Signaling Pathways Associated with Colonic Mucosa Hyperproliferation in Mice Overexpressing Gastrin Precursors

Audrey Ferrand, Claudine Bertrand, Ghislaine Portolan, Guanglin Cui, Jane Carlson, Lucien Pradayrol, Daniel Fourmy, Marlene Dufresne, Timothy C. Wang, and Catherine Seva

Abstract
MTI/G-Gly mice and hGAS mice, overexpressing glycine-extended gastrin (G-Gly) and progastrin, respectively, display colonic mucosa hyperplasia, hyperproliferation, and an increased susceptibility to intestinal neoplasia. Here, we have used these transgenic mice to analyze in vivo the modulation of intracellular signaling pathways that may be responsible for the proliferative effects of gastrin precursors. The expression, activation, and localization of signaling and cell-to-cell adhesion molecules were studied using immunofluorescence and Western blot techniques on colonic tissues derived from MTI/G-Gly, hGAS, or wild-type FVB/N mice. These analyses revealed an up-regulation of Src tyrosine kinase and related signaling pathways [phosphatidyl inositol 3′-kinase (PI3K)/Akt, Janus-activated kinase (JAK) 2, signal transducer and activator of transcription (STAT) 3, and extracellular-signal regulated kinases (ERK)] in both MTI/G-Gly and hGAS mice compared with the wild-type control animals as well as an overexpression of transforming growth factor-α (TGF-α). In contrast, overexpression of the gastrin precursors did not affect the activation status of STAT1 nor the expression and the distribution of adhesion proteins (focal adhesion kinase, cadherins, and catenins). We report for the first time that the transition from a normal colonic epithelium to a hyper-proliferative epithelium in MTI/G-Gly and hGAS mice may be a consequence of the up-regulation of Src, PI3K/Akt, JAK2, STAT3, ERKs, and TGF-α. Deregulation of cell adhesion, a late event in tumor progression, does not occur in these transgenic models. (Cancer Res 2005; 65(7): 2770-7)

Introduction
Hyperproliferation and hyperplasia of the colonic mucosa are associated with an increased risk of colon cancer development and likely represent early stages in the sequence of events leading to adenocarcinoma. The factors involved in this hyperproliferative response remain largely unknown. However, numerous data suggest that precursors of gastrin, a gastrointestinal peptide hormone, could contribute to neoplastic progression in colon. As with many peptide hormones, gastrin is synthesized as a large precursor molecule, progastrin, which is processed to gastrin after the cleavage of the NH2-terminal signal peptide. Progastrin then undergoes posttranslational modifications leading to a processing intermediate of gastrin, glycine-extended gastrin (G-Gly), which serves as a substrate for the synthesis of the mature amidated gastrin (1). High concentrations of gastrin precursors, progastrin, and G-Gly, have been observed in colon tumors and in blood of patients with colorectal cancer (2, 3). These precursors represent 90% to 100% of the gastrin peptides produced by colon tumor and are found in 80% to 90% of colorectal polyps in human (4). Numerous groups have shown that G-Gly and progastrin act as growth factors for nontransformed cells and colon cancer cell lines (5–8). Furthermore, transgenic mouse models overexpressing progastrin (hGAS mice) or G-Gly (MTI/G-Gly mice) have confirmed in vivo these observations (9, 10). Histologic studies and bromodeoxyuridine incorporation on colon from these transgenic mice have shown hyperplasia of the colonic mucosa and increased epithelial proliferation. In addition, hGAS mice treated with a chemical carcinogen, azoxymethane, display an increased predisposition to develop preneoplastic lesions or even colonic adenocarcinoma compared with wild-type control mice (11). Taken together, these studies show the role of progastrin and G-Gly in early events potentially involved in colonic carcinogenesis, namely hyperproliferation and hyperplasia, but also suggest that progastrin may play role of co-carcinogen for colonic epithelial cells. However, the in vivo molecular mechanisms involved in progastrin and G-Gly mitogenic effects remain largely unknown.

