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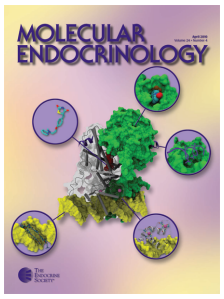

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In Situ Analysis of Human Menin in Normal and Neoplastic Pancreatic Tissues: Evidence for Differential Expression in Exocrine and Endocrine Cells

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Multiple endocrine neoplasia type 1 (MEN1) is a hereditary syndrome linked to mutations in the *MEN1* gene, which encodes a 610-amino-acid nuclear protein termed menin. Because of the lack of a suitable detection protocol, the *in situ* expression pattern of menin in human tissues remains to be determined. In this study, we have developed an antimenin monoclonal antibody and an indirect immunofluorescence/laser-scanning microscopy protocol for analyzing menin expression in frozen tissue sections. Because neuroendocrine pancreatic tumors represent a key feature of MEN1, we focused this study on nontumoral pancreas and a small panel of neuroendocrine pancreatic tumors. Results showed that menin was readily detected in nontumoral exocrine cells. In contrast, most islet cells expressing insulin, glucagon, or somatostatin showed considerably weaker levels of menin expression; however, a subpopulation of pancreatic polypeptide-positive cells exhibited a signal comparable with that detected in adjacent exocrine cells. Sporadic endocrine tumors showed variable levels of menin expression, whereas a *MEN1*−/− gastrinoma scored negative. This report thus provides the first description of the expression pattern of menin in human pancreas *in situ* and lays the groundwork for further studies of other tissues and tumors. (*J Clin Endocrinol Metab* 88: 3893–3901, 2003)

MULTIPLE ENDOCRINE NEOPLASIA type 1 (MEN1) is a hereditary polyendocrine syndrome that is transmitted as an autosomal dominant, highly penetrant trait (1). MEN1 is characterized by neoplastic-hyperplastic lesions of parathyroid, anterior pituitary, and enteropancreatic neuroendocrine tissue; involvement of other tissues, such as the adrenals and dermal structures, is less frequent (reviewed in Refs. 2 and 3). A gene in the 11q13 locus was found to be mutated in the large majority of MEN1 patients; expression of this gene gives rise to a 10-exon, 2.8-kb mature mRNA that codes for a 610-amino-acid nuclear protein named menin (4–6).

Genetic analyses have revealed a large number of mutations within the *MEN1* gene in MEN1 tumors and in a subset of sporadic parathyroid adenomas, insulinomas, gastrinomas, and pulmonary carcinoids (reviewed in Ref. 3). Most mutations are nonsense or frameshift types resulting in premature truncation of the protein (reviewed in Ref. 3), suggesting that menin acts as a tumor suppressor. In line with such a role, menin was shown to suppress tumorigenicity of Ras *in vitro* (7). Furthermore, knockout mice carrying germline monoallelic deletions of the *MEN1* gene develop endocrine lesions resembling human MEN1 (8).

The *MEN1* mRNA was detected by Northern blotting in a wide spectrum of adult human tissues (4, 5). Immunoblot assays of human fetal tissues revealed menin expression in many organs, except in liver, lung, pancreas, and skin (9). The *MEN1* mRNA and menin protein were detected by RT-PCR and immunoblotting in primary and secondary hyperparathyroid lesions (10). Immunoblot analyses indicated that expression of menin is increased in sporadic pituitary adenomas, compared with normal pituitary tissue (11). Based on both RT-PCR and immunoblotting, menin expression was reported to be down-regulated, but not abolished, in MEN1 tumors, including cases carrying biallelic ablative mutations of the *MEN1* gene (10).

Results obtained using techniques that analyze homogenized tissues (*e.g.* immunoblot, Northern blot, and RT-PCR) can be difficult to interpret because of the presence of stromal components. *In situ* RNA hybridization, a technique that overcomes such problems, demonstrated expression of the *MEN1* transcript in a wide array of adult human tissues, with the strongest signal detected in placenta (12). To date, limited information is available on the pattern of menin protein expression *in situ* in normal tissues and in sporadic and MEN1 tumors, mostly because of the lack of an effective antibody and detection protocol. This report describes an antimenin monoclonal antibody and indirect immunofluo-

Abbreviations: C126men, Portion of *MEN1* coding for the C-terminal 126 amino acids of menin; GST, glutathione-S-transferase; IF/LSM, immunofluorescence/laser-scanning microscopy; MEN1, multiple endocrine neoplasia type 1; NSE, neuron-specific enolase; PP, pancreatic polypeptide.

rescence/laser scanning microscopy (IF/LSM) protocol developed to detect human menin *in situ*. We provide the first description of the protein's expression in frozen sections of normal pancreas and endocrine pancreatic tumors, which revealed interesting differences in expression levels among exocrine and islet cell populations.

Patients and Methods

Patients and tissues

Nontumoral samples. Nontumoral pancreatic tissues were obtained from a type II diabetes patient (sample no. 3) and from patients who underwent surgical resection of sporadic pancreatic tumors [an insulinoma (sample no. 1), a nonsecreting endocrine tumor (sample no. 2), a well-differentiated sporadic gastrinoma (sample no. 7B), a well-differentiated sporadic nonfunctioning endocrine tumor (sample no. PF28), two ductal pancreatic adenocarcinomas (no. PF14 and PF30), a pseudopapillary carcinoma (no. PF26), and an adenocarcinoma of the papilla of Vater (sample no. PF35)].

