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Somatostatin stimulates menin gene expression by inhibiting protein kinase A

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Mensah-Osman E, Zavros Y, Merchant JL. Somatostatin stimulates menin gene expression by inhibiting protein kinase A. *Am J Physiol Gastrointest Liver Physiol* 295: G843–G854, 2008. First published August 28, 2008; doi:10.1152/ajpgi.00607.2007.—Somatostatin is a potent inhibitor of gastrin secretion and gene expression. Menin is a 67-kDa protein product of the multiple endocrine neoplasia type 1 (MEN1) gene that when mutated leads to duodenal gastrinomas, a tumor that overproduces the hormone gastrin. These observations suggest that menin might normally inhibit gastrin gene expression in its role as a tumor suppressor. Since somatostatin and ostensibly menin are both inhibitors of gastrin, we hypothesized that somatostatin signaling directly induces menin. Menin protein expression was significantly lower in somatostatin-null mice, which are hypergastrinemic. We found by immunohistochemistry that somatostatin receptor-positive cells (SSTR2A) express menin. Mice were treated with the somatostatin analog octreotide to determine whether activation of somatostatin signaling induced menin. We found that octreotide increased the number of menin-expressing cells, menin mRNA, and menin protein expression. Moreover, the induction by octreotide was greater in the duodenum than in the antrum. The increase in menin observed in vivo was recapitulated by treating AGS and STC cell lines with octreotide, demonstrating that the regulation was direct. The induction required suppression of protein kinase A (PKA) since forskolin treatment suppressed menin protein levels and octreotide inhibited PKA enzyme activity. Small-interfering RNA-mediated suppression of PKA levels raised basal levels of menin protein and prevented further induction by octreotide. Using AGS cells, we also showed for the first time that menin directly inhibits endogenous gastrin gene expression. In conclusion, somatostatin receptor activation induces menin expression by suppressing PKA activation.

MEN1; SSTR2A; gastrin; forskolin; octreotide

SOMATOSTATIN IS A PARACRINE inhibitor of gastrin secretion (24, 56) and gastrin gene expression (7, 18). In the stomach, somatostatin is secreted from specialized neuroendocrine cells called D cells in addition to being secreted from a variety of other cell types including inflammatory cells (34). Somatostatin binds to one of five seven-transmembrane G protein-coupled receptors (SSTRs) to activate signaling cascades through the inhibitory G (G_i) protein (41, 42). In the upper gastrointestinal tract, somatostatin function is predominantly mediated through the SSTR2A receptor, which is highly expressed on neuroendocrine cells (19). Activation of G_i suppresses cAMP, which subsequently binds the regulatory subunit of protein kinase A (PKA) to induce its phosphorylation and translocation to the nucleus. This event leads to phosphorylation of the cAMP response element binding protein (CREB) at Ser133 (43). In addition to its role as a potent inhibitor of gastrin, somatostatin also exhibits antiproliferative effects by activating cyclin-dependent kinase inhibitors such as $p27^{Kip1}$ (3, 32, 52).

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant syndrome characterized by tumor formation in endocrine cell populations located in the anterior pituitary, islet pancreas, and proximal gut, e.g., duodenal gastrinomas (4, 5). Germline and sporadic mutations in the MEN1 locus cause both familial MEN1 and sporadic neuroendocrine tumors. Therefore, the MEN1 locus has been designated a tumor suppressor gene. Menin is the 67-kDa nuclear protein product of the MEN1 gene that inhibits cell proliferation by activating cyclin-dependent kinase inhibitors $p27^{Kip1}$ and $p18^{Ink4c}$ (15, 29, 39). Menin also interacts with a number of transcription factors such as JunD and NF-κB proteins (2, 17, 22). Additionally, menin associates with chromatin and the nuclear matrix to recruit histone deacetylase complexes (HDACs) through association with mSin3A, a general transcriptional corepressor (30, 40). Yet, despite its impact on the cell cycle and potential link to endocrine neoplasias, no studies to date describe the mechanisms by which menin expression is regulated in the neuroendocrine cells of the gut, especially those that are the precursors for gastrinomas.

Since hypergastrinemia is the hallmark of MEN1-induced gastrinomas and somatostatin is the major negative regulator of gastrin, we hypothesized that somatostatin is a positive regulator of menin. We demonstrate here that somatostatin induces menin expression through the SSTR2A receptor by suppressing PKA. Moreover, we find that the regulation of menin expression by the somatostatin analog octreotide is more robust in the duodenum than in the antrum and thus contributes to our understanding of why duodenal neuroendocrine cells might be more vulnerable to the consequences of mutations in the menin protein.

MATERIALS AND METHODS

Animal husbandry. Wild-type (WT, *C57Bl/6*) and somatostatin-deficient ($SOM^{-/-}$, *C57Bl/6* background) (35) mice were maintained in individual, sterile microisolator cages in nonbarrier mouse rooms (conventional housing). Mice homozygous for the floxed menin allele were purchased from Jackson Laboratories (FVB;129S-$Men1^{tm1.1Ctre}$/J, stock no. 004066) and bred to generate a conditional homozygous null allele in the intestine ($Men^{\Delta Int}$) by crossing to the villin-Cre mouse (36). The study was performed with the approval of the University of Michigan Animal Care and Use Committee, which maintains an American Association of Assessment and Accreditation of Laboratory Animal Care facility.

WT mice were injected with octreotide intraperitoneally at a concentration of 30 μg/kg for 26 h then euthanized prior to analysis of the gastric contents to determine the hydrogen ion concentration by base titration and extraction of the tissue for immunoblot analysis and for immunohistochemistry.

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Immunohistochemistry. Briefly, longitudinal sections of the stomach antrum and duodenum were fixed in 4% paraformaldehyde-PBS then embedded in paraffin, and 3-μm sections were prepared. The slides were deparaffinized by heating to 100°C in 0.01 M sodium citrate for antigen retrieval prior to blocking nonspecific antigenic sites for 30 min with 20% normal donkey serum diluted in PBS and 0.1% Triton X-100. The slides were incubated for 1 h with a 1:200 dilution of rabbit menin antibody (Bethyl Laboratories, Montgomery, TX) or 1 h with a 1:800 dilution of rabbit anti-gastrin antibody, then overnight with a 1:200 dilution of goat or rabbit anti-SSTR2A antibody conjugated to horseradish peroxidase. The antigen-antibody complexes were detected by use of a diaminobenzidine immunohistochemistry detection kit. For immunofluorescence, an overnight incubation with a 1:200 of rabbit anti-menin, a 1:400 dilution of goat anti-SSTR2A, or a 1:800 dilution of rabbit anti-gastrin antibodies followed by a 1-h incubation with a 1:200 dilution of FITC- or Texas red-conjugated anti-rabbit or anti-goat IgG secondary antibody was used. Intestinal tissue sections from a 1-mo-old conditional homozygous null mouse ($Men^{\Delta Int}$) was used as a negative control for immunohistochemistry with the menin antibody. The number of menin-stained cells was quantified morphometrically by counting 10 well-oriented antral glands or villi per animal then expressing the result as the average number of cells counted per gland or villus.

Measurement of gastric acidity. After euthanasia, the stomachs of WT mice injected with octreotide or PBS were rinsed with 2 ml of saline. The hydrogen ion concentration was determined by base titration with 0.005 N NaOH via a pH-STAT control titrator (PHM290, Radiometer Analytical), and the result was expressed as microequivalents (μeq) of acid.

Cell culture and transfection. The human AGS adenocarcinoma and mouse STC neuroendocrine cells lines (ATCC, Manassas, VA) were cultured in DMEM supplemented with 10% fetal calf serum (Life Technology). AGS cells were transfected with PKA-specific small-interfering RNA (siRNA) oligonucleotides using Lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen). One microgram of a reporter construct containing 3,373 bp of the human gastrin promoter ligated upstream of the pGL3B luciferase reporter plasmid (Promega, Madison, WI) was cotransfected into AGS cells with 1 μg of the menin expression vector cells using Lipofectamine 2000 in 24-well plates. Lysates were prepared and luciferase activity measured using the Dual-Luciferase Assay System (Promega) according to the manufacturer's protocol on an automated Luminometer Auto Lumat LB953 (Berthold). The luciferase activity was normalized to *Renilla* activity.