The nonreceptor tyrosine kinase p60-Src is well established as an oncogenic and several studies have linked Src to colon cancer. Increased Src expression and activation have been described in colon cancer cell lines and in primary colon cancer resection specimens (12). Moreover, in vivo studies comparing p60-Src activation levels between nonmalignant and malignant polyps have shown that the activation of tyrosine kinase is likely an early event in the colonic tumor process (13, 14). In addition, another attractive candidate is the phosphatidyl inositol 3′-kinase (PI3K)/Akt pathway, which is involved in the regulation of many cell processes including proliferation and survival. LY294002, a PI3K specific inhibitor, has been shown to inhibit growth and to induce apoptosis in several human colon cancer cell lines (15). Moreover, phosphorylation (on Ser473) of Akt, also named protein kinase B, a transducer and activator of transcription (STAT) proteins in oncogenesis has been extensively studied. In particular, STAT3 is now recognized as an oncogene implicated in many cancers, and may be required in Src-dependent oncogenesis (17).
Thus, the aim of the current study was to investigate whether progastrin and glycine-extended gastrin might regulate in vivo proteins known to play a crucial role in cell proliferation and colon cancer development. For this purpose, expression and activation of signaling molecules were studied using immunofluorescence and Western blot analysis on colon tissue from MTI/G-gly, hGAS, and wild-type FVB/N mice. Here, we report an up-regulation of Src and related signaling pathways [PI3K/Akt, Janus-activated kinase (JAK) 2, STAT3, and extracellular-signal-regulated kinases (ERK)] in the colonic mucosa of hGAS and MTI/G-gly mice compared with the wild-type FVB/N mice as well as an overexpression of transforming growth factor-α (TGF-α). In contrast, expression and localization of adhesion molecules were not affected.

Materials and Methods

**Animals.** hGAS and MTI/G-Gly (both in a FVB/N background) and control FVB/N mice were sex matched and 10 mice of each experimental group (22-24 weeks old) were used in this study. Mice were reared in routine animal facility of the University of Massachusetts Medical School and maintained on a 12:12 hours light-dark cycle. All the experiments were done during the daytime.

**Antibodies.** Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Chemicon (Temecula, CA); p120-catenin, β-catenin (immunofluorescence and Western blot), and STAT3 (Western blot) were from Transduction Laboratories (Lexington, KY); phospho-Tyr397 Src (immunofluorescence and Western blot) and phospho-Tyr397 focal adhesion kinase (FAK) were from BioSource (Camarillo, CA); FAK, JAK2, STAT3, TGF-α, STAT1 (immunofluorescence), ERK2, Src (immunofluorescence and Western blot), and vimentin were from Santa Cruz Biotechnology (Santa Cruz, CA); EGF, Akt, P-Akt Ser473 (immunofluorescence), and phospho-ERK (immunofluorescence and Western blot) were from Cell Signaling (Beverly, MA); JAK2 (immunofluorescence), phospho-JAK2, and phospho-Tyr205 STAT3 (immunofluorescence and Western blot) were from UBI (Hauppauge, NY); and E-cadherin (immunofluorescence and Western blot) was from New England Biolabs (Beverly, MA). A10 lysate (vimentin antibody positive control) was provided by Santa Cruz.

**Immunofluorescence staining.** For signaling molecules, TGF-α and epidermal growth factor receptor (EGFR), we used formaldehyde-fixed, paraffin-embedded tissues. Antigen retrieval was done on dewaxed sections by microwaving slides in 10 mmol/L citrate buffer (pH 6). After serum blocking and application of primary antibodies, the detection was done using Alexa Fluor 488 goat anti-rabbit antibody (Molecular Probes, Eugene, OR). Control slides, where the primary antibody was replaced by diluted nonimmune rabbit IgG, were checked for nonspecific reactivity before assessment of the staining. For adhesion molecules, immunofluorescence staining was done on tissues embedded in Optimal Cutting Temperature compound (TissueTek, Miles, Elkhart, IN) and stored at −80°C until used. Cryosections were immunostained as previously described (18). Slides were analyzed on a Nikon E400 microscope (Nikon Manufactory, Badhoevedorp, The Netherlands) with a Sony DXC 950 camera and Visiolab 2000 software. For comparisons, identical volumes of antibody mix were used for all samples. Immunofluorescence intensity was analyzed using the image analyzer Biocom (Lyon, France).

**Isolation of colonic epithelial cells and Western blot analysis.** Colonic epithelial cells were isolated as previously described (19). Briefly, mice colonic cells were isolated by shaking the entire everted colon in a dispersing solution containing EDTA. Cells were lysed and analyzed by Western blot as previously described (20).