Tumor samples. The following tumor samples were analyzed. Case no. 10A was a sporadic gastrinoma (maximum diameter, 1.6 cm) of the uncinate process of the pancreas, microscopically infiltrating the duodenum and peripancreatic fat, resected from a 70-yr-old male with Zollinger-Ellison syndrome; it scored positive in immunohistochemistry for chromogranin, neuron-specific enolase (NSE), and gastrin. Case no. 12A was a massive lymph node metastasis (2.2 cm) arising from a small (<2 mm) gastrinoma in the duodenal wall of a 51-yr-old woman who, in the past, had undergone a total parathyroidectomy and suffered from hypergastrinemia; although her clinical and family history were consistent with MEN1 syndrome, no germ line mutations were found in the coding portion of *MEN1*. Case no. 14B was a sporadic, well-differentiated insulinoma (maximum diameter, 1.7 cm) resected from the pancreatic head of a 39-yr-old male who presented hypoglycemic syndrome; it was chromogranin +, NSE +, insulin +, Ki 67 less than 0.9%, with no evidence of mitosis. Case no. 19B was a sporadic, well-differentiated insulinoma (maximum diameter, 2.7 cm) resected from the pancreatic head of a 24-yr-old male with hypoglycemic syndrome; it was chromogranin +, NSE +, insulin, Ki 67 = 2.1%, with no signs of mitosis. Case no. 21A was a sporadic, well-differentiated insulinoma (maximum diameter, 0.8 cm) resected from the pancreatic head of a 20-yr-old male with hypoglycemic syndrome; it was chromogranin +, NSE +, somatostatin +, insulin + (only 30% of cells). Case no. 13A was a sporadic duodenal somatostatinoma (maximum diameter, 2.0 cm) that presented infiltration of the duodenal wall and massive lymph node metastases; it was resected from a 53-yr-old male with no clinical symptoms; the sample showed diffuse positivity for somatostatin and NSE (>90% of cells); 20% of the cells were positive for chromogranin. Case no. 15A was a duodenal gastrinoma (maximum diameter < 5 mm) resected from a 37-yr-old male MEN1 patient whose clinical history included a prolactin-secreting pituitary adenoma, parathyroid hyperplasia, hypergastrinemia, stomach and lung carcinoids, pancreatic PPomas, and a family history of MEN1; in addition to the duodenal gastrinoma, the pathologic picture included multiple duodenal microadenomas, multiple gastric neuroendocrine tumors, multiple pancreatic nonfunctioning neuroendocrine tumors, periduodenal lymph node metastases, and liver metastasis of neuroendocrine origin. The duodenal tumor scored NSE + and gastrin + by immunohistochemistry. Genetic analysis revealed a germ-line 11-bp deletion in exon 9 of *MEN1* and loss of the second *MEN1* allele in the tumor.

Informed consent for tissue analyses was obtained from living patients or from relatives. Tissues were snap-frozen in dry ice immediately after surgery and stored at −80 C.

Genetic analyses

DNA was isolated from frozen tissues by proteinase K digestion and phenol/chloroform extraction. Exons 2–10 of *MEN1* and adjacent splice junctions were amplified by PCR as 15 partially overlapping fragments (5, 13). Sequence analysis was performed on both DNA strands using an ABI PRISM 310 DNA sequencer and the ABI PRISM BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA).

Generation of a menin-specific monoclonal antibody

The portion of *MEN1* coding for the C-terminal 126 amino acids of menin (C126men) was PCR-amplified from human genomic DNA using primers M1 (5′-ccgggagtccaagccagaggag-3′) and M2 (5′-agttcagaggcctttgcgctgc-3′) (synthesized by Life Technologies, Glasgow, Scotland). The resulting product was cloned into pBluescript KS+ (Stratagene, La Jolla, CA) and then subcloned as a BamHI-EcoRI fragment into the pGEX-2T vector (Amersham Pharmacia Biotech, Uppsala, Sweden) "in frame" with the glutathione-S-transferase (GST) gene. GST-C126men fusion protein was expressed in *Escherichia coli* strain BL21 after induction with isopropyl β-D-thiogalactopyranoside. The bacteria were lysed in 50 mM Tris-HCl (pH 8.0), 25% sucrose, 1 mM EDTA, 0.5% Triton X-100, and 100 μg/ml lysozyme (Roche, Basel, Switzerland) followed by centrifugation at 17,500 × *g* at 4 C for 10 min. GST-C126men was purified from the resulting supernatant, by affinity chromatography, using glutathione-Sepharose (Amersham Pharmacia Biotech), as described elsewhere (14), followed by SDS-PAGE; pulverized gel bands containing the protein were used to immunize BALB/c mice. Serological reactivity was tested by dot blot using GST-C126men and by IF using a HeLa-derived cell line (HLtat, Ref. 15) transiently transfected with a eukaryotic expression plasmid coding for the C-terminal 125 amino acids of menin (pLs-C125men-AU). Splenocytes from 1 positive animal were fused to NSO mouse myeloma cells, selected in HAT medium and cloned by limiting dilution (16). Culture supernatants were screened for menin reactivity using dot blot, IF, and immunohistochemistry on paraffin-embedded sections of HLtat cells transfected with pLs-C125men-AU. Of over 500 clones, only 1 (*i.e.* mAb C126) proved to be positive in all these assays and was inoculated ip into severe combined immunodeficiency (SCID) mice to generate ascites. Procedures involving mice and their care were monitored by a University of Padova veterinarian and conformed to institutional guidelines that comply with Italian and international laws and policies.

Immunohistochemistry

HLtat cells were transiently transfected with the menin cDNA expression vector pCMVsport A11 (kindly provided by S. J. Marx and S. C. Chandrasekharappa). One day later, the cells were detached with trypsin, rinsed in PBS, paraffin-embedded, and analyzed by immunohistochemistry as described elsewhere (17). In brief, sections were subjected to three cycles of microwave pretreatment at 750 W for 5 min and then incubated with a 1:10 dilution of normal horse serum for 30 min at room temperature, and treated with the Vector Avidin/Biotin Blocking Kit (Vector Laboratories, Inc., Burlingame, CA). Sections were then incubated overnight at 4 C with mAb C126 (1:100) followed by 30-min incubations at room temperature with biotinylated horse antimouse antibody (1:300; Vector Laboratories, Inc.) and avidin-biotin-peroxidase complex (Vectastain Elite Kit; Vector Laboratories, Inc.). Peroxidase activity was detected using diaminobenzidine (1 mg/ml; Sigma, St. Louis, MO) and 0.01% H_2O_2. The cells were counterstained with hematoxylin, dehydrated, and mounted with Entellan (Merck, Darmstadt, Germany).

