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Synergistic activation of the murine gastrin promoter by oncogenic Ras and β-catenin involves SMAD recruitment

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Abstract

While Wnt and Ras signaling pathways are activated during progression of colorectal cancers, many of their important downstream targets remain to be elucidated. The gastrin gene encodes for a family of peptide growth factors that are commonly upregulated in colorectal neoplasia. Previously, we showed that the Wnt signaling pathway moderately stimulates the gastrin promoter. To determine whether Ras signaling can cooperate with Wnt signaling in transcriptional regulation of gastrin gene expression, we have analyzed the response of murine gastrin promoter–reporter gene constructs to combinations of oncogenic stimulation in transient transfection assays. We found a strong (25- to 40-fold) synergistic stimulation of the gastrin promoter by the combination of oncogenic β-catenin and K-ras overexpression. Deletion analysis localized the response element to an area between −140 and −110 bp upstream in the murine gastrin promoter. Electrophoretic mobility shift assays detected a complex containing β-catenin/TCF, AP1, and SMAD3/4 transcription factors that bound to a DNA element through AP1 and SMAD binding sites. Gastrin promoter activation could be further enhanced or suppressed by the co-expression of wild type SMAD4 or dominant negative mutant of SMAD4, respectively, and abrogated by the PI3K inhibitor, LY20004, but not by the MEK inhibitor, PD98059. Taken together, our data strongly suggest that oncogenic Wnt and Ras signaling pathways can synergistically induce gastrin expression, possibly contributing to neoplastic progression.

Keywords: Gastrin; Gene regulation; Oncogene; Ras; Wnt; SMADs; Colorectal cancer

Gastrin is synthesized and secreted by endocrine cells (G cells) located primarily in the gastric antrum. In addition to its well-defined role in gastric acid secretion, gastrin also contributes to growth and proliferation of the gastrointestinal mucosa. Overexpression of amidated gastrin results in proliferation and gastric mucosal hypertrophy in INS-GAS transgenic mice [1]. In addition, the gastrin gene is frequently expressed and upregulated in numerous cancers, including colorectal, pancreatic, ovarian, and lung cancers [2], where it is thought to contribute to tumor growth and progression. In colorectal cancer, gastrin is incompletely processed and secreted as the precursor forms, progastrin and glycine-extended gastrin, which have been shown to induce colonic proliferation both in vitro and in vivo [2]. Deletion of the gastrin gene results in decreased colonic proliferation, while overexpression of the incompletely processed gastrin leads to accelerated colonic neoplasia in response to azoxymethane (AOM) or Apc/Min-dependent polyposis [3]. Studies have also shown increased plasma progastrin

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levels in patients with colorectal cancer [4]. Taken together, previous studies strongly support a role for incompletely processed gastrins in tumorigenesis, but the pathways leading to gastrin gene expression in cancer have not been well defined.

The Apc/β-catenin and K-ras genes are commonly mutated during the pathogenesis of colorectal cancers. Activation of the Apc/β-catenin pathway occurs early in colorectal cancer pathogenesis, and work from our laboratory has demonstrated that gastrin is regulated in part by the Wnt pathway. Induced Apc expression was found to be capable of suppressing gastrin gene expression, while overexpression of activated β-catenin stimulated the human gastrin promoter activity by twofold [5]. However, many additional pathways appear to modify Wnt signaling, and studies from other laboratories have suggested that K-ras activation correlates with gastrin gene expression in colon cancer, and overexpression of activated K-ras can induce the human gastrin promoter weakly [6].

In the present study, we explored whether Wnt and K-ras pathways can cooperate in the stimulation of the murine gastrin promoter. We co-transected a minimal murine gastrin promoter-luciferase reporter gene along with constructs expressing oncogenic β-catenin and an activated form of (GTP-bound) Ras. Our findings indicate that the oncogenic Wnt/β-catenin and K (Ha)-ras proteins synergistically transactivate the murine gastrin promoter via PI-3 kinase dependent pathway.