**Primary culture of isolated colonic epithelial cells from FVB/N mice.** The method used for colonic epithelial cell preparation is a modified Booth procedure (21). Here, after resection, colons from wild-type FVB/N mice were washed four times with culture medium (DMEM containing 15% serum, 1 mg/ml, penicillin, and streptomycin) to flush out luminal contents and reduce the intestinal flora. Colons were slit open longitudinally, washed again, and incubated for 30 minutes in 30 ml culture medium in a 50 ml falcon tube. After shaking, colons were allowed to settle under gravity for 30 seconds and the top 20 ml were carefully removed. Colons were transferred into a Petri dish and colonic epithelium was gently scraped away. Tissue separation was verified under microscope. Epithelium was dissociated by pipetting and vigorous shaking in 30 ml culture medium. After this step, we followed Booth method (21) using DMEM supplemented with 15% serum and containing 1 mg/ml penicillin and streptomycin as culture medium. For Western blot analysis on primary culture of isolated colonic epithelial cells, cells were plated on dishes coated with collagen purified from rat tail. Forty-eight hours after plating, cells were treated for 48 hours with peptides (10^−8 mol/L) and then lysed.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on primary culture.** Approximately 50,000 cells/well were plated into 96-well plate that had been previously coated with collagen purified from rat tail. Forty-eight hours after plating, cells were treated for 48 hours with peptides (10^−8 mol/L) and inhibitors (10 mmol/L) when indicated. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Sigma, St. Louis, MO) was used to measure proliferation as previously described (22).

**Results**

**Src status in MTI/G-gly and hGAS mice.** p60-Src expression was analyzed on colonic tissue sections by immunofluorescence methods using antibodies that detect specifically total p60-Src protein irrespective of its phosphorylation status. As shown in Fig. 1 and Table 1, tissues derived from hGAS and MTI/G-Gly mice showed high levels of Src expression compared with control FVB/N. Using a phospho-tyrosine-specific Src antibody that detects activated Src proteins, we also showed, in both transgenic models, a significant increase of Src activation (Fig. 1; Table 1). Src overexpression and activation were then confirmed by Western blot analysis done on lysates from isolated colonic epithelial cells from different animals (Fig. 2).

**Regulation of signal transducer and activator of transcription 3 and Janus-activated kinase 2 in MTI/G-gly and hGAS mice.** We analyzed, by immunofluorescence microscopy on colonic tissue sections, the expression levels of STAT3 using antibodies specific for STAT3 protein. We detected a significant over-expression of STAT3 in hGAS and MTI/G-Gly mice compared with wild-type controls (Fig. 1; Table 1). In addition, colonic samples from transgenic mice also showed intense staining with antibodies specific for the phosphorylated active form of STAT3 (PY-STAT3), whereas there was a faint PY-STAT3 signal in control mice (Fig. 1). The significant up-regulation of STAT3 in the animal models overexpressing gastrin precursors (Table 1) was confirmed by Western blot analysis using the same specific STAT3 or PY-STAT3 antibodies (Fig. 2). In contrast to STAT3, STAT1 seems to play a role in cell growth arrest and thus is generally considered as a tumor suppressor. Immunofluorescence staining of STAT1 with either anti-STAT1 or anti-PY-STAT1 antibodies in hGAS or MTI/G-Gly showed no change in expression levels compared with those observed in wild-type controls (Fig. 1).

The JAKs are well known to directly activate the STAT proteins. Among the four members of the JAK family, JAK2 is well described for its involvement in cell growth. Therefore, we tested whether gastrin precursors regulate JAK2 expression and activation in vivo. Colonic tissue sections from both transgenic mice models analyzed by immunofluorescence microscopy using an anti-JAK2 antibody showed a strong JAK2 overexpression compared with FVB/N controls (Fig. 4; Table 1). Similarly, MTI/G-Gly and hGAS mice colonic tissue sections showed high levels of staining for the phosphorylated active form of JAK2 (Fig. 1; Table 1) as opposed to...
In vitro adaptor/regulatory subunit p85 of a catalytic subunit p110 constitutively associated with an 
G-Gly regulate the PI3K/Akt pathway activation in colonic 
and G-Gly mice. ERKs activation by gastrin peptides in vitro has previously been described by several groups (20, 24). However, there is currently no in vivo information about the status of the ERK pathway in gastrin precursors signaling. Immunofluorescence analysis was done using antibodies specifically directed against ERK proteins. Colonic mucosa of hGAS and MTI/G-gly mice showed a higher level of total ERK expression compared with control FVB/N mice (Fig. 3; Table 1) as confirmed by Western blot analysis (Fig. 2). In addition, we analyzed in these animals ERK activation by immunofluorescence or Western blot using antibodies specific for dually phosphorylated (active) ERKs. Results show a strong immunoreactivity in transgenic mice compared with control mice (Figs. 2 and 3). Overall, these results are consistent with an up-regulation of the ERK pathway in the colonic mucosa of mice overexpressing progastrin or G-Gly.