IF/LSM

Frozen tissues were cryosectioned into 5-μm sections, air-dried for 30 min, fixed in phosphate-buffered 4% paraformaldehyde for 5 min, rinsed twice in PBS, and then incubated for 4 min in 70% ethanol and for 5 min in 96% ethanol. The tissues were either analyzed immediately or stored in 96% ethanol at 4 C for no longer than 24 h, because prolonged storage in ethanol after paraformaldehyde treatment resulted in a substantial decrease in menin reactivity. IF was performed by incubating the tissues with normal goat serum (1:70) in PBS for 30 min at room temperature and then overnight at 4 C with a 1:80 dilution of mAb C126, followed by a 1:500 dilution of Alexa 488-conjugated goat antimouse secondary antibody (Molecular Probes, Eugene, OR). Dual labeling experiments were carried out by staining with 0.1 μg/ml propidium iodide or with rabbit antibodies specified in the figure legends, followed by secondary antibodies conjugated to either Texas red (Jackson Labora-

tories, Bar Harbor, ME) or Alexa 546 (Molecular Probes). LSM was carried out with a Zeiss LSM 510 microscope (Carl Zeiss, Jena, Germany) using Argon (488 nm) and Helium-Neon (543 nm) lasers with objectives and settings as specified in the figure legends. Laser intensity, pinhole aperture, and photomultiplier parameters were standardized to allow comparison of signals obtained in different samples. Fluorescence signals were analyzed using a 505- to 530-nm band-pass filter for Alexa 488 and a long-pass 560-nm filter for the propidium iodide, Texas red, and Alexa 546 signals. Quantitations were carried out with Zeiss's Profile software tool. After IF analysis, sections were subjected to standard hematoxylin-eosin staining, and fields matching IFs were analyzed by LSM using three lasers (Ar 488 nm, HeNe 543, HeNe 633) and visualized by red, blue, and green false-color overlay.

The specificity of the IF pattern obtained with mAb C126 was verified in a competition assay carried out by preadsorbing mAb C126 ascites with glutathione-Sepharose-attached GST or GST-C126men (approximately 5 μg), for 5 h at 4 C, before incubation with tissue sections.

Immunoblots

The ability of mAb C126 to recognize menin in immunoblots was tested as follows. Hltat cells were transfected with either pCMVsport A11 or a control plasmid (pBluescript KS+) and lysed by shearing five times through a 26-G needle in a solution containing 50 mM Tris (pH 6.8), 2% sodium dodecyl sulfate, 1% β-mercaptoethanol, 5% glycerol, 0.05% bromophenol blue, and a protease inhibitor cocktail (Complete; Roche). Resulting lysates were loaded in triplicate on a 10% T/2.6% C denaturing polyacrylamide gel along with prestained size markers (Cell Signaling Technology, Beverly, MA), electrophoresed, and electrotransferred to nitrocellulose. Membranes were cut into strips, blocked in 1% nonfat dried milk (Sigma), and incubated with either mAb C126 (diluted 1:1,000) or with commercially available antibodies raised against the N terminus or C terminus of menin (Santa Cruz Biotechnology, Santa Cruz, CA) followed by horseradish peroxidase-conjugated sheep antimouse (Amersham Pharmacia Biotech) or donkey antigoat (Santa Cruz Biotechnology) antibodies. The strips were developed using chemiluminescence reagents (Supersignal Pico; Pierce, Rockford, IL), realigned, and exposed to Hyperfilm MP (Amersham Pharmacia Biotech). For immunoblots of pancreatic tissue samples, frozen sections (25-μm thick) were solubilized as described above, and analyzed by SDS-PAGE/immunoblotting using a mouse antitubulin antibody (Sigma). After balancing the sample quantities based on the tubulin signal, SDS-PAGE/immunoblotting was repeated with mAb C126 as described above, with the exception that Supersignal femto was used as developer. All antigen-antibody reactions and washing steps were carried out in the presence of 0.05% Tween 20.

Results

Generation of a menin-specific monoclonal antibody

A 126-amino-acid C-terminal portion of menin was expressed as a GST-fusion protein, purified, and used as an immunogen to generate a monoclonal antibody (mAb C126, see *Materials and Methods*) recognizing menin *in situ*. As shown in Fig. 1, immunohistochemistry (A) and IF/LSM (B) analyses, carried out using mAb C126, yielded a strong nuclear signal in cells transfected with a menin expression plasmid. The nuclear localization of menin is more evident in B, which shows an overlay IF/LSM image obtained using mAb C126 (*green signal*) and an antibody against the nuclear export protein CRM-1, added to visualize the nucleus (*red signal*).

Pilot studies using mAb C126 and IF/LSM on frozen tissue sections revealed a strong signal in exocrine pancreatic cells (C). The specificity of this signal was assessed in control IF/LSM using: 1) an irrelevant primary antibody, which did not yield a detectable signal (data not shown); 2) a MEN1 duodenal gastrinoma (sample no. 15A) with a germ-line 11-bp deletion in exon 9 and an allelic loss of the *MEN1* gene, which scored negative with mAb C126 (sample no.15A, D); and 3) competition assays showing abrogation of the mAb C126 signal by GST-C126men but not by unfused GST recombinant protein (E and F, respectively).

As shown in Fig. 1G, mAb C126 recognized a band of approximately 68 kDa in immunoblots of cells transfected with the menin expression vector pCMVsport-A11 (Hltat, lanes +); this band, whose apparent size is consistent with that published for menin, was also detected by two commercially available antimenin antibodies raised against C-terminal and N-terminal portions of the protein. Immunoblots of lysates prepared from three normal pancreas samples and a sporadic insulinoma confirmed that mAb C126 recognized the 68-kDA menin band in all of the samples (Fig. 1G, pancreatic tissues).

In situ *detection of menin in nontumoral pancreas by IF/LSM*

Figure 2 shows results of IF/LSM analyses carried out on a frozen section of human nontumoral pancreas (sample no. 1). Menin was detected using mAb C126 and Alexa 488-conjugated antimouse antibody (*green signal*, A) and nuclei were identified by propidium iodide staining (*red signal*, B). The area shown includes exocrine cells and three islets (indicated by *arrows*), whose identification was facilitated by staining the section with hematoxylin/eosin after IF (D). Results showed strong antimenin reactivity in most exocrine cells (A); overlap of the menin and propidium iodide signals produced a yellow-green color in the overlay image (C), thus demonstrating nuclear accumulation of endogenous menin in human tissues, and confirming results obtained with ectopic expression systems (Fig. 1 and Ref. 6). Interestingly, in contrast to adjacent exocrine cells, the islets exhibited substantially lower levels of menin expression, yielding a *red-orange signal* in the overlay (C). An area spanning the islet-exocrine boundary (*white square* in C) was examined at higher magnification (E) and analyzed with the Profile software tool (F) to quantitate the menin and propidium iodide signals across several exocrine and islet cells (see *arrow* in E). Results of this analysis are plotted in a graph showing the relative intensities of the signals in the two channels (y-axis) against the distance, in micrometers, from the origin of the *arrow*. Results confirmed the overlap of the menin and propidium iodide peaks, and demonstrated that menin expression in islet cells was about 4- to 5-fold lower than in exocrine cells (*e.g.* compare two exocrine nuclei corresponding to the two peaks at about 5 and 30 μm, with islet cell nuclei at about 40, 55, 62, and 68 μm). Similar results were observed in the majority of the islets in all the other nontumoral tissues examined (samples no. 2, 3, PF14, PF26, PF28, PF30, and PF35; see *Materials and Methods*). In addition to exocrine/islet borders, we also analyzed exocrine areas more distant from islets and observed no major difference in the expression levels of menin, compared with exocrine cells adjacent to islets (data not shown).