Flow cytometry. Primary mouse cells were dissociated from two antrums or two duodenums by using dispase in three separate experiments. Briefly, the antrums and duodenums were dissected into 2-mm pieces and subjected to enzymatic digestion in 20 ml of RPMI medium containing 1 mg/ml dispase II (Roche Molecular Biochemicals, Indianapolis, IN) for two 30-min incubations at 37°C with vigorous agitation to dissociate cells. The primary cells were collected, washed, and then permeabilized with Cytoperm (BD Biosciences, San Diego, CA). The cells were then incubated with a 1:100 dilution of rabbit anti-SSTR2A (Chemicon International, Temecula, CA), a 1:100 dilution of goat anti-gastrin, or 1:100 dilution of goat anti-menin (Santa Cruz Biotechnology, Santa Cruz, CA). A goat anti-goat antibody was used as a control. Phytoerythrin-tagged goat and FITC-tagged rabbit antibodies were used to detect the two proteins by double-fluorescence flow cytometry. To study the effect of a somatostatin agonist on menin expression, primary cells dissociated from the antrum or duodenum were treated in separate incubations with 100 nM octreotide for 24 h before performing the quantification of SSTR2A-positive cells expressing menin.

Immunoblot analysis. Protein was extracted from stomachs removed from WT mice injected with octreotide or PBS by homogenizing the tissue in lysis buffer (300 mM NaCl, 30 mM, Tris, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1% Triton X-100, pH 7.4) supplemented with protease inhibitors (EDTA-free complete tablets, Roche). Immunoblot analysis was performed using a 4–20% SDS-polyacrylamide gradient gel (Invitrogen, Carlsbad, CA). The nylon membranes were blocked by incubating with 0.5× Uniblock (Analytical Genetic Testing Center) or KPL's milk diluent/blocking solution (Gaithersburg, MD) for 1 h at room temperature prior to a 1-h incubation with rabbit anti-menin antibody (Bethyl Laboratories) at a dilution of 1:1,000. In a separate experiment, AGS cells transfected with menin siRNA were lysed. Extracts resolved on a gradient gel were subjected to immunoblot analysis using anti-gastrin rabbit antibody at a dilution of 1:400 (Santa Cruz). The membranes were washed three times for 10 min then incubated with a 1:2,000 dilution of Alexa Fluor 680 goat anti-rabbit (Molecular Probes) for an additional hour. Proteins were visualized and quantified by using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE) and then expressing the data in pixels per millimeter squared.

PKA activity. PKA activity was determined on AGS cell lysates by a nonradioactive assay performed according to the manufacturer's protocol (Assay Designs, EKS-390A, Ann Arbor, MI). Extracts (50 μg) from cells treated with 100 nM octreotide were incubated with the tetramethylbenzidine substrate; the amount of product generated was detected at an absorbance at 450 nm and then used to calculate the amount of PKA activity per microgram of protein. The time course of PKA activity suppression by octreotide was determined empirically by performing time course assays between 0 and 48 h. Optimal PKA suppression occurred at 16 h. Means ± SE for three independent experiments performed in triplicates are shown.

PCR analysis. Menin cDNA was reverse transcribed from total RNA extracted using TRIzol reagent (Invitrogen). The extracted tissue was removed from octreotide or PBS-injected WT stomachs. AGS cells were treated with 100 nM octreotide or 1 μg/ml α-amanitin to block RNA polymerase II-dependent transcriptional initiation according to the manufacturer's protocol (Invitrogen). Quantitative PCR was performed on a Bio-Rad iCycler (Hercules, CA) with SYBRgreen. Threshold cycles were normalized to the threshold cycles for GAPDH. The reactions were carried out in a total volume of 25 μl, containing 10 × PCR buffer with $MgCl_2$, 10 nM of deoxynucleotide triphosphates, 200 nM of primers, 5 μl of cDNA, 100 nM of *Taq* polymerase GOLD, and 2.5 μl of SYBRgreen (Molecular Probes). The primers for menin were forward primer: TCATTGCTGCCCTCTATGCC; reverse primer: TCCAGTTTGGTGCCTGTGATG. The primers for GAPDH were forward primer: TTCACCACCATGGAGAAGGC; reverse primer: GGCATGGACTGTGGTCATGA (Invitrogen). The primers for human gastrin were forward primer S2: GCCCAGCCTCTCATCATC; reverse primer A2: GCCGAAGTCCATCCATCC. The PCR amplification was performed in duplicate with the following conditions: 94°C for 10 min, followed by 35 two-temperature cycles 94°C for 1 min and 55°C for 1 min.

Statistics. The unpaired *t*-test was used to analyze the in vivo mouse studies or one-way ANOVA was used to analyze the cell culture experiments via a commercial software (GraphPad Prism, GraphPad Software, San Diego, CA). A P value <0.05 was considered significant.

RESULTS

Somatostatin modulates gastrin and menin. Gastrin is an important gastrointestinal hormone that is secreted from neuroendocrine cells (G cells) of the gastric antrum and duodenum (9). In the antrum, somatostatin inhibits gastrin (11, 57), but little is known regarding the effect of somatostatin on G cells in the duodenum where MEN1-associated gastrinomas develop because of loss of menin protein or function in subjects harboring the mutation. To determine whether modulating

somatostatin receptor activity influenced the number of duodenal gastrin-expressing (G) cells, we examined the duodenums of somatostatin-deficient mice by immunohistochemistry. We observed that the number of gastrin-expressing cells in the antrums of $SOM^{-/-}$ mice was slightly elevated (Fig. 1*A*), but in the duodenum G cells were more dramatically elevated (Fig. 1*B*). Using morphometric analysis, we found that the change in G cell numbers was greater in the duodenum owing to the presence of fewer G cells at baseline compared with the antrum (Fig. 1*C*). The plasma gastrin concentration for a $SOM^{-/-}$ mouse is greater than 400 pmol/l compared with baseline levels for WT mice (30–100 pmol/l) (55), which is consistent with a greater number of G cells that we detected by immunohistochemical staining of the duodenum. Thus we concluded that duodenal G cells, like those in the antrum, are also regulated by somatostatin.

Since mutations in the menin locus result in duodenal gastrinomas that secrete excess gastrin (5) and $SOM^{-/-}$ mice also produce excess gastrin (Fig. 1*B*), we postulated that somatostatin might modulate menin gene expression. Therefore, we examined whether menin protein levels would be low in $SOM^{-/-}$ mice. As predicted, we found that menin levels