The two other mitogen-activated protein kinase (MAPK) cascades, JNK and p38-MAPK, were initially identified as two pathways mediating cellular stress induced by UV, proinflammatory cytokines, heat, and osmotic shocks. More recent studies have shown that JNK and p38-MAPK are also involved in the regulation of cell proliferation by growth factors or cytokines. We therefore analyzed, by immunofluorescence assays, the expression and the phosphorylation status of these two proteins. In contrast to what we observed for the ERK pathway, the regulation of JNK and p38-MAPK was not affected in transgenic mice compared with control animals (data not shown).

Table 1, the immunofluorescence staining obtained with the antibody directed against the phosphorylated Ser473 of Akt (P(S473)-Akt) showed an increased phosphorylation of this protein in both transgenic mice models compared with wild-type FVB/N controls that seems correlated to p85 overexpression. In contrast, total Akt protein expression was unchanged in all mice models (Fig. 3; Table 1).

The extracellular-signal regulated kinase pathway in MTI/ G-gly and hGAS mice. ERKs activation by gastrin peptides in vitro has previously been described by several groups (20, 24). However, there is currently no in vivo information about the status of the ERK pathway in gastrin precursors signaling. Immunofluorescence analysis was done using antibodies specifically directed against ERK proteins. Colonic mucosa of hGAS and MTI/G-gly mice showed a higher level of total ERK expression compared with control FVB/N mice (Fig. 3; Table 1) as confirmed by Western blot analysis (Fig. 2). In addition, we analyzed in these animals ERK activation by immunofluorescence or Western blot using antibodies specific for dually phosphorylated (active) ERKs. Results show a strong immunoreactivity in transgenic mice compared with control mice (Figs. 2 and 3). Overall, these results are consistent with an up-regulation of the ERK pathway in the colonic mucosa of mice overexpressing progastrin or G-Gly.

The two other mitogen-activated protein kinase (MAPK) cascades, JNK and p38-MAPK, were initially identified as two pathways mediating cellular stress induced by UV, proinflammatory cytokines, heat, and osmotic shocks. More recent studies have shown that JNK and p38-MAPK are also involved in the regulation of cell proliferation by growth factors or cytokines. We therefore analyzed, by immunofluorescence assays, the expression and the phosphorylation status of these two proteins. In contrast to what we observed for the ERK pathway, the regulation of JNK and p38-MAPK was not affected in transgenic mice compared with control animals (data not shown).

The phosphatidyl inositol 3-kinase/Akt pathway in MTI/ G-gly and hGAS mice. Class I PI3Ks are heterodimers composed of a catalytic subunit p110 constitutively associated with an adaptor/regulatory subunit p85. In vitro studies previously done in our laboratory provided evidence that G-Gly in particular activates the p85/p110 PI3K (23). In hGAS and MTI/G-gly mice, we observed, by immunofluorescence using an anti-p85 antibody, an increase of this protein expression that might reflect an up-regulation of the p85/p110 PI3K pathway (Fig. 3; Table 1).

Akt, also known as protein kinase B, is a well-established downstream effector of PI3K. To investigate whether progastrin and G-Gly regulate the PI3K/Akt pathway activation in colonic mucosa, in vivo immunofluorescence studies were carried out using antibodies specific for total and activated Akt. As shown in Fig. 3 and wild-type mice. Results obtained by indirect immunofluorescence staining were correlated to those obtained by Western blot analysis using isolated colonic epithelial cells from transgenic or control mice (Fig. 2).