Thorough examination of these samples revealed a subpopulation of neuroendocrine cells expressing menin at levels comparable with or higher than those in exocrine cells. An

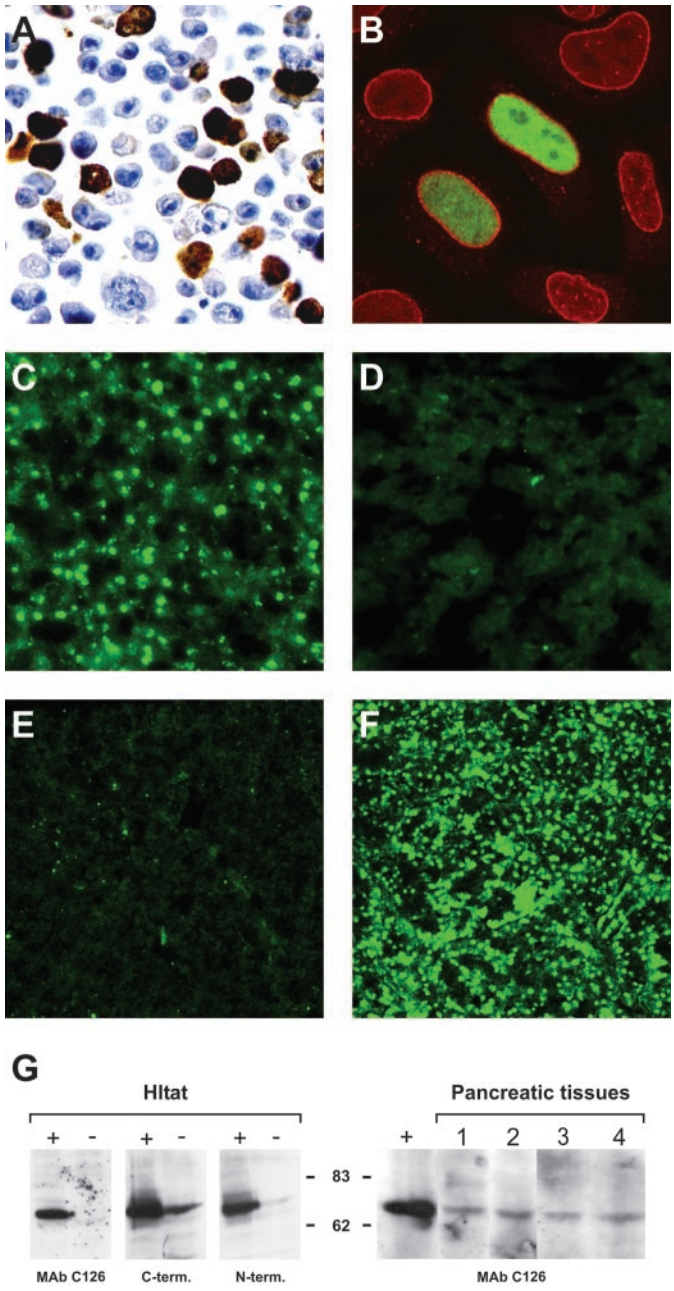

FIG. 1. Detection of menin using mAb C126. A and B, HLtat cells were transiently transfected with the menin cDNA expression vector pCMVsport A11. A shows IHC (×40 original magnification) carried out as detailed in *Materials and Methods*; transfected cells show an intense nuclear staining for menin. B shows a IF/LSM image (×63 original optical magnification/×2 scanning zoom) obtained using mAb C126 and a rabbit serum raised against the nuclear export protein CRM1 (1:300 dilution, kindly provided by Dr. M. Neumann) followed by Alexa 488-conjugated antimouse and Texas red-conjugated antirabbit secondary antibodies. Shown is an overlay image demonstrating menin staining (*green signal*) surrounded by the nuclear envelope labeled by CRM1 (*red signal*). C and D, Antimenin IF/LSM of normal exocrine pancreas (obtained from sample no. 10A) and a MEN1 gastrinoma (sample no. 15A), respectively, obtained using mAb C126 followed by Alexa 488-conjugated antimouse antibody (×20 original optical magnification, ×2 scanning zoom). The menin signal is readily detected in the normal exocrine pancreas (C), whereas the MEN1 gastrinoma shows very faint background staining, with no evidence of nuclear accumulation of the signal (D). E and F, IF/LSM on normal exocrine pancreas (sample no. 10A) carried out after preadsorbing MAb C126 with either GST-C126men or unfused GST, respectively (×20 original optical magnification). The specificity of the nuclear staining pattern of menin is demonstrated by competition with GST-C126men (E) but not by unfused GST (F). The *left-hand portion* of G shows an immunoblot analysis of menin in HLtat cells transfected with either carrier DNA (−) or with the menin expression vector pCMVsport-A11 (+). As described in *Materials and Methods*, identical immunoblot strips were incubated with mAb C126 and commercial polyclonal antibodies raised against C-terminal (C-term) or N-terminal (N-term) portions of menin. Lanes + show that mAb C126 and the C- and N-terminal antibodies recognize the 68-kDA menin protein produced from pCMVsport-A11. The *right-hand* portion of G shows a composite image of an immunoblot analysis of pancreatic tissues carried out using mAb C126. The following lysates were examined: lane +, HLtat cells transfected with pCMVsport-A11; lane 1, normal pancreas no. PF14; lane 2, normal pancreas no. PF30; lane 3, normal pancreas no. PF35; lane 4, sporadic insulinoma no. 21A. Results showed that mAb C126 recognizes a protein in pancreatic tissues that comigrates with the 68-kDa menin band produced from pCMVsport-A11. The positions of 83-kDa and 62-kDa size marker proteins are indicated between the two blots.

example of this observation is presented in Fig. 3, which shows dual labeling of sections of sample no. PF35 with mAb C126 and an antibody against synaptophysin, a marker for neuroendocrine cells, with strong menin reactivity detected in some synaptophysin-positive cells (*top panels*) and weak reactivity in others (*bottom panels*). As expected, synaptophysin was detected mainly in the cytoplasm and was excluded from nuclei.

