase in proliferation occurs as early as 2–3 mo of age. Published reports from a number of groups have suggested that an increased rate of colonic proliferation enhances the risk of progression to colon cancer (16).

A striking finding of the present study was that the intermediate markers of colon carcinogenesis were significantly increased in hGAS mice vs. those in WT mice in response to AOM. The progastrin-expressing hGAS mice had significantly increased numbers of ACFs, a surrogate marker for progression to colon cancer, compared with WT mice. The hGAS mice also showed a trend toward increased multiplicity of their ACFs, which has also been recognized to correlate with increased colon cancer risk (reviewed in Refs. 26 and 45). Because the total number and multiplicity of ACFs is now believed to correlate strongly with the risk for developing adenomas and adenocarcinomas (18, 22, 26, 45), and because ACFs display genetic mutations known to be associated with colon carcinogenesis in humans and rodents (as recently reviewed in Refs. 26 and 45), it is reasonable to speculate that the significantly higher number and multiplicity of ACFs measured in the hGAS mice will translate into the formation of higher

### Table 2. Percent labeling index of normal, uninvolved, and aberrant crypts from WT, hGAS, and INS-GAS animals

<table>
<thead>
<tr>
<th>Animals</th>
<th>Control Normal</th>
<th>AOM Treated</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Uninvolved</td>
</tr>
<tr>
<td>WT</td>
<td>0.044 ± 0.0004</td>
<td>0.084 ± 0.0004</td>
</tr>
<tr>
<td>INS-GAS</td>
<td>0.042 ± 0.0005</td>
<td>0.066 ± 0.0011</td>
</tr>
<tr>
<td>hGAS</td>
<td>0.102 ± 0.0007*</td>
<td>0.125 ± 0.0007*</td>
</tr>
</tbody>
</table>

Values for normal and uninvolved crypts are means ± SE from 60–80 crypts per animal group, wherein >10–20 crypts per animal were counted from all animals in experiment 4. Values for aberrant crypts are means ± SE from 15–19 crypts per group of animals, wherein at least 2–3 aberrant crypts per animal were counted. *P < 0.05 vs. corresponding WT and INS-GAS values.

### Table 3. Concentration of amidated gastrin and nonamidated progastrin in serum of mice

<table>
<thead>
<tr>
<th>Peptide Expt</th>
<th>Concentration (fmol/ml)</th>
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<tr>
<td></td>
<td>Progastrin</td>
</tr>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td>h-GAS</td>
<td>8,640 ± 5,600* (6)</td>
</tr>
<tr>
<td>WT Means ± SE</td>
<td>&lt;40 (4)</td>
</tr>
<tr>
<td>INS-GAS</td>
<td>41.7 ± 37 (6)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are number of mice studied. Values in INS-GAS mice probably reflect baseline (nonpeak) values, because serum was always collected during daytime (10 AM–12 noon). Food-stimulated gastrin levels in rodents are known to be at peak during nighttime (33). Amidated gastrins, G17 or G34. *P < 0.05 vs. corresponding WT and INS-GAS values.  †P < 0.05 vs. corresponding INS-GAS values. 

1. G396 NONAMIDATED GASTRINS IN COLON CARCINOGENESIS
numbers of adenomas and adenocarcinomas in hGAS mice vs. WT animals; our preliminary studies confirm this possibility (48).

Interestingly, the INS-GAS mice, which over-express amidated gastrin, showed no increase in ACFs compared with control mice. There was in fact a negative correlation between plasma levels of amidated gastrins and number of ACFs (Fig. 5), suggesting that amidated gastrins might even be protective. In contrast, there was a positive correlation between progastrin levels and the number of ACFs in the INS-GAS mice, consistent with a cancer-promoting role for these incompletely processed forms of gastrin. The number of ACFs in hGAS mice did not strongly correlate with progastrin levels in the mice (which may be explained by the extremely high levels in most of these mice) possibly beyond the dose-dependent range, which may have resulted in maximal stimulation of the proliferative pathways. It remains to be seen whether a >100-fold increase in levels of amidated gastrins can similarly result in increasing predisposition to preneoplastic changes in colonic mucosa in response to AOM. Based on the results presented in Fig. 5, however, that seems less likely. The present studies with hGAS mice thus provide the first evidence that high levels of circulating nonamidated gastrins can potentially function as cocarcinogens and significantly augment the carcinogenic potential of agents such as AOM.