![Figure 1](image_url) Status of p60 Src, STAT3, STAT1, and JAK2 in colonic mucosa of FVB/N, MTI/G-Gly, and hGAS mice. Sections of paraffin-embedded colonic mucosa were immunostained with the indicated antibodies. Micrographs from representative fields of the stained sections were taken (original magnification ×400).

Table 1. Quantitative immunofluorescence analysis of signaling and adhesion proteins in colonic mucosa of FVB/N, MTI/G-Gly, and hGAS mice

<table>
<thead>
<tr>
<th>Signaling proteins</th>
<th>Mice</th>
<th>hGAS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTI/G-gly</td>
<td></td>
</tr>
<tr>
<td>p60-Src</td>
<td>339% ± 5 (*<strong>), 355% ± 11 (</strong>)</td>
<td></td>
</tr>
<tr>
<td>PY418-Src</td>
<td>222% ± 9 (<em>)     275% ± 8 (</em>)</td>
<td></td>
</tr>
<tr>
<td>STAT3</td>
<td>268% ± 2 (**)    242% ± 10 (*)</td>
<td></td>
</tr>
<tr>
<td>PY-STAT3</td>
<td>320% ± 4 (<strong><strong>)  459% ± 6 (</strong></strong>)</td>
<td></td>
</tr>
<tr>
<td>JAK2</td>
<td>292% ± 2 (*<strong>), 328% ± 11 (</strong>)</td>
<td></td>
</tr>
<tr>
<td>PY-JAK2</td>
<td>218% ± 5 (<strong><strong>)  412% ± 6 (</strong></strong>)</td>
<td></td>
</tr>
<tr>
<td>p85</td>
<td>198% ± 11 (*)    293% ± 7 (**)</td>
<td></td>
</tr>
<tr>
<td>Akt</td>
<td>102% ± 4 (NS), 108% ± 2 (NS)</td>
<td></td>
</tr>
<tr>
<td>PS473-Akt</td>
<td>200% ± 2 (<strong>)    250% ± 2 (</strong>)</td>
<td></td>
</tr>
<tr>
<td>ERK</td>
<td>261% ± 4 (<strong>)    465% ± 15 (</strong>)</td>
<td></td>
</tr>
<tr>
<td>P-ERK</td>
<td>213% ± 5 (<strong>)    378% ± 10 (</strong>)</td>
<td></td>
</tr>
<tr>
<td>TGF-α</td>
<td>215% ± 6 (<em>), 366% ± 6 (</em>)</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE:** Results of immunofluorescence quantification are expressed as percentages of the control values (FVB/N). Data from five experiments (five different animals in each group) are presented as means ± SE. Statistical analysis (Student’s t test) was done using GraphPad Prism. (**), P < 0.001; (***), 0.001 < P < 0.001; (*), 0.01 < P < 0.05; (NS), P > 0.05. Abbreviation: NS, not significant.
Regulation of transforming growth factor-α in MTI/G-gly and hGAS mice. The EGF-related peptide, TGF-α, is frequently co-expressed with EGFR in colon cancer. In addition, several reports indicated that TGF-α autocrine activation of the EGFR is likely an important mechanism contributing to hyperproliferation of colon epithelial cells (25). Indirect immunofluorescence assays with anti-TGF-α or anti-EGFR antibodies were done on colon tissue from the different animal models. In hGAS mice, we detected a marked increase of TGF-α expression compared with wild-type FVB/N controls whereas the up-regulation observed in MTI/G-Gly animals was significantly less important. In contrast, EGFR staining was identical in all tissue sections (Fig. 3).

Adhesion in colonic mucosa of MTI/G-gly and hGAS mice. FAK was initially discovered as a tyrosine-phosphorylated protein in Src-transformed cells. This tyrosine kinase, which is overexpressed in a variety of human cancers, might contribute to tumor progression by controlling cell survival but also focal adhesion and cell migration (26). Analysis by immunofluorescence of FAK expression and activation using antibodies for total or activated FAK revealed no difference between hGAS or MTI/G-Gly mice and control FVB/N animals (Fig. 3).

Loss of cell adhesion is an early event in the epithelial/mesenchymal transition, which occurs during tumor progression. Catenins, including α-, β-, and p120-catenin, are proteins that mediate cell-to-cell adhesion by connecting the transmembrane glycoprotein, cadherins, to the actin network. Recently, Holland et al. (24, 27) have shown in vitro, on gastric or colonic epithelial cells, that G-Gly and progastrin are capable to dissociate the E-cadherin/β-catenin complex through signaling pathways that include the PI3K. In addition, it was reported that an elevated Src kinase activity in colon cancer cells disturbs membrane localization of E-cadherin and cell-to-cell junctions (28).