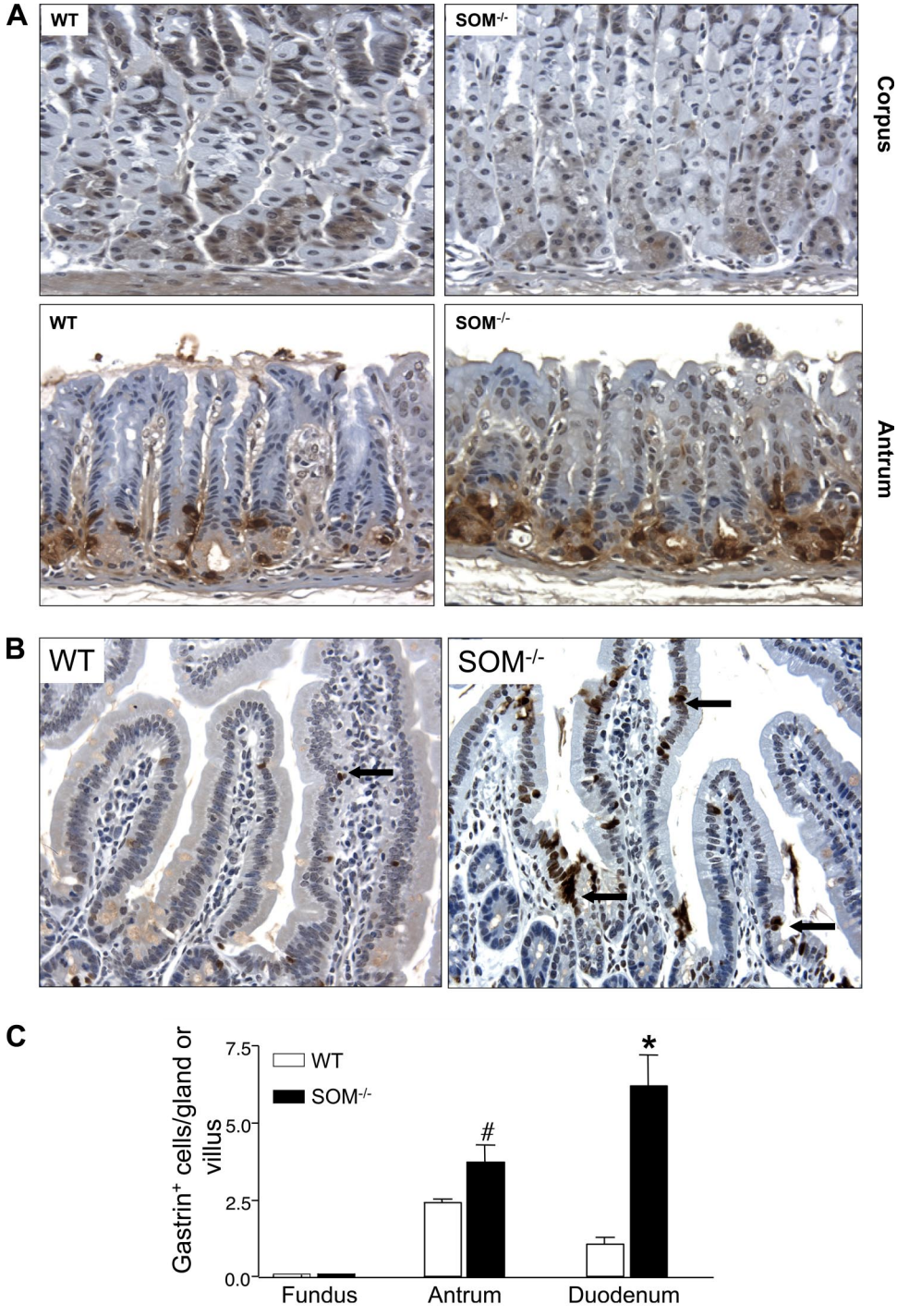

Fig. 1. Increased number of gastrin (G) cells in SOM-null mice. *A*: representative immunohistochemical staining for gastrin in the fundus and antrum of wild-type (WT) or $SOM^{-/-}$ mice (×400). *B*: representative immunohistochemical staining in the duodenum of WT or $SOM^{-/-}$ mice (×400). *C*: morphometric analysis of the number of G cells per gland or villus. Results are expressed as means ± SE for 4 mice per group. #$P = 0.051$, *$P < 0.05$ compared with WT.

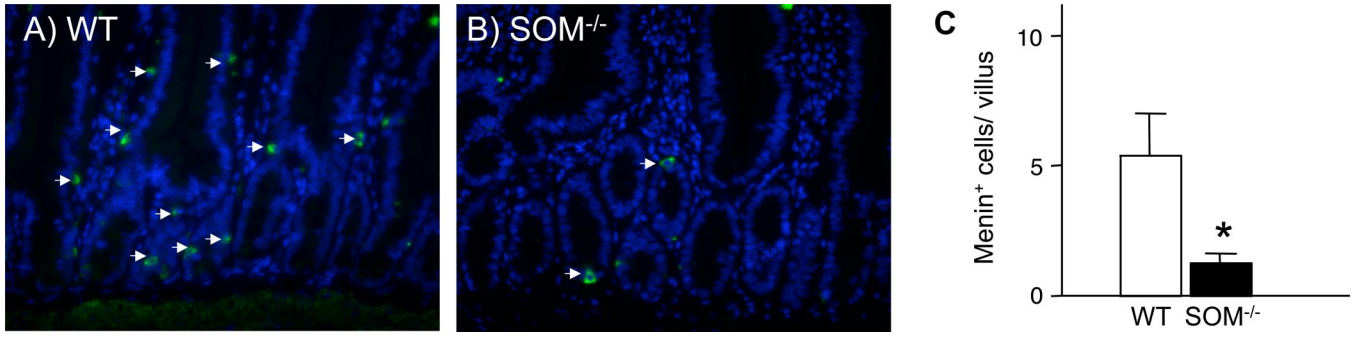

Fig. 2. Reduced menin-positive cells in duodenums of SOM-null mice. Representative immunohistochemical staining for menin in the duodenum of WT (*A*) or SOM$^{-/-}$ (*B*) mice. Blue = DAPI; green = FITC-labeled menin-positive cells indicated with arrows. *C*: morphometric analysis was used to quantify the number of menin-positive cells per villus in the duodenum. The results are expressed as means ± SE for 4 mice per group. *$P < 0.05$ compared with WT.

were markedly reduced in the duodenums of SOM$^{-/-}$ mice (Fig. 2). Immunohistochemical staining with menin antibodies revealed that the antrum exhibited primarily a nuclear pattern of expression whereas the duodenum exhibited a cytoplasmic pattern of staining in cells of the villi (Fig. 3) and at the base of the crypts (Figs. 2*A* and 3, *C* and *D*). Indeed, Ratineau et al. (45) previously showed by in situ hybridization that menin gene expression in the proximal small intestine is located in cells at the crypt base as well as in cells scattered throughout the villi. Thus our immunolocalization of menin protein correlated well with the prior localization of the mRNA. Menin has several nuclear localization signals, suggesting that its transcriptional activity might be regulated by its ability to shuttle in and out of the nucleus (31). Therefore, we concluded that the pattern of menin staining in WT mice differs depending on the location (antrum vs. duodenum). To confirm the fidelity of the immunohistochemical staining, we compared the pattern of staining in the WT mice to the pattern of staining in a 1-mo-old mouse homozygous for the menin allele in the intestine (Men$^{\Delta Int}$) (8). Indeed, the immunofluorescence confirmed that menin staining was absent in epithelial cells of the antrum and

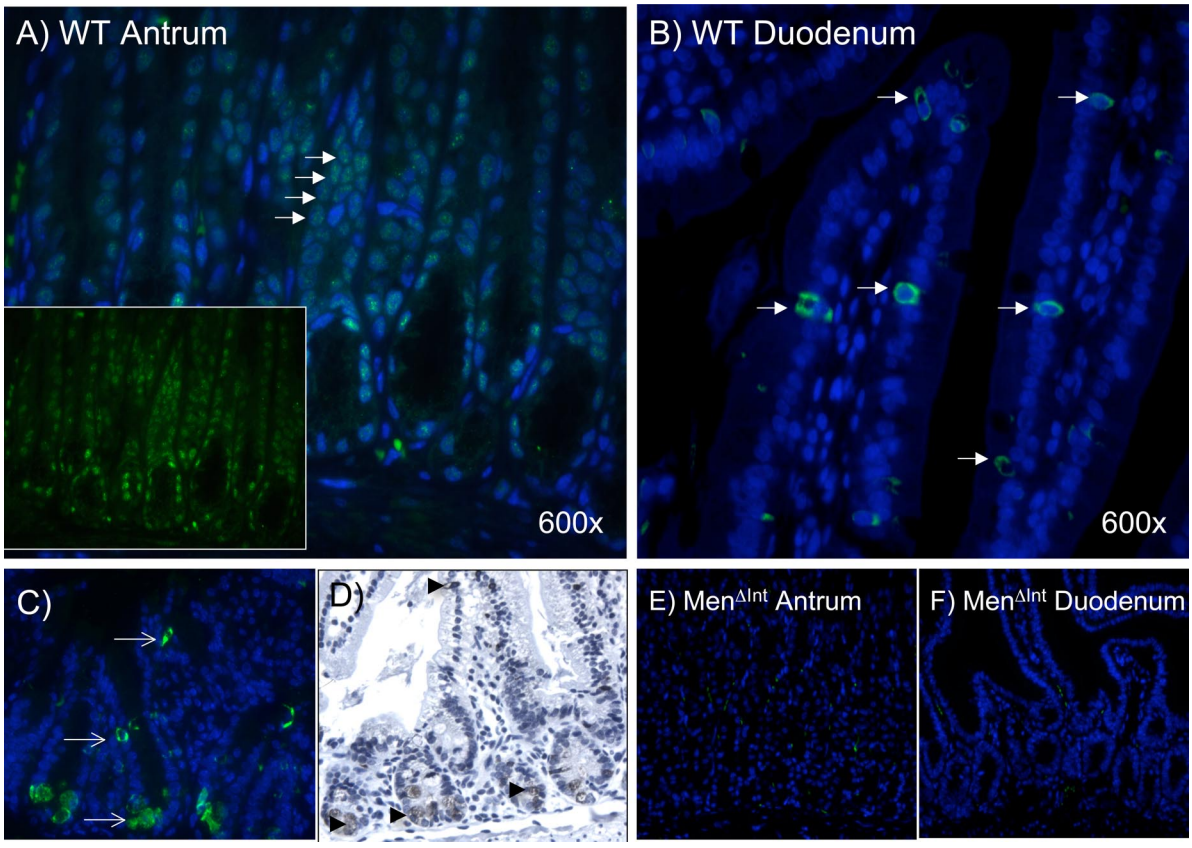

Fig. 3. Immunolocalization of menin in the antrum vs. the duodenum. Representative immunohistochemical staining for menin in the antrum (*A*; *inset* = FITC-labeled cells alone) or duodenum (*B*) of WT mouse, duodenum of WT mouse showing crypt base (*C*; ×400). *D*: light micrograph of duodenal crypt. Menin-positive cells are indicated (arrowheads). Antrum (*E*) or duodenum (*F*) of a Men$^{\Delta Int}$ mouse. Blue = DAPI nuclear stain; green = FITC-labeled menin indicated with arrows. Note that menin was not detected in the intestinal epithelial cells of the Men$^{\Delta Int}$ mice.

duodenum of a menin conditionally null mouse (Fig. 3, *E* and *F*).