Materials and methods

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin (from Gibco, Grand Island, NY) at 37 °C in humidified atmosphere (5% CO_2). The promoter luciferase reporter constructs of murine gastrin gene, a detailed protocol for transfection of reporter constructs, and the result's treatment were described elsewhere [7]. Additional reporter constructs were constructed in the same way by using custom-made oligonucleotides corresponding to the putative SMAD3/4 binding element and AP1 binding element of the murine gastrin promoter (see figures).

The integrity of all constructs was verified by DNA sequencing. CMV early promoter-driven 5′ Flag-tagged SMAD3 and SMAD4 mammalian expression vectors, pCF-S3 and pCD-D43F(R), were kind gifts from Dr. Toshi Shioda (Harvard Medical School). Oncogenic human β-catenin vector and K-ras were kind gifts of Dr. Dan Chung (Harvard Medical School). Preparation of Nuclear Extracts and electrophoretic mobility shift assay (EMSA) were done as described previously [7]. Antibody block shift experiments were performed by incubating various oligonucleotides and nuclear complex with 2 μl of polyclonal antiserum against SMAD3, SMAD4, c-Jun, c-Fos, and β-catenin. Oligonucleotides representing the wild type or mutant SMAD3 site alone, mutant AP1 site alone, and double mutant of both SMAD3 and AP1 site of the mouse gastrin promoter are shown in figures.

Results

Wnt and Ras pathways synergize in activation of the murine gastrin promoter

In order to investigate possible interactions between Ras and Wnt signaling in gastrin regulation, we generated a 1 kb murine gastrin-luciferase reporter gene construct and used it in transient co-transfection studies with overexpression constructs for oncogenic β-catenin and K-ras (G12V). These studies showed that overexpression of K-ras alone led to mild (∼2-fold) stimulation of the murine gastrin promoter (Fig. 1A, second bar). In accordance with our previous data [7], overexpression of oncogenic β-catenin alone had little effect on mouse gastrin promoter activity (Fig. 1A, third bar). However, overexpression of both oncogenic β-catenin and K-ras resulted in powerful activation of gastrin promoter (>25-fold, Fig. 1A, fourth bar). These data suggested a strong synergy between K-ras and Wnt signaling in the regulation of the murine gastrin promoter.

In order to identify the cis-acting element, we generated a series of gastrin promoter deletion constructs and examined their activation by the oncogenic K-ras (G12V) and β-catenin in the transient transfection assay of HeLa cells (Fig. 1B). Deletion of 5′ gastrin promoter region between −1000 and −140 bp did not affect the basal promoter activity or the synergistic activation by K-ras (G12V) and β-catenin. However, further 5′ promoter deletions (down to −125 and −110 bp) strongly decreased the synergistic activation of the promoter with a minimal effect on basal promoter activity (Fig. 1B). Additional shortening of gastrin promoter (to −30 bp) did not reveal any additional changes in the synergistic activation by K-ras (G12V) and β-catenin (Fig. 1B). Thus, this analysis demonstrates that a cis-acting region in the murine gastrin promoter located between −140 and −110 bp is responsible for activation by Ras and Wnt pathways.

A closer inspection of the identified promoter region for transcription factor binding sites revealed a potential motif for the AP1 transcription factor, a known mediator of the Ras pathway, located between −126 and −120 bp. No TCF consensus-binding site was found in the −140 to −110 region. Instead, analysis of the gastrin promoter revealed a CAGA motif, a potential SMAD binding element or SBE [8], in close proximity to AP1-like site (Fig. 2A).

To evaluate the role of these cis-acting sites in the response to Ras/Wnt signaling, we mutated the AP1 or SMAD sites in the murine gastrin promoter and tested the constructs for activation by β-catenin and K-ras (G12V). We found that mutation of the AP1-like binding site (TGACA to TGATT), as well as mutation of the SBE (CAGA to TACA) attenuated the observed synergistic effect (Fig. 2B, lanes 3 and 4). Thus, these