To search for possible functional correlates for the different levels of menin expression in islet cells, we then carried out dual labelings to analyze menin expression in different neuroendocrine cell subpopulations producing insulin, glucagon, pancreatic polypeptide (PP), and somatostatin, using rabbit antibodies against these hormones. Results demonstrated low levels of menin expression in insulin-, somatostatin-, and glucagon-secreting cells (Fig. 4, *upper panels* labeled I, G, and SS, respectively). In contrast, high levels of menin expression were found in PP-positive cells. Interestingly, menin expression among PP-positive cells seemed to be heterogeneous, with higher-expressing cells more frequently located at the islet periphery (see *arrows* in PP panels E, F, and G). These observations were made for nontumoral samples no. 1, PF26, PF28, and PF35; other samples were not informative, because they did not score positive for all three hormones.

In situ *expression of menin in neuroendocrine pancreatic tumors*

Figure 5 shows IF/LSM analysis of a sporadic gastrinoma of the head of the pancreas (sample no. 10A), with menin (A) and propidium iodide (B) signals detected as described above. The field shown in A, B, C, and E spans an area that includes normal exocrine tissue with typical acinar structure (upper left-hand and center portions of field) and the tumor mass consisting of closely packed cells (*bottom-center* and *right-hand portions* of the field). An area spanning the boundary between exocrine and tumor cells (*white square* in C) was analyzed at a higher magnification (D), and menin *vs.* propidium iodide signals were quantitated across several exo-

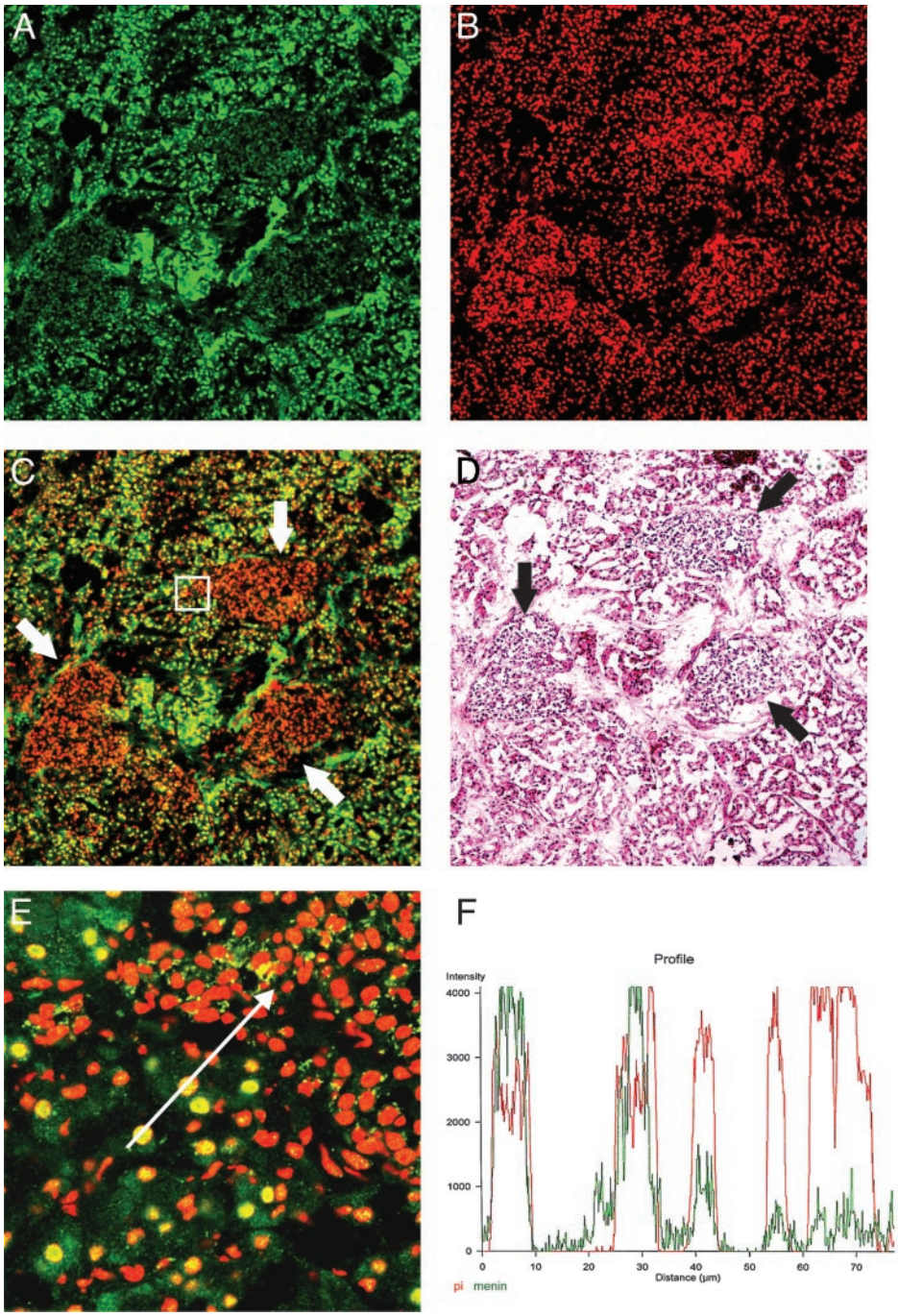

FIG. 2. IF/LSM analysis of menin expression in nontumoral human pancreas. IF/LSM of nontumoral pancreas (obtained from sample no. 1) was carried out using mAb C126 and Alexa 488-conjugated antimouse antibody to detect menin (*green signal*, A), and propidium iodide to identify nuclei (*red signal*, B); C shows the overlay image, with islets indicated by *arrows*, and D shows the same field after hematoxylin-eosin staining (×10 original magnification). Results show a colocalization of the menin and PI signals. An area spanning an islet-exocrine boundary (*white square* in C) was examined at higher magnification (×63 original magnification, E) and quantitated using the Zeiss Profile software tool to compare the intensities of the menin and propidium iodide signals along a line traced across several exocrine and islet cells (see *arrow*, E). Results are presented as a graph (F) showing the relative intensities of the signals in the menin (*green*) and PI (*red*) channels (y-axis) against the distance (in micrometers) from the origin of the *arrow*. Exocrine nuclei (first two peaks at 5 and 30 μm) exhibited a menin signal that was 4- to 5-fold higher than that in islet cells (next 4 peaks at about 40, 55, 62, and 68 μm).

crine and tumor cells (indicated by *arrow*) as described above. Results (F) showed the nuclei of two exocrine cells (first two peaks at about 10 and 35 μm) with a menin signal of relative intensity of 4,000. In contrast, nuclei of tumor cells (the series of peaks from 45–120 μm) showed a relative intensity of 1,000–2,000. Similar differences in menin expression between exocrine and tumor cells were observed for a sporadic insulinoma (sample no. 14B). Additional IF/LSM analyses of one gastrinoma (sample no. 12A), two insulinomas (samples no. 19B and 21A), and one somatostatinoma (sample no. 13A) revealed that menin was expressed at variable levels; however, the absence of normal exocrine cells in these tissue sections precluded a direct comparison of the relative levels of menin expression.