Octreotide stimulates menin expression in vivo. Next, we examined whether somatostatin receptor activation induced menin expression in vivo. Octreotide, a somatostatin analog that preferentially binds to the SSTR2A receptor (23, 49, 50), suppressed basal gastric acid levels in mice as expected (Fig. 4*A*). Moreover, we found that octreotide increased menin protein levels in both the antrum and duodenum (Fig. 4*B*) and increased the number of cells in the duodenum that express menin (Fig. 4*C*). Although menin is a ubiquitous nuclear factor (1), we concluded from these results that menin is highly expressed in somatostatin-responding cells, i.e., cells expressing the somatostatin receptors, e.g., SSTR2A. Most of the SSTR2A cells in the antrum are gastrin positive, but there are fewer double-positive cells in the duodenum (19, 20). To further quantify the number of somatostatin-responding cells that increased their expression of menin, we performed flow cytometry. First, we sorted for the population of SSTR2 cells that is gastrin positive to quantify the SSTR2 cells that are G cells (Fig. 5). The population of gated cells depicted by the overlap between R4 (SSTR2+) and R5 (gastrin+) represents the double-positive SSTR2 and gastrin population (bold rectangle). In the antrum, this population was ~43%, whereas in the duodenum this population was ~14% (Fig. 5, *A* and *B*). Next, we quantified the number of SSTR2A-positive cells that express menin after octreotide treatment of primary cells dissociated from the antrum or duodenum (Fig. 5, *C* and *D*). Indeed, we found that octreotide induced primary cells of the duodenum to a greater extent than in the antrum. The number of menin-positive cells was lower in the duodenum than in the antrum at baseline but was dramatically induced with octreotide treatment (Fig. 5*D*). Although this result might represent induction of the SSTR2A receptor, the morphometric analysis demonstrated that there was indeed an absolute increase in the total number of menin-positive cells correlating with the increase in menin protein levels (Fig. 4*C*).

We confirmed that SSTR2A receptors colocalized with menin-expressing cells in the antrum (data not shown) and duodenum by colocalizing the SSTR2A receptor with menin by immunofluorescence (Fig. 6, *A–C*). It has been reported that neuroendocrine cells in the gut, especially G cells, preferentially express the SSTR2A receptor (19, 20, 46). Therefore, we examined whether some of the SSTR2A-expressing cells in the duodenum also produce gastrin and found that the few scattered G cells indeed expressed the somatostatin receptor (Fig. 6, *D* and *E*). Next, we examined whether the menin-positive cells, presumably responding directly to octreotide, also expressed gastrin. Unexpectedly, gastrin colocalized with menin in the antrum but not in the duodenum (Fig. 7). In addition,

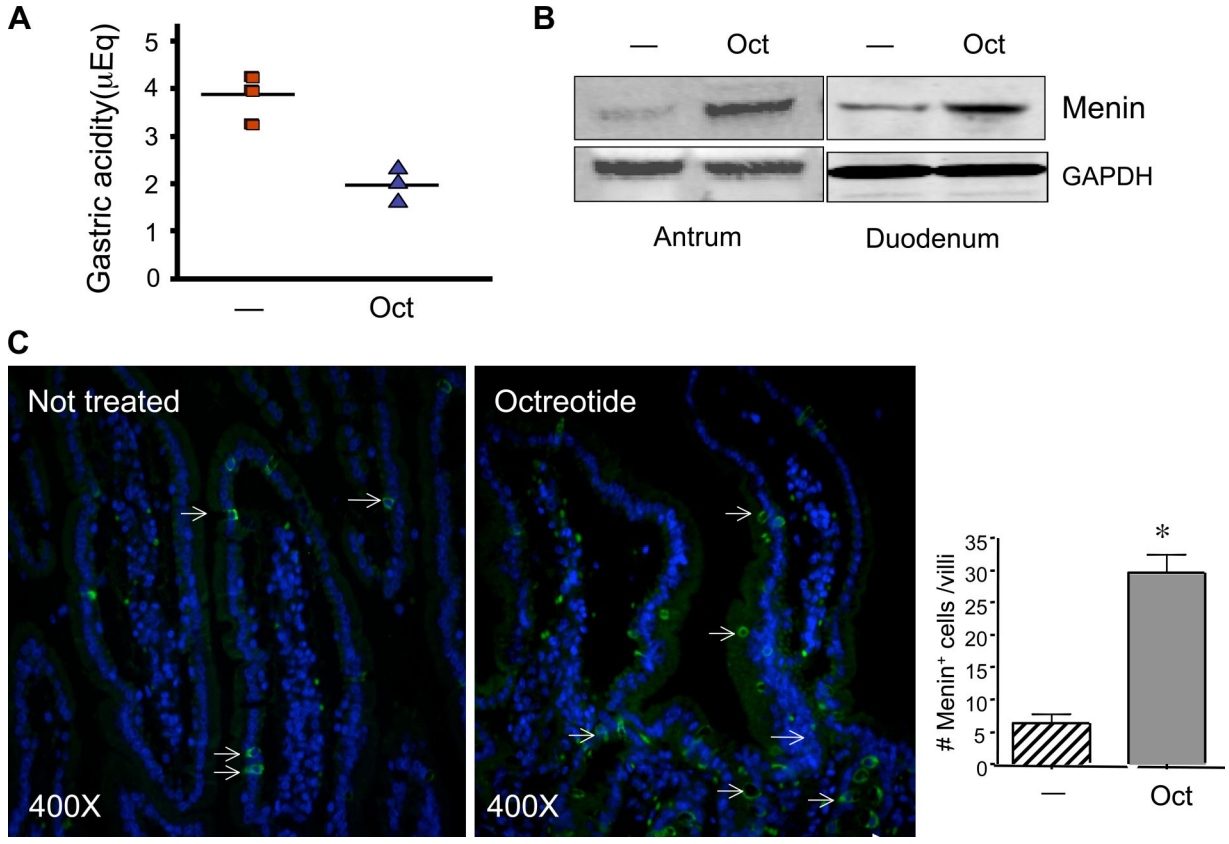

Fig. 4. Octreotide (Oct) stimulates menin expression in vivo. Octreotide was infused into mice for 26 h before death. *A*: gastric acid was measured on stomach washings by base titration. *B*: an immunoblot was performed on tissue extracts from the antrum and duodenum of octreotide-treated and untreated mice (—). Antibody (1:1,000 dilution) was used to detect menin protein in 100 μg of tissue lysates. GAPDH was probed as the loading control. *C*: immunofluorescence of menin staining in untreated and octreotide-treated mice. Menin (FITC, green); nuclear staining using DAPI. Arrows indicate menin-positive cells. *Right*: mean number ± SE of menin-positive cells per villi for 3 mice.