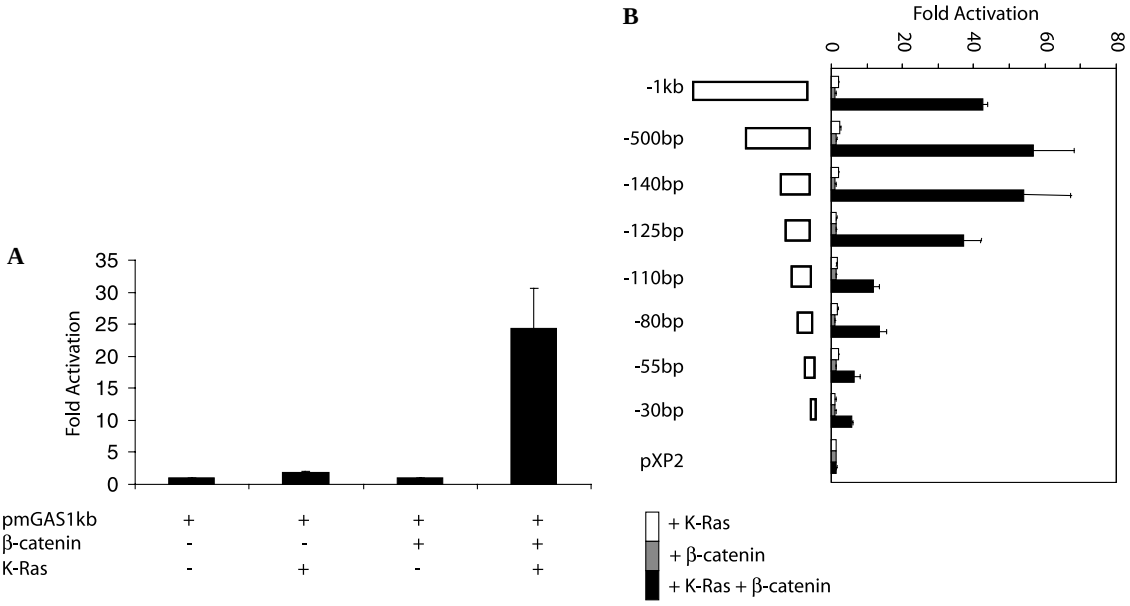

Fig. 1. Synergistic cooperation of K-ras and Wnt/β-catenin in the oncogenic regulation of the murine gastrin gene promoter in HeLa cells. (A) Reporter construct containing ~1 kb of mouse gastrin promoter fused with luciferase was transfected alone or in combination with expression constructs for the oncogenic β-catenin or K-ras (G12V), or their combination. Reporter firefly luciferase activity was normalized to reference signal of the co-transfected *Renilla* luciferase-expressing vector (100 ng). Results are presented as fold activation of normalized signal of pmGas1kb-luc construct alone that was arbitrarily chosen as 1. (B) Localization of the Ras-responsive element in mouse gastrin promoter by deletion analysis. The reporter constructs containing fragments of gastrin promoter (from −1 kb, −500, −140, −125, −110, −80, −55 or −30 to +50 bp) were transfected alone or with the oncogenic β-catenin and/or K-ras(G12V) into HeLa cells. Reporter signals were analyzed as in (A).

studies suggested the involvement of the SMAD family of transcriptional regulators in mediating the response to Ras and Wnt activation.

Ras/Wnt synergistic element of murine gastrin promoter is dependent on PI-3 kinase pathway

Ras interacts and activates several known downstream effectors including Raf, Rac, and PI3K kinases. In order to identify the downstream Ras effector mediating the synergistic interaction with β-catenin on gastrin promoter activation, we examined additional Ras mutants. We first tested whether another Ras family member, Ha-ras, and its regulatory loop mutants defective in interaction with Raf/MEKK or PI-3 effectors, are able to synergize with β-catenin in the gastrin promoter activation. The activated signaling competent Ha-Ras (G12V) mutant and the Raf interaction deficient mutant, Ha-Ras (G12V, E37G) [9], were able to synergize with β-catenin to activate the gastrin promoter (Fig. 3A). In the same assay, the Ha-Ras (G12V, T35S) mutant that is known to hyperactivate ERK/MAPK pathway [10] was ~2-fold less efficient than oncogenic Ha-Ras. Thus, Ras-mediated MAPK kinase activation through the Raf/MEKK pathway is not essential for the observed gastrin promoter activation. Indeed, we found a huge gastrin promoter stimulation by the Ha-Ras (G12V C40) mutant which is known [11] to strongly activate the PI-3/Akt kinase pathway (Fig. 3A).