As described in *Materials and Methods*, in addition to *MEN1* −/− gastrinoma 15A, the status of the *MEN1* gene was verified for gastrinoma no. 12A and insulinomas no. 14B, 19B, and 21A. Results showed that sample no. 21A contained only the wild-type sequence. Likewise, no mutations were found in the *MEN1* sequence for sample no. 14B, which exhibited heterozygosity for the D418D polymorphism. Therefore, we did not obtain evidence suggesting allelic loss in samples no. 14B or 21A. In contrast, sample no. 19B exhibited the R171Q polymorphism in the absence of the wild-

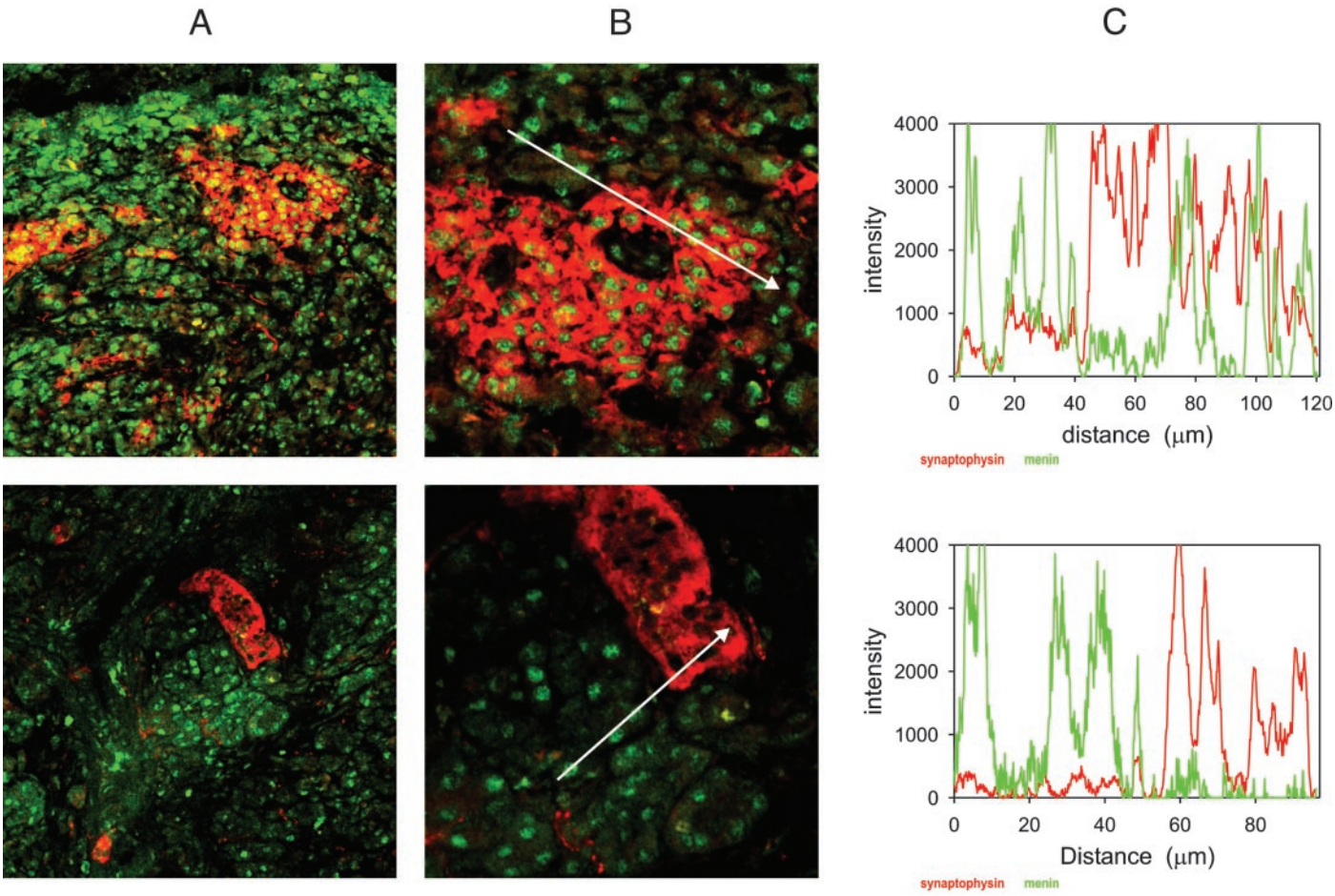

FIG. 3. Expression of menin in islet cells: dual menin/synaptophysin IF/LSM. Dual IF/LSM of nontumoral pancreas (obtained from sample no. PF35) was carried out using antimenin mAb C126 and rabbit antisynaptophysin antiserum (Dako, Glostrup, Denmark; 1:20 dilution) followed by Alexa 488-conjugated antimouse antibody (*green*, menin signal) and Alexa 546-conjugated antirabbit antibody (*red*, synaptophysin signal). Shown are overlay images of sample no. PF35 showing different levels of menin expression in insular cells in different areas of the sample [*compare upper* and *lower panels*, ×10 (A) and ×40 (B) original magnification]. C shows Profile quantitation of signals across exocrine cells (*left-hand portion of arrows*) and endocrine cells (*right-hand portion of arrows*), demonstrating heterogeneous menin expression in different islets.

type sequence, suggesting loss of one *MEN1* allele. Though patient no. 12A had a clinical and family history suggestive of MEN1, there was no germ-line mutation in the MEN1 coding region; genetic analysis of the tumor was not informative.