The possible relationship between hypergastrinemia and colon cancer has been a confusing and controversial area (21, 25, 27, 28, 34, 43, 44). In a majority of these studies only amidated gastrin was measured, which may have contributed to the discrepancy in results. Whereas most human colon cancers express gastrin mRNA (reviewed in Ref. 2), gastrin peptides are poorly processed in colon tumors and colon cancer cell lines (2, 6, 41) and are largely secreted as progastrin and Gly-extended gastrin (2, 6, 41). In a recent study, circulating forms of both amidated and nonamidated gastrins were measured (4). Nonamidated gastrins were significantly higher (by almost 2- to 5-fold) in patients with colorectal carcinomas, compared with levels in control patients (4). More recently, a good correlation between ras mutations and gastrin gene expression was demonstrated, suggesting that gastrin gene may be an important downstream target of ras (23). Based on these recent reports (4, 23) it can be speculated that colon tumors positive for ras mutations are likely to express and secrete nonamidated gastrins.

In a recent prospective study, amidated gastrin and Gly-gastrin were measured in the serum of a large number of patients, some of whom eventually developed colorectal carcinomas (46). The results suggested that in a subpopulation of patients (~8.6%), high levels of serum gastrin may have contributed to the development of colorectal carcinomas (46); serum levels of progastrin were not measured in this study. However, other studies (21, 25, 27, 28) have not shown a clear association between levels of amidated gastrin and colorectal cancer. We now know that amidated gastrin and nonamidated gastrins can potentially exert proliferative effects on several cell types, including gastrointestinal cancer cells in vitro (6, 10, 35, 38, 40, 41), suggesting that the difference in the cocarcinogenic potential of amidated gastrins (INS-GAS mice) and nonamidated gastrins (hGAS mice), as observed in the present in vivo studies, probably reflects other contributing factors. We speculate that the receptor subtypes...
that mediate the mitogenic actions of amidated and nonamidated gastrins have an important role in the 
carcinogenic potential of gastrinlike peptides, as discussed 
discussed below.

The CCK-B receptor (CCKB-R) represents the major 
receptor for amidated gastrins (50) and has been vari- 
ously reported to be either expressed or not expressed 
in the colons of many species (5, 9, 20). The CCKB-R 
does not bind progastrin or Gly-extended forms of 
gastrin (50) and is unlikely to be responsible for prolifer- 
ative effects of progastrin observed in the colon of 
hGAS mice. Several candidate receptors have been 
described, including a gastrin-binding protein (CCKC-R) 
(2), a gastrin preferring receptor (GP-R) (38), and a 
Gly-gastrin binding receptor (GG-R) (35); the latter two 
receptors have not been cloned. So, whereas any one or 
more of these receptors can potentially mediate mito- 
genic effects of nonamidated gastrins, the mitogenic 
effects of amidated gastrins can potentially be medi- 
ated via CCKB-R and GP-R (since both these receptor 
subtypes bind amidated gastrins with high affinity) 
(38, 50). However, besides mediating mitogenic effects, 
CCKB-R can also mediate growth inhibitory–(7) and 
morphogenic-differentiation effects (7, 14, 36). Thus the 
contribution of the CCKA-R to the total mitogenic 
effects of nonamidated gastrins, the mitogenic 
effects of amidated gastrins observed in the colon of 
hGAS mice. Several candidate receptors have been 
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(38, 50). However, besides mediating mitogenic effects, 
CCKB-R can also mediate growth inhibitory–(7) and 
morphogenic-differentiation effects (7, 14, 36). Thus the 
significant difference in the carcinogenic potential of 
nonamidated gastrins and amidated gastrins (as ob-
erved in the present study) may reflect the conflicting 
growth-inhibitory vs. growth-stimulatory effects of 
amidated gastrins (mediated via different receptor 
subtypes).