In this study, we have investigated whether the adhesion complexes were differentially regulated in hGAS and MTI/G-Gly mice compared with the FVB/N mice. Western blot analysis (Fig. 4A) did not indicate any change in the expression of adhesion proteins between hGAS, MTI/G-Gly, or FVB/N mice. In agreement with these results, stainings for E-cadherin, β-catenin, or p120-catenin in hGAS and MTI/G-Gly mice were identical to that observed in control mice (Fig. 4B).

Involvement of Src, Janus-activated kinase 2, and phosphatidylinositol 3′-kinase pathways in cell proliferation induced by gastrin precursors. To further explore the mechanisms that may be responsible for the effect of gastrin precursors on colonic mucosal proliferation, we used primary colonic epithelial cells isolated from FVB/N mice as described in Materials and Methods. After 4 days of primary culture, the preparation of isolated colonic epithelial cells was controlled by Western blot analysis using two different markers: E-cadherin, a marker of differentiated epithelial tissues, and vimentin, a general marker of cells originating in the mesenchyme. As expected, the colonic primary culture cells do not express the vimentin but display a high E-cadherin expression, in contrast to what is observed for the control A10 cells (thoracic
Discussion

The development of cancer is thought to be dependent on the progressive perturbation or usurpation of normal signaling pathways involved in cell proliferation, thus conferring a growth advantage to the cells. This study is the first to analyze the expression and the activation of signaling molecules in transgenic mice models with colonic hyperplasia that overexpress incompletely processed forms of gastrin (G-Gly and progastrin). Our results indicate that transition from a normal aorta myoblast cells; Fig. 5A). In these conditions, proliferation rates of the primary culture cells, as determined by MTT assays after progastrin or G-Gly stimulation (Fig. 5B), confirmed the growth effects of these peptides observed in transgenic mice. JAK2 and Src are the two tyrosine kinases potentially involved upstream of the signaling pathways activated in colonic mucosa of MTI/G-Gly and hGAS mice. These tyrosine kinases are also activated in primary colonic epithelial cells isolated from FVB/N mice in response to G-gly or progastrin (Fig. 5C). Therefore, we tested in this model the involvement of JAK2, Src, but also the PI3K pathway on the colonic epithelial cells proliferation induced by G-Gly or progastrin. Pretreatment of the colonic epithelial cells with inhibitors for Src (PP2), JAK2 (AG490), or PI-3K (LY294002) reverse the effects of gastrin precursors, indicating the potential role of these signaling molecules in colonic mucosal proliferation induced by progastrin or G-Gly (Fig. 5D).

The Src family kinases play a key role in carcinogenesis, controlling multiple signaling pathways involved in such diverse processes as cell growth, migration, and survival. In a variety of carcinomas, including those derived from the colon, the expression and activation of Src is markedly increased. Moreover, high levels of activation have been observed in colonic polyps and adenomas, indicating that deregulation of Src kinases may also be an early event in colonic carcinogenesis (13, 14). In both MTI/G-Gly and hGAS mice, we showed increased Src protein expression and activity compared with control FVB/N mice. This deregulation of Src kinases might be a causal factor in the hyperproliferation of the colonic mucosa observed in these mice, as well as in the predisposition of these animals to develop colon adenomas and carcinomas in response to a chemical carcinogen. Whereas one study has reported the in vitro activation of Src by the gastrin precursor, progastrin (8), this study is the first to show in vivo the up-regulation of Src by incompletely processed forms of gastrin (G-Gly and progastrin).