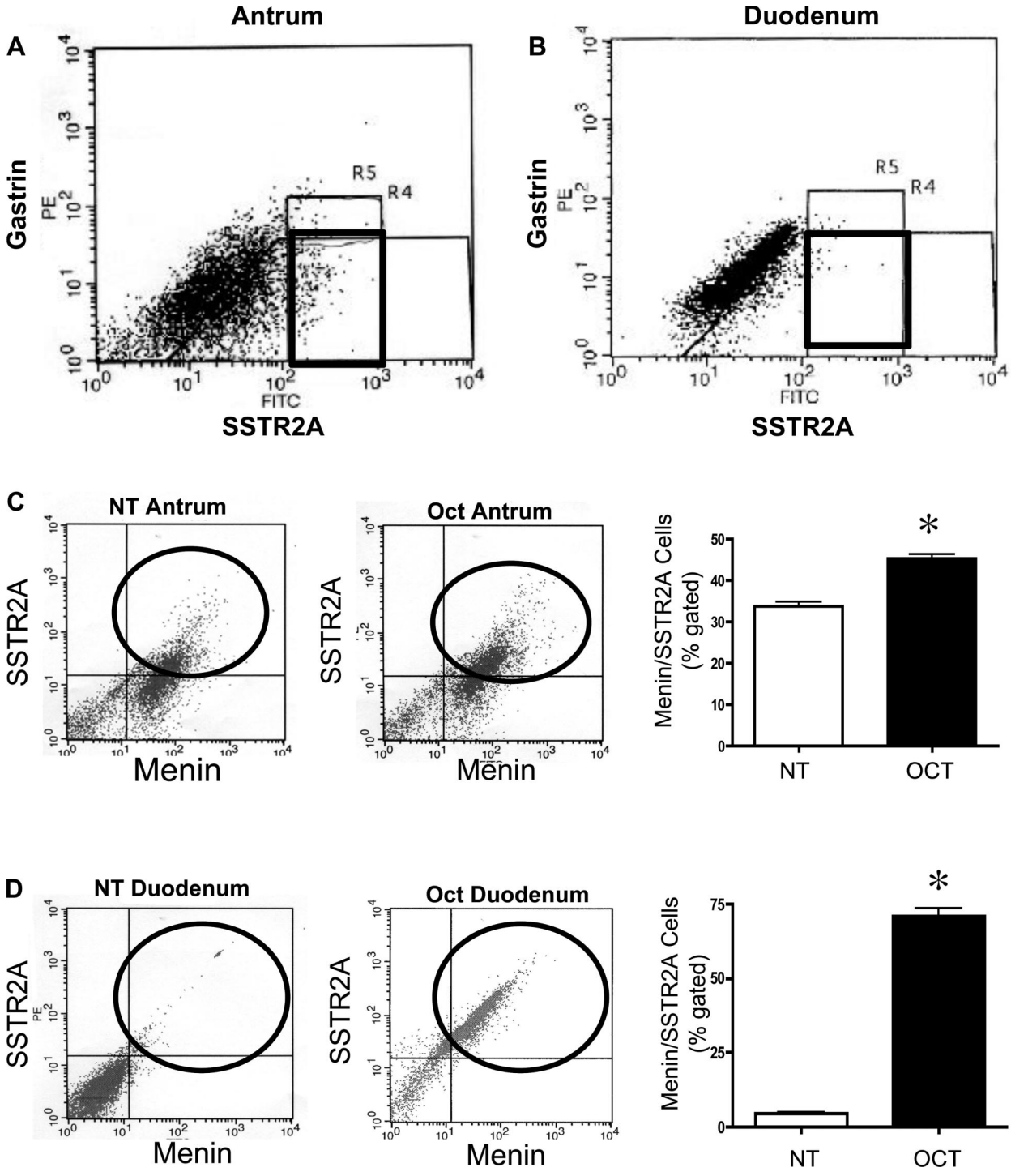

Fig. 5. Antral and duodenal SSTR2A+ cells induced by octreotide. Antral (*A*) or duodenal (*B*) cells were dissociated with dispase/EDTA, permeabilized, and then incubated with a 1:100 dilution of goat anti-gastrin and a 1:100 dilution of rabbit anti-SSTR2A antibodies for analysis by flow cytometry. The dissociated and permeabilized cells were also incubated with fluorescently tagged IgG to control for nonspecific antibody adherence. The percentage of double-positive cells detected is shown. R5 and R4 = percentage of gated cells from the antrum that are both gastrin and SSTR2A positive (42.7%, bold rectangle). R5 and R4 = percentage of gated cells from the duodenum that are both gastrin and SSTR2A positive = (14.4%, bold rectangle). R4 = SSTR2A-expressing cells, vertical rectangle; R5 = gastrin-expressing cells, horizontal rectangle. Dissociated antral (*C*) and duodenal (*D*) cells were cultured and treated with vehicle or 100 nM octreotide for 24 h, permeabilized, and then incubated with a 1:100 dilution of goat anti-menin and a 1:100 dilution of rabbit anti-SSTR2A antibodies for analysis by flow cytometry. The overlap of SSTR2 and menin-positive cells is circled in bold. Mean percent overlap was graphed as a function of treatment. *$P = 0.05$ compared with untreated (NT) for antral cells and $P < 0.05$ for duodenal cells; $N = 3$ individual mouse antral and duodenal preparations. The dissociated and permeabilized cells were also incubated with fluorescently tagged IgG to control for nonspecific antibody adherence. The percentage of cells detected with the following controls was subtracted from the percentage of cells detected with anti-SSTR2A or anti-menin antibodies. IgG-phycoerythrin (PE): 0.08%; IgG-FITC: 0.00%; IgG-PE/IgG-FITC: 0.06%.

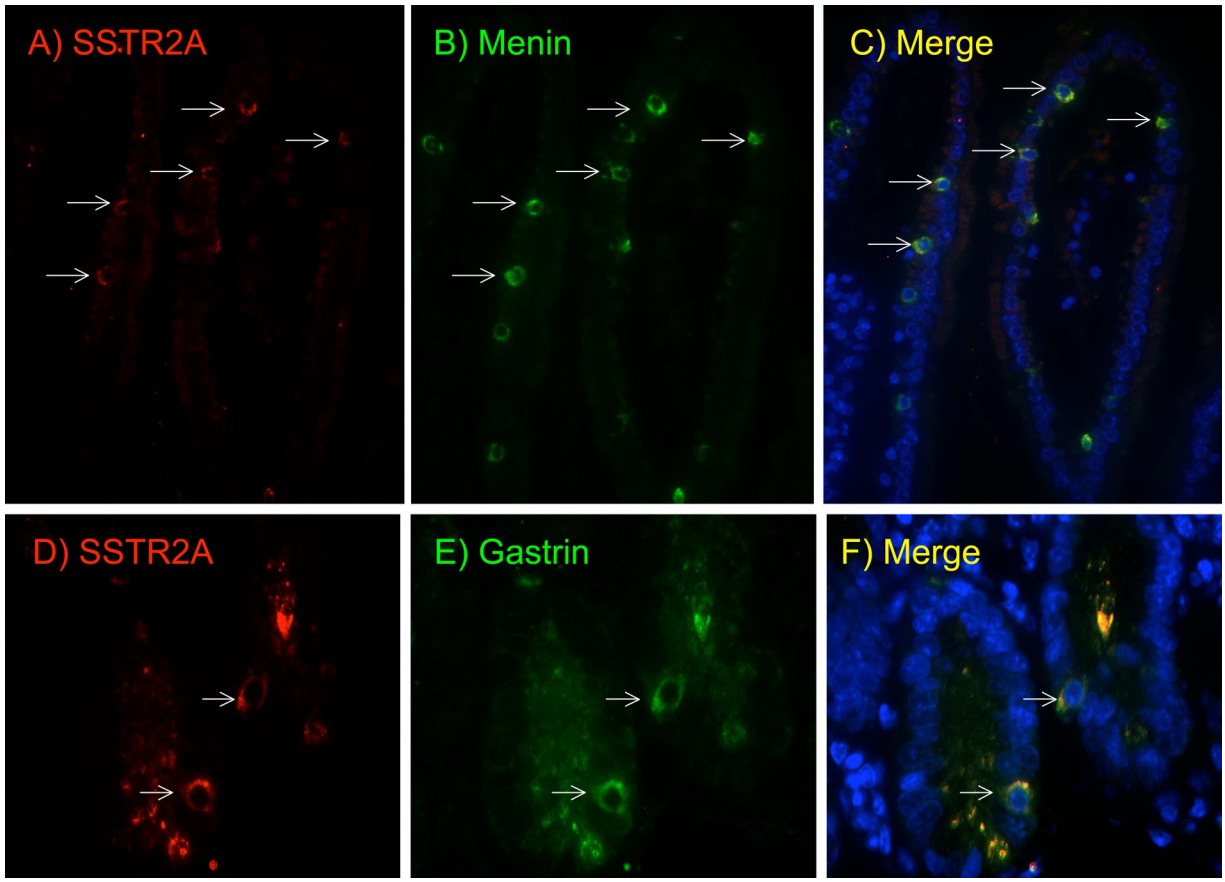

Fig. 6. Menin colocalizes with SSTR2A in the duodenum. SSTR2A antibody was colocalized with menin (*A–C*) (magnification ×400) or gastrin (*D–F*) antibody (magnification ×600) on WT mouse duodenal tissue sections. A 1-h incubation with a 1:200 (menin), 1:400 (SSTR2A), or 1:800 (gastrin) dilution followed by incubation with FITC- or Texas red-conjugated secondary antibody was performed. Arrows indicate the cells colocalizing with SSTR2A or gastrin.

there were a number of menin-positive cells in the antrum that were negative for gastrin consistent with the known ubiquitous distribution of the protein. Collectively, these results showed that octreotide treatment (somatostatin signaling) stimulates menin in vivo. G cells clearly express SSTR2 receptors and are thus able to respond to somatostatin. However, G cells comprise a subpopulation of the menin expressing cells in the antrum but not in the duodenum. Although we cannot exclude the presence of very low levels of menin, the relative absence of menin in the duodenal G cells suggests that menin does not directly regulate gastrin expression in the intestinal endocrine cell but instead directly regulates gastrin in the stomach antrum. Therefore menin-expressing cells apparently regulate duodenal G cells through a paracrine mechanism given their close proximity.