To confirm the latter observation, we treated transfected cells with pharmacological inhibitors of PI3K or MAP kinases. We observed a complete abrogation of the synergistic promoter stimulation after treatment with LY294002, a PI3K inhibitor, but not with PD98059, a MAP kinase inhibitor (Fig. 3B). Taken together, these experiments strongly suggest that the K-ras and Wnt synergy was preferentially mediated through the PI3-kinase pathway but not the Raf–MEKK–MAPK pathway.

The K-ras and Wnt synergy of the murine gastrin promoter activation is mediated by SMADs

As shown above, mutation of the SBE resulted in decreased gastrin promoter activation by the combination of K-ras and β-catenin stimulation (Fig. 2B). Consequently, we explored further the possible role of SMAD3 and SMAD4 as mediators of the observed synergy by testing an effect of co-expression of wild type SMAD4 or a dominant negative SMAD4, in combination with oncogenic β-catenin and K-ras (G12V). We found that wild type SMAD4 could potentiate the promoter activation by 1.7-fold (Fig. 4A, seventh bar), while dominant-negative SMAD4 abrogated promoter activation (Fig. 4A, eighth bar). To define further the

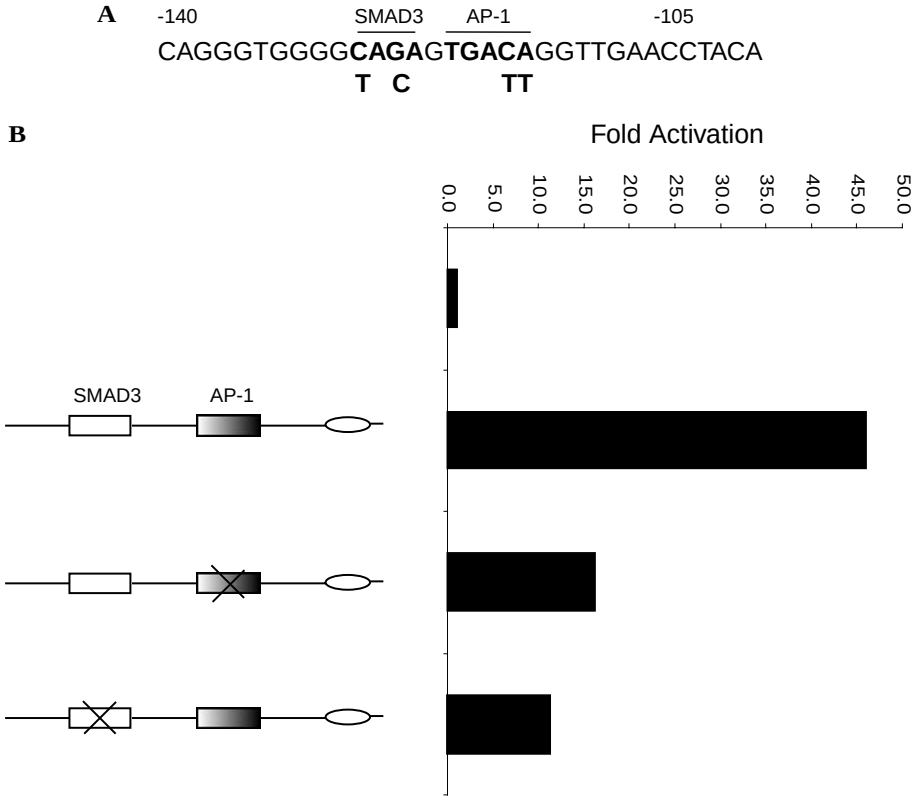

Fig. 2. Small region of mouse gastrin promoter confers Ras/Wnt responsiveness through AP1 and SMAD consensus-binding sites. (A) Sequence from −140 to −105 bp of mouse gastrin promoter that contains AP-1 like (TGACAG) and SMAD3/4 (CAGA) consensus motifs is shown on the top. Mutations of these sites that were used for functional assays are shown on the bottom. (B) Reporter assay of pmGas 140-luc constructs containing WT or mutated AP-1 like or SMAD consensus sites in the −140 to −110 bp K-ras/Wnt responsive region. Then correspondent point mutations of consensus AP-1 or SMAD binding sites (as shown in A) were introduced by site directed mutagenesis. Signal of pmGas 140-luc construct alone (top bar) was arbitrarily chosen as 1.