STAT3 belongs to a family of transcription factors that play a variety of roles in normal cellular processes. However, several studies have proposed that aberrant activation of STAT3 might be involved in the initiation and progression of human cancers through up-regulation of genes involved in cell cycle progression or apoptosis inhibition. STAT3 has been shown to possess oncogenic potential and its constitutive activation has been detected in a wide variety of human tumors (17). However, to our knowledge, there is currently little or no information with respect to the up-regulation of STAT3 in colonic neoplasia. In the colonic mucosa of MTI/G-Gly and hGAS mice, we observed an overexpression and increased activation of STAT3. In addition, the high level of JAK2 kinase activity observed in these mice compared with control FVB/N mice may be one mechanism leading to constitutive activation of STAT3 and, consecutively, to cell proliferation in these models. Among the other tyrosine kinases that may be responsible for the hyperactivation of STAT3 in our mice models, Src is a good candidate. STAT3 has been shown to directly associate with Src, and seems to increase the oncogenic potential of this kinase. In addition, STAT3 is constitutively active in many cell lines transformed by v-Src (29–31). Akt phosphorylation, which occurs downstream of the PI3K pathway, has been shown to be involved in antiapoptotic effects, cell survival, and proliferation. In particular, this pathway has been involved in intestinal epithelial proliferation (32). Akt may also contribute to tumor progression and development in human cancers. Indeed, in various tumoral cells, including those from colonic origin, high levels of Akt phosphorylation on Ser473 have been detected and correlated with increased cellular proliferation (16). In addition, inhibition of the PI3K/Akt pathway by a specific PI3K inhibitor causes apoptosis in several human colonic cancer cells in vitro as well as in vivo (15). Our results strongly support a crucial role of this signaling pathway in the regulation of cell proliferation induced by incompletely processed forms of gastrin. Indeed, we show, in the colonic mucosa of MTI/G-Gly and hGAS mice, an up-regulation of the p85 regulatory subunit of PI3K and Akt.

The role of the ERK pathway in the regulation of cellular growth is well documented. This signaling cascade has been implicated in the proliferative effects induced by tyrosine kinase receptors, cytokine receptors, and G protein–coupled receptors. The ERKs are activated by many oncogene proteins, and increased ERK phosphorylation has...
been observed in several human cancers and may contribute to the neoplastic phenotype (33, 34). However, there is currently very little information regarding the role of the ERK pathway in the early stage of carcinogenesis. Detection of high levels of ERK expression and activation in the colonic mucosa of MTI/G-Gly and hGAS mice strongly supports that ERK activity is one of the factors contributing to cell proliferation induced by gastrin precursors in vivo. Recently, Varro et al. (35) have shown that mature amidated gastrin stimulates the ERK pathway and gastric cell proliferation through release of heparin binding EGF and the paracrine stimulation of EGFRs. Because we observed in MTI/G-Gly and hGAS mice an overexpression of TGF-α, we can make the hypothesis that ERK activation by gastrin precursors may also be the result of EGFR transactivation.

Our study shows that both gastrin precursors, G-Gly and progastrin, induce in vivo signaling pathways that could explain their effects on proliferation of the colonic mucosa. Whether these effects are mediated through the same receptor remains an open question. At present, the receptors for these precursor peptides have not been cloned. However, in previous reports, binding studies have allowed the characterization of at least two pharmacologically different receptors, distinct from the CCK1 and CCK2 receptors: one receptor, specific for G-gly, which does not bind the mature form of gastrin nor the specific antagonists of the gastrin/CCK2 receptor (6, 36), and a second receptor, which binds both gastrin precursors and amidated gastrin (7). Both of these pharmacologic forms have been shown to mediate in vitro the proliferative effects of gastrin precursor peptides. The current study has revealed some differences in signaling. Thus, whereas both G-gly and progastrin resulted in strong up-regulation of Src, JAK2, and STAT3 activation, progastrin stimulation resulted in significantly greater activation of ERK and Akt pathways as well as in overexpression of TGF-α. These results strongly support the existence of more than one additional receptor for gastrin precursors.

Besides its role in cell adhesion, β-catenin is also a transcription factor interacting with the LEF/TCF family, which regulates the expression of target genes potentially involved in cell proliferation (37). Deregulation of cell-to-cell adhesion and accumulation of β-catenin in the nucleus are frequently observed in colon cancer but it is likely that these events seem late in the tumor progression. Indeed, alteration of cell-to-cell adhesion and in particular down-regulation of E-cadherin expression have been correlated to migration and metastatic potential of cancer cells. In addition, formation of active complexes LEF/TCF/β-catenin in the nucleus