To determine whether the induction of menin represented a direct effect of somatostatin signaling, we treated two gut-derived cell lines: AGS (human gastric adenocarcinoma cells) and STC (a mouse duodenal neuroendocrine cell type) (13, 33). We found that octreotide induced menin protein expression in a dose-dependent manner (Fig. 8). Octreotide induced menin mRNA within 12 h (Fig. 9*A*) and the induction was blocked by the RNA polymerase II inhibitor amanitin (Fig. 9*B*). Thus induction of menin gene expression by activation of the somatostatin signaling pathway was direct and due to induction of transcriptional initiation.

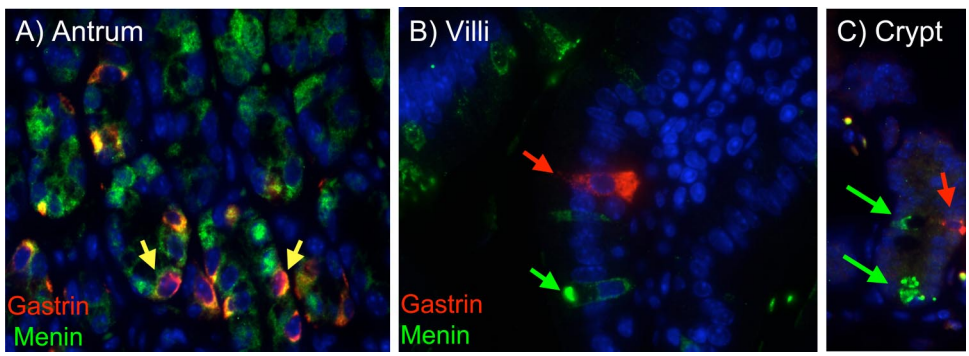

Fig. 7. Menin colocalizes with gastrin in the antrum, not in the duodenum. Menin antibody was colocalized with gastrin antibody on WT mouse tissue sections in either the antrum (*A*; ×600), duodenal villi (*B*; ×600), or duodenal crypt (*C*; ×400). A 1-h incubation with a 1:200 (menin) or 1:800 (gastrin) dilution followed by incubation with FITC- or Texas red-conjugated secondary antibody was performed. Arrows indicate the cells expressing menin (green) or gastrin (red) or both (yellow).

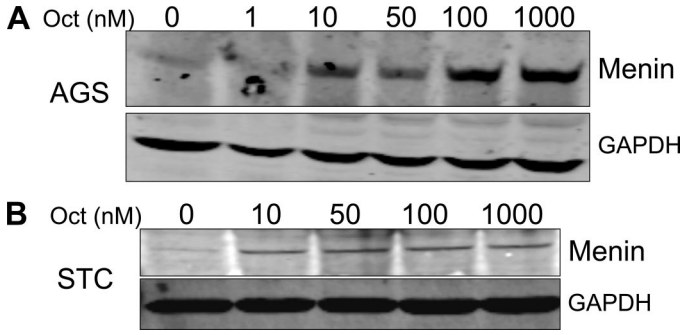

Fig. 8. Octreotide stimulates menin expression in cell lines. *A*: AGS cells were serum starved for 24 h and then treated with increasing concentrations of octreotide for 24 h. *B*: STC cells were treated with increasing concentrations of octreotide for 48 h. Whole cell extracts were analyzed by immunoblot. Protein (100 μg) was resolved on a gradient gel, transferred to nylon, and then probed for menin (1:1,000 dilution) and GAPDH.

Reduced levels of protein kinase A increase menin expression. Somatostatin binds to the receptor SSTR2A and recruits G_i proteins that inhibit the cAMP-PKA signaling network (43). Therefore we examined whether this signaling pathway might also mediate the induction of menin. We used the PKA inducer forskolin to assess whether PKA activation suppresses menin expression. Forskolin, through the activation of PKA, inhibited menin (Fig. 10*A*) and suppression of PKA using siRNA oligos increased basal levels of menin protein (Fig. 10*B*). The amount of menin protein after knockdown of PKA protein with siRNA oligos was equivalent to the levels observed with octreotide treatment, demonstrating that constitutive PKA activity is likely responsible for the lower levels of menin at baseline (Fig. 10*B*; compare *lanes 2* and *3*). After siRNA treatment there was little PKA protein available to be regulated by octreotide; thus no further changes in menin protein levels were observed (Fig. 10*B*, *lane 4*). Since the levels of PKA protein remained unchanged after octreotide treatment, we showed directly by assaying for enzyme activity that PKA was inhibited by octreotide (Fig. 10*C*). We concluded from these results that modulation PKA activity is likely the mechanism of octreotide regulation.

Menin inhibits gastrin gene expression. Since somatostatin signaling increased menin protein levels and menin colocalizes with gastrin in the antrum, we examined whether menin directly regulated gastrin gene expression in the AGS gastric cell line. Reduction of menin increased progastrin peptide levels and indeed this antibody detected a decrease in progastrin peptide after siRNA was used to suppress the gene (Fig. 11*A*). Using quantitative RT-PCR, we established that suppression of menin with siRNA oligonucleotides dramatically induced endogenous gastrin mRNA in AGS cells (Fig. 11*B*). Using a human gastrin reporter plasmid, suppression of menin stimulated transcription 2.5-fold (Fig. 11*C*). By contrast, overexpression of wild-type menin in AGS cells suppressed 90% of gastrin reporter activity (Fig. 11*D*). Collectively, these results demonstrated that menin is a transcriptional repressor of gastrin gene expression.

DISCUSSION

Although the menin gene is mutated and the protein eventually lost or dysfunctional in both sporadic and MEN1-type gastrinomas (4), whether menin regulates gastrin expression has never been examined. On the basis of the observation that hypergastrinemia is a convergent phenotype observed in the absence of either somatostatin or menin (gastrinomas), we hypothesized that menin might be a transcriptional mediator of somatostatin receptor activation. Indeed, we found that duodenal gastrin varies inversely with somatostatin peptide levels and that duodenal menin levels are induced by somatostatin to a greater extent than in the antrum. This result is consistent with the predilection of the duodenum to develop MEN1