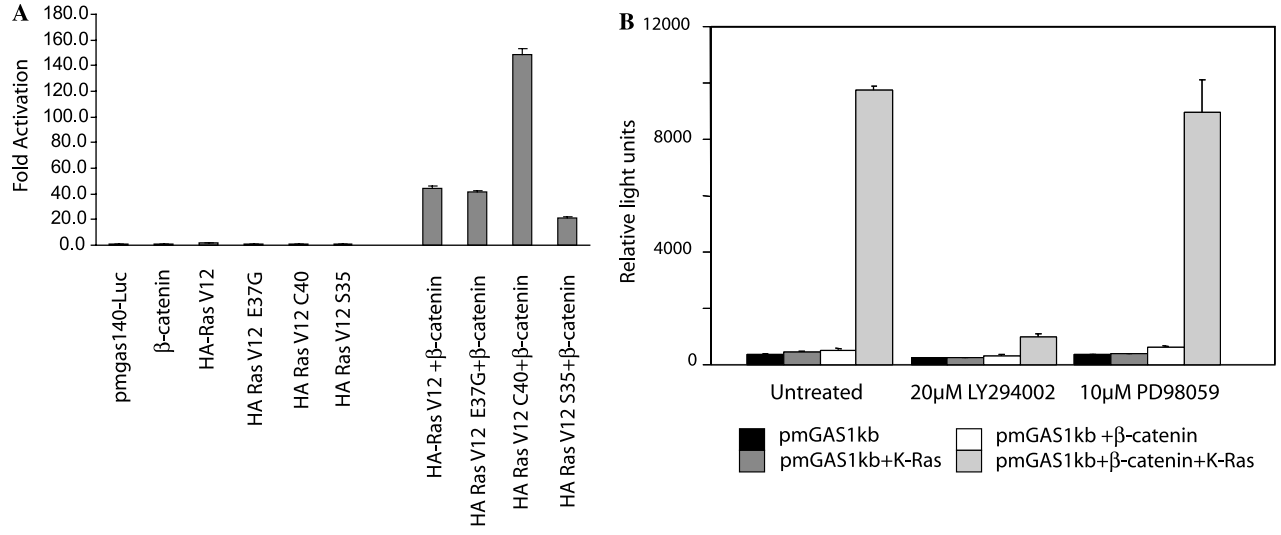

Fig. 3. Identification of downstream mediators of Wnt and K-ras mediated synergistic stimulation. (A) Synergistic effect of different K-ras mutants on murine gastrin promoter activation by the oncogenic β-catenin in the reporter assay of HeLa cells. Ha-ras mutant, which decreases Ras–Raf interaction and MAPK/JNK activation (E37G), overactivates MAPK (G12V, T35S) or PI3K (G12V, 40C). (B) Inhibition of PI3K, but not of MAPK, abrogates the activation of murine gastrin promoter by the oncogenic β-catenin and K-ras (G12V) in HeLa cells. Transfected cells were treated or not by 20 μM LY294002, an inhibitor of PI3 kinase, or 10 μM PD98059, an inhibitor of MAPK, for 20 h before measurements of the reporter luciferase signal of the pmGas1 kb-luc reporter construct.