**Figure 5.** Involvement of Src, JAK2, and PI3K pathways in cell proliferation induced by gastrin precursors. A. Western blots for E-cadherin and vimentin were done on lysate from colonic epithelial cells isolated from FVB/N wild-type mice after 4 days primary culture (ICEC: isolated colonic epithelial cells). A10 cells (thoracic aorta myoblast cells) were used as positive control for vimentin. B-D, colonic epithelial cells isolated from FVB/N wild-type mice were stimulated with the indicated peptides (10⁻⁷ mol/L) for 48 hours. When indicated, cells were preincubated with 10 μmol/L of PP2, AG 490, or LY 290042. Proliferation rates were determined by MTT assay (B and D). Western blots for PY-JAK2 and PY418-Src were done on lysate of colonic epithelial cells (C). Columns, mean; bars, SE (n = 5). Statistical analysis (Student’s t test) was done using GraphPad Prism. ***P < 0.001; **0.001 < P < 0.01; *0.01 < P < 0.05; ns P > 0.05.
results, in most cases of human colorectal cancers, from mutations of the APC tumor supressor or of beta-catenin itself. These observations may explain why the localization and expression of adherens junction molecules were unchanged in the colonic mucosa in MTI/G-Gly and hGAS mice, which exhibit a hyperproliferation of the colonic cells but do not develop tumors.

Several studies have suggested that abnormal proliferation of the colonic mucosa in patients contributes to increased colon cancer risk (38–45). In addition, a recent study suggests that the risk for developing colorectal cancer is increased in patients with hypergastrinemia (46). Nonamidated gastrins, such as progastrin and G-Gly, are now recognized as factors inducing proliferation of colonic epithelial cells (10). Because the gastrin precursors are overexpressed by preneoplastic lesions, such as colonic polyps, it can be speculated that they might contribute to the initiation phases of colon carcinogenesis by up-regulating signaling pathways involved in cellular proliferation and cell survival. However, although progastrin and G-Gly are capable of stimulating colonic epithelial cell proliferation in vitro, their overexpression alone in hGAS or MTI/G-Gly mice does not lead to a tumor phenotype. A “second hit,” most likely as genetic alterations, such as a mutation of K-ras, APC, or p53, is likely necessary for tumor initiation. Interestingly, hGAS mice treated with a chemical carcinogen, azoxymethane, known to induce colon carcinogenesis, are likely necessary for tumor initiation. Interestingly, hGAS mice does not lead to a tumor phenotype. A “second hit,” most likely as genetic alterations, such as a mutation of K-ras, APC, or p53, is likely necessary for tumor initiation. Interestingly, hGAS mice treated with a chemical carcinogen, azoxymethane, known to induce colon carcinogenesis, is overexpressed in hGAS mice, which regulates gastrin gene expression in colon cancer cells and might contribute to the maintenance loop of the gastrin precursors (48).

In summary, this article provides the first evidence that the up-regulation of several signaling pathways, including Src, PI3K/Akt, JAK2/STAT3, and ERKs, as well as an overexpression of growth factors, such as TGF-α, is associated with colonic mucosa hyperproliferation in transgenic mice overexpressing progastrin or glycine-extended gastrin.

Acknowledgments

Received 3/19/2004; revised 11/16/2004; accepted 1/4/2005.

Grantsupport: Institut National de la Sante et de la Recherche Medicale, Association pour la Recherche contre le Cancer grants 4514 and 5860, Ligue contre le Cancer, and NIH grant RO1 K052778-06 ACI 1A067G.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Aline Kowalski-Chauvel, Dana Frederick, and Michael Langlois for their interest and encouragement on this project, Jean-Pierre Esteve for his help in the quantification of immunofluorescence studies, and Etienne Holland and Marjorie Fanjul for their helpful suggestions.

References

33. Ding XZ, Tong WG, Adrian TE. Multiple signal pathways are involved in the mitogenic effect of (9S)-HETE in human pancreatic cancer. Oncology 2003;65:285–94.
34. Davidson B, Givant-Horwitz V, Lazarovic I, et al. Matrix metalloproteinases (MMP). EMMPRIN (extracellular matrix metalloproteinase inducer) and


Signaling Pathways Associated with Colonic Mucosa Hyperproliferation in Mice Overexpressing Gastrin Precursors

Audrey Ferrand, Claudine Bertrand, Ghislaine Portolan, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/7/2770

Cited articles
This article cites 44 articles, 18 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/7/2770.full#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/7/2770.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.