Discussion

At face value, our observation of relatively low levels of menin expression in the majority of islet cells is somewhat unexpected, given that pancreatic tumors in MEN1 patients arise from endocrine cells and the proposed role of menin as a tumor suppressor. However, these levels must be physiologically relevant in neuroendocrine cells, because complete loss of menin expression (*e.g.* in MEN1 sample no. 15A, shown in Fig. 1D) is associated with tumor formation. The observation that menin is differentially expressed in exocrine *vs.* neuroendocrine pancreatic cells extends previous *in situ* hybridization data published by Ikeo *et al.* (12), which suggested differences in expression between exocrine and islet cells at the RNA level.

Ikeo *et al.* (12) also provided evidence that the *MEN1* mRNA is more abundantly expressed in actively proliferating cells. It is thus conceivable that expression of menin in islet cells might be tightly regulated in response to proliferative stimuli. Our observation of high levels of menin expression in a subpopulation of pancreatic peptide-producing cells (Fig. 4) indeed suggests tight regulation of menin expression and a possible functional diversity within this cell population. The recent finding that overexpression of menin in rat insulinoma cells inhibits expression from the insulin promoter, diminishes activation of insulin production in response to glucose, and reduces proliferation (18) indicates that menin might also play a role in determining the secretion status of islet cells.

Our IF/LSM screening of endocrine pancreatic tumors (see Table 1; three gastrinomas, three insulinomas, and one somatostatinoma) revealed menin signals of varying intensities, except in *MEN1* −/− gastrinoma no. 15A, in which menin was not detected. Genetic analysis of sporadic insulinomas no. 14B, 19B, and 21A failed to detect biallelic loss of *MEN1*, which is in line with the presence of a detectable menin signal.

Although the present study was focused on a limited num-

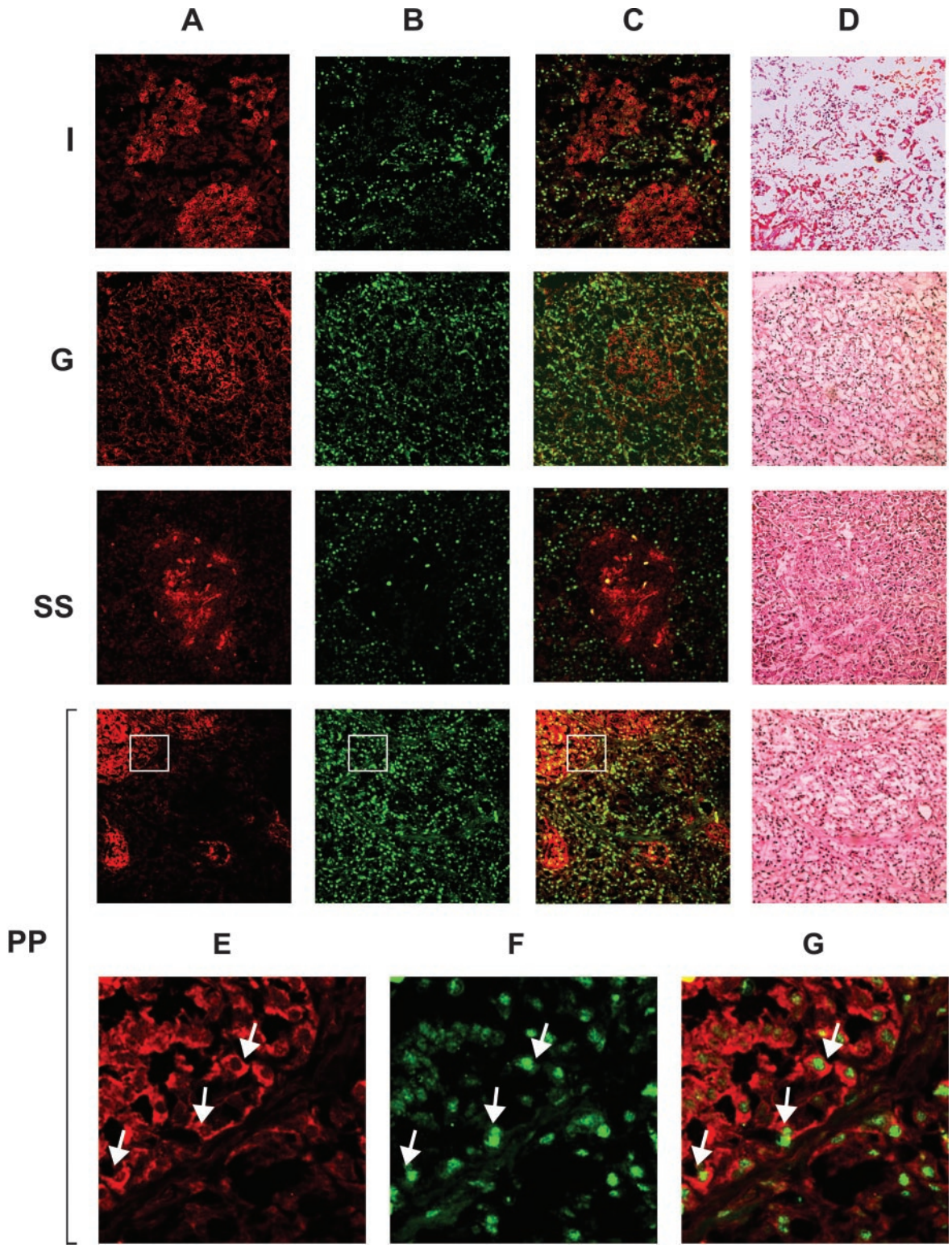

FIG. 4. Differential expression of menin in islet cell populations: dual IF/LSM using antibodies against menin and islet hormones. Dual staining analysis of nontumoral pancreas was carried out using antimenin mAb C126 and Alexa 488-conjugated antimouse antibody (*green signal*, B). The different islet cell populations were analyzed using rabbit antibodies (Dako) recognizing insulin (I, 1:50 dilution), glucagon (G, 1:100 dilution), somatostatin (S, 1:200 dilution), and PP (1:600 dilution), followed by Alexa 546-conjugated antirabbit antibodies (*red signal*, A). C shows overlay images, and D shows hematoxylin-eosin staining of the same fields (×10 original magnification). Menin is expressed at relatively higher levels in exocrine and PP-positive cells; other islet cell populations show considerably lower menin expression. PP panels E, F, and G show a ×63 magnification of the portion of the field indicated by the *white square*, including an islet (*upper left*)/exocrine (*lower right*) boundary. *Arrows* indicate cells with higher expression of menin within the PP-positive cell population.