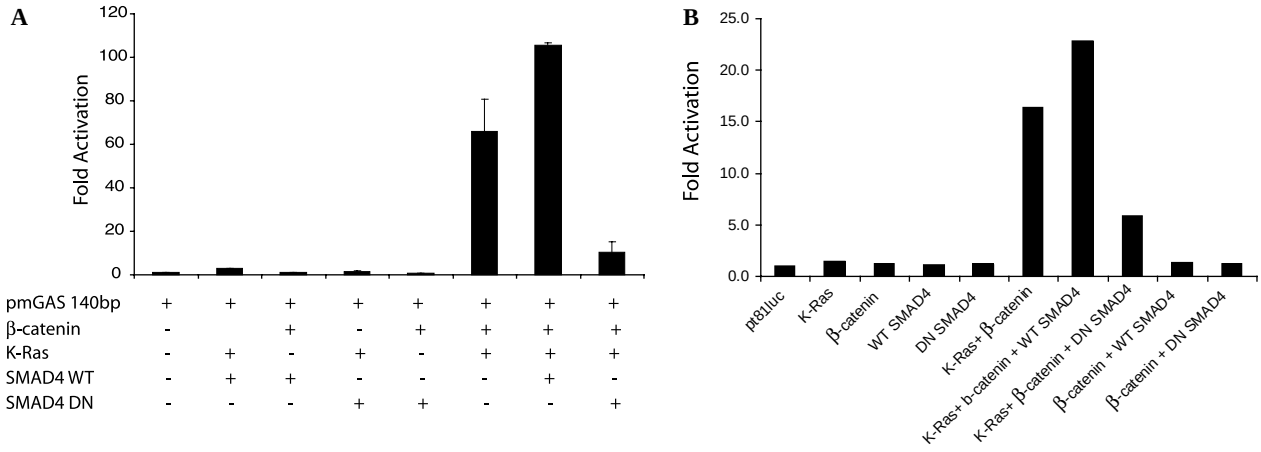

Fig. 4. Synergistic stimulation mediated by β-catenin and K-ras involves transcription factor SMAD. (A) Stimulatory effect of SMAD4 co-expression on the activation of murine gastrin promoter by the Wnt and Ras pathways. pmGas140bp-luc reporter construct was either transfected alone or in different combinations with the β-catenin, K-ras, wild type SMAD4 (WT SMAD4), and dominant negative mutant of SMAD4 (DN SMAD4) expressing constructs. Reporter signal was normalized as in Fig. 1A. (B) SMAD4 and Ras/Wnt responsive elements are co-localized in the same −140 to −110 bp region of murine gastrin promoter. pT81-luc reporter construct containing −140 to −110 bp region of mouse gastrin promoter was transfected alone or in combinations with indicated expressing constructs. Results were treated and presented as in Fig. 1A.

cis-acting region necessary for SMAD-dependent regulation, we examined heterologous reporter construct pT81 containing the −110 to −140 bp region upstream of the TK promoter (Fig. 4B). We found that this small region was sufficient to mediate the SMAD effect. Taken together, these observations strongly suggest a role for SMAD transcription factors in the stimulation of gastrin promoter by K-ras and β-catenin through the SBE/AP1 response element.

In order to analyze more directly the nuclear proteins binding the Ras/Wnt response element, we next performed EMSA. Using the WT response element as a probe, we detected one major DNA–protein complex (Fig. 5A, lane 2), which can be eliminated by the addition of 25- to 100-fold excess of unlabeled probe in the binding reaction (Fig. 5A, lanes 3–5). Next, when using competitors with mutated AP1 or SMAD sites, we observed a loss of specific competition in the assay (Fig. 5B, lanes 6–11). Interestingly, this competition defect was more prominent for oligonucleotide with the mutated SMAD site (lanes 6–8) than for one with mutated AP1 site (lanes 9–11). Antibodies to SMAD3 or SMAD4 inhibited DNA binding and complex formation (Fig. 5B compare lanes 4, 5 versus 3), while control antibodies (unrelated IgG) did not affect the complex formation (lane 3). Antibodies to c-fos also blocked the formation of specific complexes (Fig. 5B, lane 6), thus indicating the presence of c-fos protein in the complex. Similar EMSA experiments were performed using anti-TCF and anti-LEF antibodies (Fig. 5C, lanes 5 and 6), which blocked specific complex formation but did not affect nonspecific bands. Overall, the EMSA data strongly suggested the involvement of SMADs, TCF, and c-fos in binding to the Ras/Wnt response element in the mouse gastrin promoter.