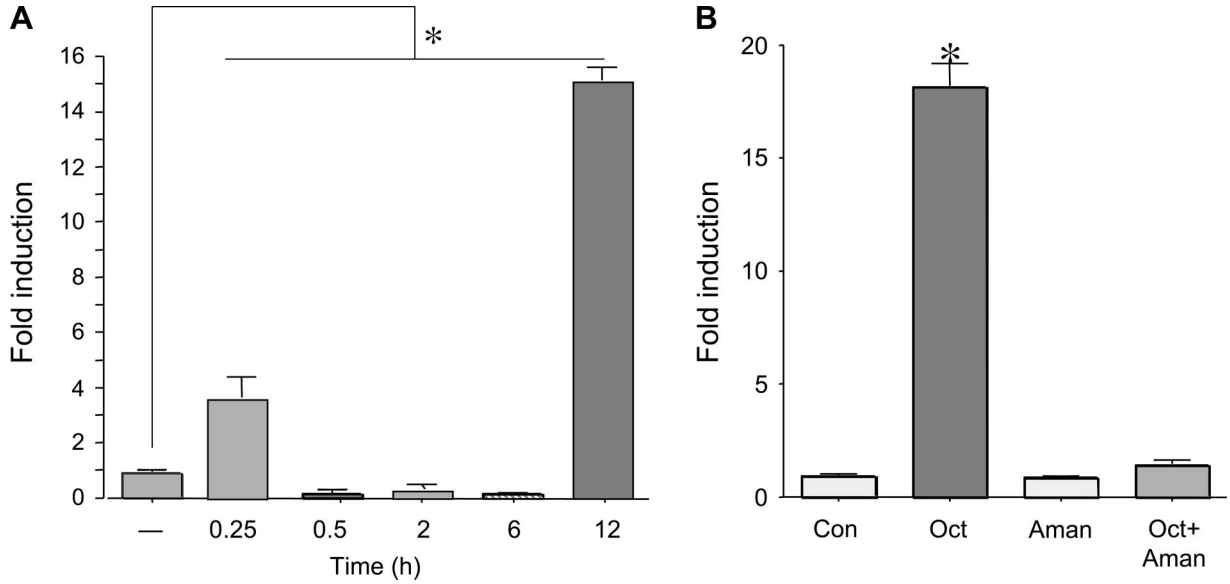

Fig. 9. Octreotide stimulates menin transcription. *A*: quantitative RT-PCR using SYBRgreen was performed on total RNA isolated from AGS cells after exposure to 100 nM octreotide for 0.25, 0.5, 2, 6, and 12 h. Shown is the fold induction compared with untreated cells of the menin-to-GAPDH ratio. $*P < 0.05$. *B*: effect of α-amanitin (Aman) on mRNA synthesis. AGS cells were treated with α-amanitin (1 μg/ml), octreotide (100 nM), or α-amanitin and octreotide for 12 h. Quantitative RT-PCR using SYBRgreen was performed by using the total RNA extracted. Shown is the fold induction compared with untreated cells of the menin-GAPDH ratio. The results were expressed as means ± SE. $*P < 0.05$. $N = 3$ independent experiments performed in duplicate.

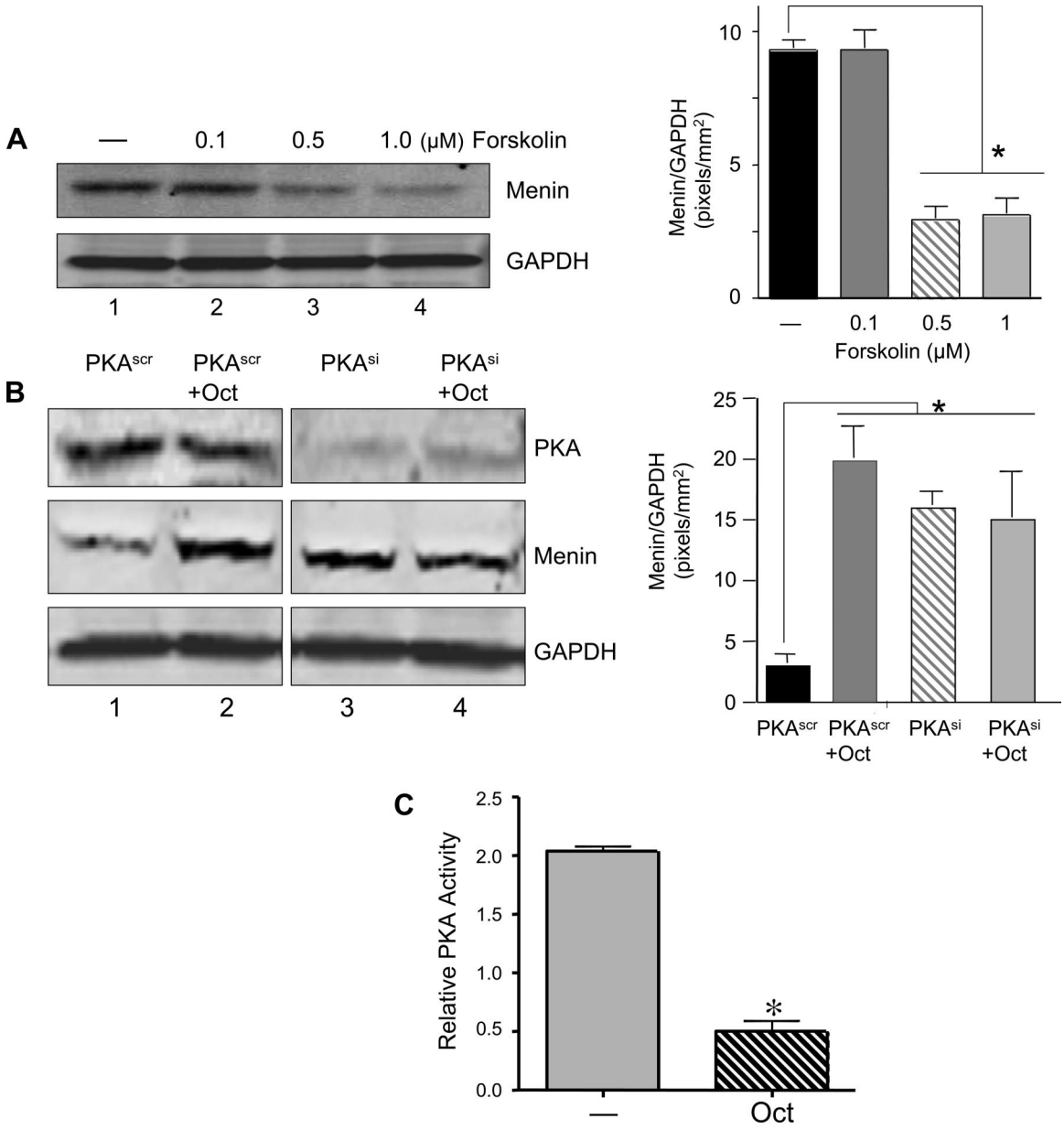

Fig. 10. Octreotide suppression of PKA induces menin. *A*: AGS cells were treated with increasing concentrations of forskolin. Whole cell extracts were analyzed by immunoblot, and 100 μg of protein was loaded and probed for menin (1:1,000 dilution) or GAPDH. *Right*: means ± SE for 3 experiments, $*P < 0.001$. *B*: AGS cells were transfected with either the scrambled (PKA^{scr}) or sequence specific interfering oligonucleotides for PKA (PKA^{si}) by use of Lipofectamine 2000 and then treated with octreotide for 48 h. Whole cell extracts were analyzed by immunoblot, and 100 μg of protein was loaded and probed for PKA, menin (1:1,000 dilution) and GAPDH. *Right*: quantification for $N = 3$ experiments ± SE. $*P < 0.05$ for the 3 treatments shown in *lanes 2–3*. *C*: lysates were prepared from AGS cells treated with or without octreotide for 16 h prior to measuring PKA activity in 50 μg of extract. Purified active PKA used as the positive control. The absorbance at 450 nm was normalized to the protein concentration prior to plotting as means ± SE relative PKA activity for 3 experiments performed in triplicate. $*P < 0.05$.

gastrinomas (4, 5). We also found that somatostatin induced menin gene expression by stimulating transcriptional initiation through a PKA-dependent mechanism. Menin can exert its effect directly on gastrin gene expression in the stomach since reducing endogenous menin levels by siRNA in AGS cells, a gastric cell line, increased endogenous gastrin mRNA and peptide. In addition, ectopic expression of menin suppressed the basal activity of a gastrin-luciferase reporter. Since menin colocalized to the G cells in the antrum, but not in the duodenum, we suggest that menin regulates duodenal gastrin by modulating the levels of a paracrine effector released from an SSTR2-positive cell within close proximity.