The present studies with hGAS mice thus provide the 
first evidence that high levels of circulating progastrin 
can potentially function as cocarcinogens and signifi-
cantly augment the carcinogenic potential of methyl-
ating agents such as AOM. We believe that the increase 
in the proliferative index of colonic crypts in the hGAS 
mice most likely rendered the colonic cells more suscep-
tible to AOM, resulting in conversion of a significantly 
higher number of colonic crypts into aberrantly prolifer-
ating crypts (demonstrating typical features of dyspla-
sia). Thus, on the basis of our results in transgenic mice 
and previous human studies (4, 46), we speculate that 
high levels of incompletely processed gastrins may 
potentially increase the risk for developing colon cancer 
in human patients.

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REFERENCES

2. Baldwin, G. S., and A. Shulkes. Gastrin, gastrin receptors and 
Expression, processing, and secretion of gastrin in patients with 
agents directed to peptide hormone receptors–defining the re-
quirements for a successful drug. Proc. Natl. Acad. Sci. USA 95: 
Gene expression in human colon cancer cells: autocrine role of 
7. Detjen, K., M. C. Fenrich, and C. D. Logsdon. Transfected 
CCK receptors mediate growth inhibitory effects on human 
8. Hollande, F., A. Imdahl, T. Mantamadiotis, G. Ciccotosto, 
D. Shulkes, and G. S. Baldwin. Glycine-extended gastrin acts 
as an autocrine growth factor in a nontransformed colon cell line. 
9. Imdahl, A., T. Mantamadiotis, S. Eggstein, E. H. Farth-
mann, and G. S. Baldwin. Expression of gastrin/gastrin/ 
CCK-B and gastrin/CCK-C receptors in human colorectal carci-
10. Iwase, K., B. H. Evers, M. F. Klareskog, T. Mantamadiotis, 
Regulation of growth of human gastric cancer by gastrin and glycine-extended 
Hamilton, B. Vogelstein, and K. W. Kinzler. Molecular deter-
mamentals of dysplasia in colorectal lesions. Cancer Res. 54: 
14. Langhans, N., G. Rindi, M. Chiu, J. F. Rehfeld, B. Ardmann, 
M. Beinborn, and A. S. Kopin. Abnormal gastric histology and 
decreased acid production in cholecystokinin-B/gastrin receptor-
15. Lawley, P. D. Carcinogenesis by alkylating agents. In: Chemical 
16. Lipkin, M. Biomarkers of increased susceptibility to gastrointes-
tinal cancer: new application to studies of cancer prevention in 
17. Maltzman, T., J. Whittington, L. Driggers, J. Stephens, and 
D. Ahnen. AOM-induced mouse colon tumors do not express 
preneoplastic lesions in the murine colon. Cancer Res. 48: 
induction of aberrant crypts in murine colons. Cancer Res. 48: 
20. McWilliams, D. F., S. A. Watson, D. M. Crosbee, D. Michaeli, 
and R. Seth. Coexpression of gastrin and gastrin receptors 
(CCK-B and delta CCK-B) in gastrointestinal tumor cell lines. 
Serum gastrin levels in patients with colorectal cancer and 
22. Moen, C. J., M. A. van der Valk, R. P. Bird, A. A. Hart, and 
P. Demanded. Different genetic susceptibility to aberrant 
23. Nakata, H., S. Wang, D. C. Chung, J. K. Westwick, and 
L. G. Tillotson. Oncogenic ras induces gastrin gene expression in 
Dockray. Identification of progastrin derived peptides in colorec-