Discussion

Mutations leading to activation of Wnt and Ras signaling pathways are common during progression of colorectal cancer. Previous reports by our group [5] and others [6] have shown that the Wnt and K-ras pathways can activate the human gastrin promoter individually. In the current study, we demonstrate strong synergistic effects of oncogenic K-ras and β-catenin on the activity of mouse gastrin promoter. Deletion analysis of the gastrin promoter identified a Wnt/Ras responsive element that could be localized to the −140 to −110 bp promoter region, which overlapped with a previously reported K-ras response region (−200 +50) [6]. This element did not contain a consensus TCF consensus site, but did contain a unique SMAD binding (SBE) site that was adjacent to an AP1-like site. Mutational analysis confirmed the importance of both the SBE and AP1-like sites in the promoter response to Wnt/Ras signaling.

Electrophoretic mobility shift assays demonstrated the formation of a complex between the Wnt/Ras response element, and c-fos, LEF/TCF, and SMAD proteins. Importantly, intact SMAD or AP1-like sites were both necessary for normal complex formation and promoter activation. While a consensus TCF4 site was not present in the Wnt/Ras response element, the data are nevertheless consistent with a model in which direct binding by SMADs and c-fos/jun to the SBE and AP-1 sites regulates the promoter. This scheme is consistent with our recent observation that the β-catenin/TCF4 heterodimer is able to activate promoters without direct DNA binding through interactions with SMAD3/4 proteins [7]. Thus, in this current model, β-catenin/TCF4 functions as a co-activator to contribute to promoter activation.