Although we focused here on SSTR2-positive cells, there is evidence that other SSTR receptors can mediate somatostatin effects. Prior studies by Martinez et al. (37) show that elevated gastric acid secretion observed in SSTR2-null mice is gastrin dependent because infusion of gastrin antibody into these mice inhibits acid output. Surprisingly, serum gastrin levels were not elevated. Thus the investigators concluded that the increase in acid secretion observed in the SSTR2-null mice was mediated

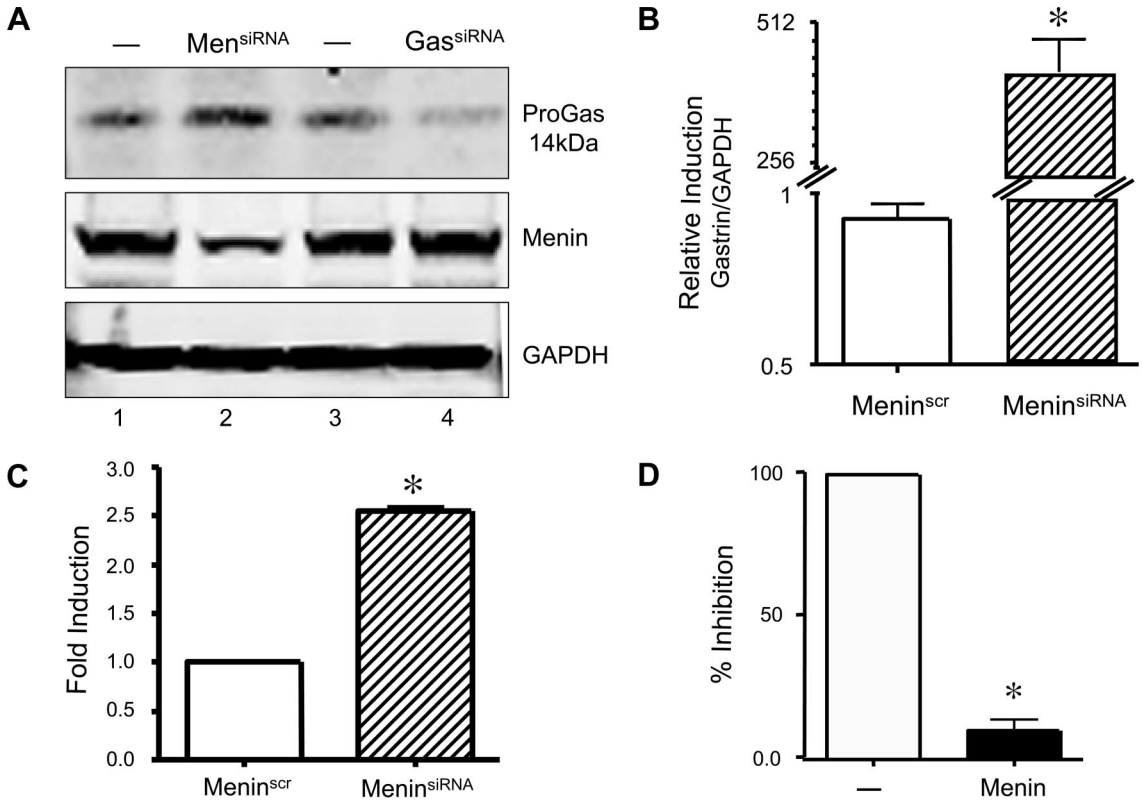

Fig. 11. Menin inhibits gastrin gene expression. *A*: Commercial small-interfering RNA (siRNA) oligos for menin (Men^{siRNA}) and gastrin (Gas^{siRNA}) (WT or scrambled) were purchased from Santa Cruz. AGS cells (10^5) were transfected with 100 nM of either oligo by using Lipofectamine for 48 h. Endogenous progastrin peptide, progastrin (ProGas), the unprocessed peptide precursor of gastrin (14 kDa), levels were compared with menin protein levels after the lysates were resolved on a 4–20% gradient gel prior to detection of the proteins by immunoblot. *B*: a similar experiment was performed as in *A* except the tissue was extracted for total RNA to quantify gastrin mRNA by quantitative RT-PCR. Shown is the relative increase in gastrin normalized to GAPDH mRNA as means ± SE for 3 independent experiments performed in duplicate. *C*: transient transformants of AGS cells expressing the 3.3-kb gastrin luciferase reporter (GASLuc) reporter were treated with 100-nM amounts of either the scrambled ($Menin^{scr}$) or menin siRNA oligo. Means ± SE for 3 experiments are shown. *D*: AGS cells were cotransfected with a 3.3-kb GASLuc reporter and a menin expression vector subcloned into the pcDNA vector. The empty vector (pcDNA) was used as the control (—). Shown are means ± SE for 5 experiments (nanomolar = nM).

through another somatostatin receptor, e.g., SSTR5. In the in vivo studies here, we showed that somatostatin signaling increased menin preferentially in a discrete population of SSTR2-positive cells but cannot exclude that somatostatin is also regulating menin through one of the other five somatostatin receptors.

Menin regulation by extracellular signals has not been well described. Aside from the present report demonstrating a role for somatostatin, only TGF-β signaling has been shown to stimulate menin (28). In that study, Kaji et al. (28) reported that TGF-β induces the expression of menin protein in a dose-dependent manner within 30 min, reaching maximum levels within 1–3 h. By contrast, our data showed that somatostatin treatment requires ~12 h to induce menin mRNA expression, suggesting that additional steps in the signaling cascade likely occur before transcription begins at the menin promoter. Moreover, since modulation of menin requires PKA activity, there is the possibility that posttranslational modification of menin, e.g., phosphorylation, might be responsible for the more rapid effects of menin on specific target genes. Nevertheless, induction of menin gene expression by somatostatin has physiological relevance since somatostatin is a well-established paracrine regulator of gastrin in the stomach as well as other endocrine peptides in the pancreas and pituitary (21). Therefore, somatostatin receptor regulation of menin will likely contribute to our mechanistic understanding of why somatostatin analogs are effective treatments for a variety of endocrine tumors (14, 47, 51).

Radiolabeled octreotide is used to diagnose and treat gastrinomas, suggesting that both normal G cells and in some instances the gastrin-expressing tumors express high levels of somatostatin receptors (6, 10, 16, 26). Of the five somatostatin receptors, octreotide has the highest affinity for the SSTR2A receptor (49, 50). Possibly the overexpression of somatostatin receptors on these neuroendocrine tumors represents activation of a feedback mechanism that emerges because of the absence of menin-regulated repressor activity resulting in the subsequent overproduction of downstream targets, e.g., gastrin. Indeed, on the basis of the immunolocalization of menin protein, we conclude that menin suppresses gastrin gene expression directly in the antral G cell but indirectly in the duodenum.

We observed that somatostatin stimulates menin by inhibiting PKA activation, implying that activation of the cAMP-PKA pathway stimulates gastrin gene expression. Indeed, we previously reported that cAMP induces gastrin gene expression and that this induction is abrogated by octreotide (50a). Somatostatin signaling through G_i normally suppresses PKA activa-

tion, releasing menin from the effects of constitutive PKA signaling. Presumably, activated PKA phosphorylates the transcription factor CREB, which in turn binds to the menin promoter. The most recent analysis of the first 1,000 bp of the menin promoter did not reveal a CREB binding site (54) and may require examination of a larger piece of promoter. Alternatively, there is the possibility that protein-protein interactions cooperating at nonconsensus DNA elements mediate PKA regulation.

The observation that G cells in the duodenum are more sensitive to changes in somatostatin levels compared with in the antrum was unexpected but supports our recent report on the regulation of the human gastrin gene in transgenic mice (38). The greater induction was due in part to the fact that gastrin-expressing cells in the duodenum are less abundant than in the antrum, amplifying the degree of change. However, the difference in responsiveness of the two G cell populations might also represent differences in their embryological origins and as a result their differences in the signaling circuitry. For example, the expression pattern of endocrine-related proteins, e.g., synaptophysin and neurogenin 3, is not the same in antral vs. duodenal neuroendocrine cells (44, 48, 53). Perhaps exploiting this distinction is the predilection of duodenal neuroendocrine cells that lose menin to permit overexpression of gastrin, resulting in a preponderance of MEN1 gastrinoma tumors to develop there (5).

In addition to its effects on hormone secretion, somatostatin exhibits significant antiproliferative effects (12). Stimulation of $p27^{Kip1}$ protein expression by somatostatin analogs (25) is a mechanism that is also targeted by menin to inhibit cell growth (29, 39). Thus a common signaling pathway appears to be shared by these two proteins, more directly linking their relationship. Our study here demonstrates that menin is a direct mediator of somatostatin signal transduction. However, what remains to be defined is how menin differentially targets specific promoters, e.g., $p27^{Kip1}$ and gastrin. Recent studies suggest that menin exerts its transcriptional control through a global effect on chromatin structure (40). Expression arrays of menin-null mouse embryo fibroblasts have implicated a role for extracellular matrix proteins (27) but still do not provide insights into the predilection for neuroendocrine cells.

Therefore, in conclusion, we provide novel evidence that somatostatin is an extracellular ligand that transcriptionally regulates the expression of menin preferentially in the duodenum. This signaling cascade is mediated by SSTR2A receptor suppression of the cAMP-PKA signaling pathway and activation of menin that subsequently modulates the gastrin promoter both directly and indirectly.

GRANTS

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