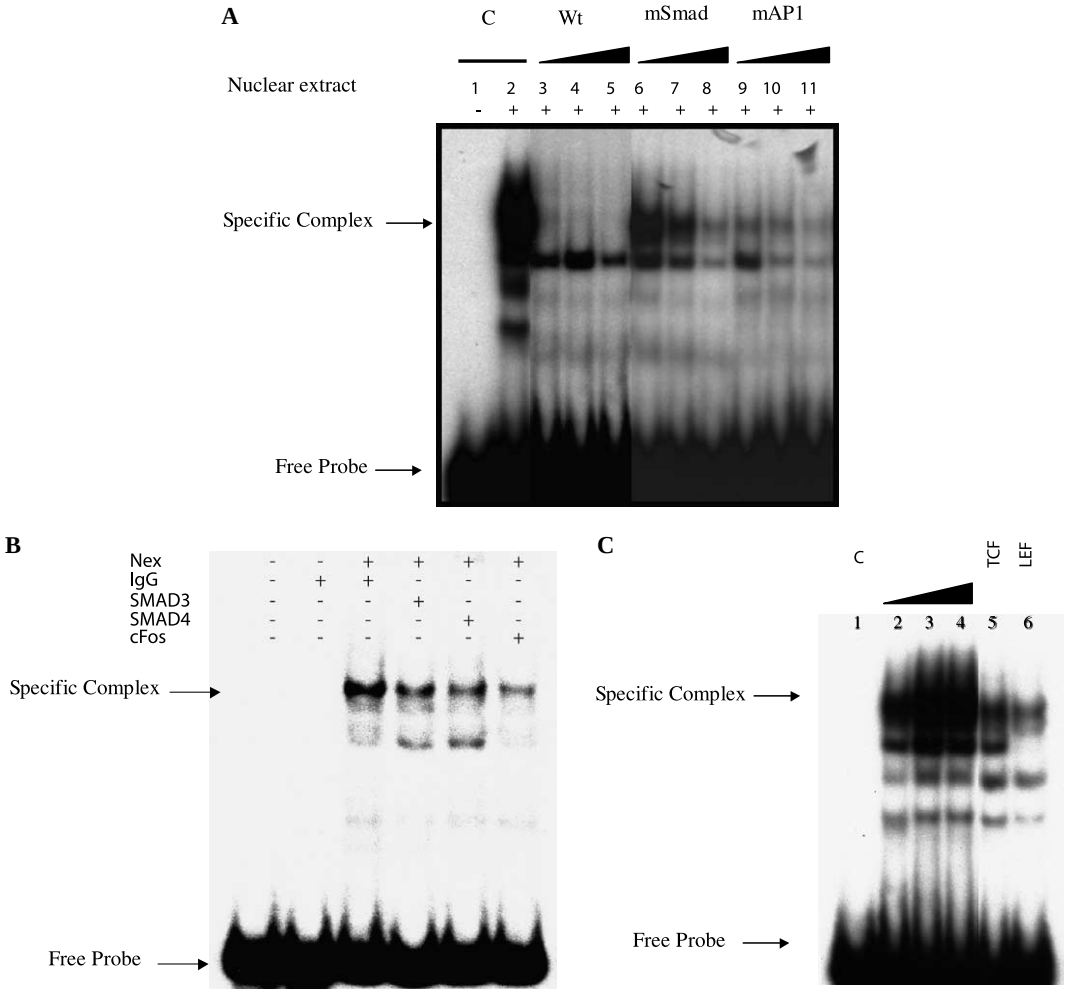

Fig. 5. SMADs, β-catenin, TCF, and AP1 bind to K-ras/Wnt responsive element in mouse gastrin promoter. (A) Electrophoretic mobility shift assay (EMSA) of HeLa nuclear protein binding to ^{32}P-labeled oligonucleotide spanning Ras/Wnt responsive element of the mouse gastrin promoter. Probe was incubated without (lane 1) or with (2–11) nuclear extracts of transfected HeLa cells in the presence of excess (25×, 50×, 100×) of the same "cold" WT or mutated competitor oligonucleotides (see Fig. 2A for sequence) as indicated above the gel. (B) Specific protein complex that binds to gastrin Ras/Wnt responsive element in EMSA contains SMADs and c-fos (AP-1 complex component) proteins. EMSA was performed as in (A), but nuclear extracts were pre-incubated with unrelated IgG or antibodies to SMAD3, SMAD4 or c-fos, as indicated above the gel. (C) EMSA using ^{32}P-labeled oligonucleotide spanning Ras/Wnt responsive element of mouse gastrin promoter. Increasing amounts of HeLa nuclear extracts (4 μg, lane 2; 6 μg, lane 3 or 8 μg, lanes 4–6) were either directly incubated with ^{32}P-labeled gastrin promoter probe or pre-incubated with the indicated antibody first and then incubated with the probe (lanes 5 and 6). Lane 1 (C) contains free-labeled probe.

In colon cancer cell lines, the Raf–Rac–MEKK pathway has been shown to be critical for K-ras-dependent activation of the human gastrin promoter, despite the absence of an obvious consensus AP-1 motif [6]. Studies by Marks et al. [12] demonstrated that EGF stimulation of gastrin transcriptional activity depends in part on c-fos expression, suggesting the involvement of AP-1. Work from Nakata et al. [6] localized the Ras-responsive element within a 200 bp region upstream of the gastrin core promoter. Our present study confirms that the Ras-responsive element is localized within the −125 to −120 bp region just upstream of the core gastrin promoter element. Moreover, this AP-1 binding, Ras-responsive motif does not fit perfectly with the known AP-1 consensus motif. Previous studies from our group suggested the involvement of Wnt signaling in the activation of human gastrin promoter, with a moderate twofold activation seen with overexpression of β-catenin [5]. Interestingly, our current results suggest that the murine gastrin gene promoter shows minimal activation with β-catenin alone, which could be the result of species-specific differences, or alternatively, could simply indicate the need for both K-ras and β-catenin to the fullest activation. Cooperation of Wnt and TGF-β, leading to synergistic activation of murine gastrin gene promoter, has also been shown by our group [7], consistent with our current results with K-ras. Interestingly, in the presence of oncogenic β-catenin, Ras preferentially activated the murine gastrin promoter through PI3K-Akt dependent signaling rather than

through the Raf–Rac–MEKK pathway. This signaling pathway is consistent with that described by others for the regulation of VEGF promoter by K-ras and Wnt in colonic neoplasia [13]. Taken together, our findings highlight the cumulative effect of Wnt and Ras transduction pathways leading to gastrin activation as an important feature in the progression of gastrointestinal cancer.

Acknowledgments

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