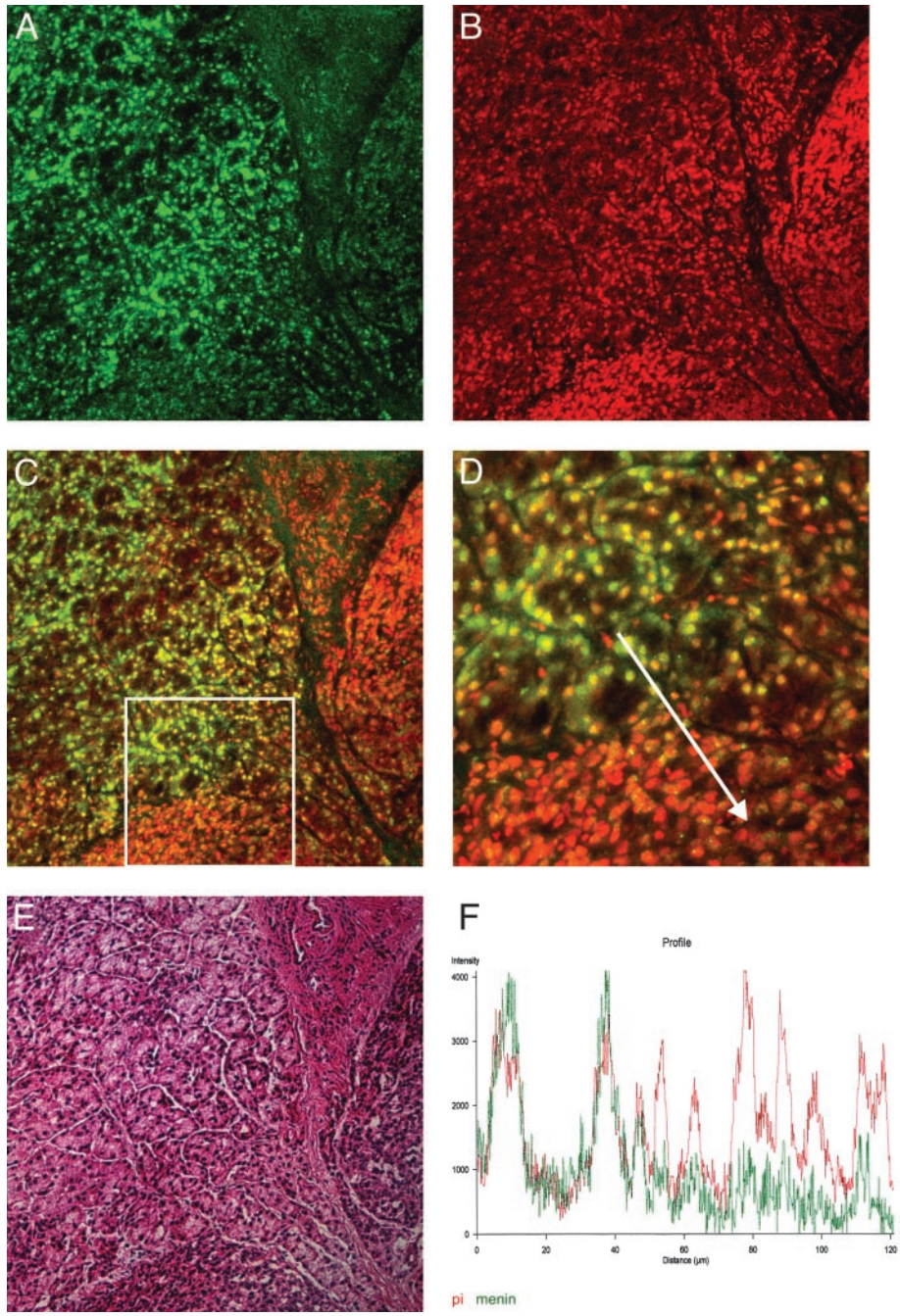

FIG. 5. IF/LSM of a sporadic gastrinoma of the head of the pancreas (sample no. 10A). The field shown in A (menin), B (propidium iodide), C (overlay), and E (hematoxylin-eosin) spans normal exocrine tissue with typical acinar structure (*upper left-hand and center portions* of field) and the tumor mass consisting of closely packed cells (*bottom-center and right-hand portions* of field). An area spanning the boundary between exocrine and tumor cells (*white square* in C) was analyzed at a higher magnification (×20 original optical magnification, ×2 scanning zoom; D), and menin *vs.* propidium iodide signals were quantitated across several exocrine and tumor cells (indicated by *arrow*) as described above. Results (F) showed the nuclei of two exocrine cells (first two peaks at about 10 and 35 μm) with a menin signal of relative intensity of 4,000. In contrast, nuclei of tumor cells (the series of peaks from 45–120 μm) showed a relative intensity of 1,000–2,000.

ber of test samples, these data suggest that, out of the context of the MEN1 syndrome, loss of menin is not strictly required for development of neuroendocrine tumors. These findings are in agreement with genetic analyses showing biallelic disruption of the *MEN1* locus in only a fraction of sporadic endocrine pancreatic tumors (19–21).

Menin is stable when expressed ectopically in tissue culture cells, with a half-life of about 10 h (22). However, our trials with mAb C126 suggest that the protein might be more susceptible to degradation in the context of primary tissues, with its detection depending on prompt freezing of the samples in dry ice, after surgery, and storage at −80 C. Furthermore, frozen sections must be subjected to IF promptly after drying-fixation, because storage of the fixed sections for more than 24 h results in substantial loss of the signal. Quantitative evaluation of menin expression with mAb C126 therefore requires careful, standardized processing of samples, which should include a component of normal exocrine cells as a positive standard within the same section.

In our hands, IF/LSM was a more rapid and robust technique than standard IHC for detecting menin with mAb C126. In addition to propidium iodide counterstaining and dual labelings with islet cell markers employed in the present study, IF/LSM would allow comparison of the levels of menin expression with those of its molecular partners [*e.g.* JunD (23), Smad3 (24), NF-κB (25), nm23 (26), and RPA-2 (27)]. Further-

TABLE 1. Summary of staining patterns of the tissues examined

Sample no.	Description	Menin
1	NTP	EX (+++); ISL (+)[a]
2	NTP	EX (+++); ISL (+)
3	NTP	EX (+++); ISL (+)
PF14	NTP	EX (+++); ISL (+)
PF26	NTP	EX (+++); ISL (+)
PF28	NTP	EX (+++); ISL (+)[a]
PF30	NTP	EX (+++); ISL (+)[a]
PF35	NTP	EX (+++); ISL (+)[a]
7B	NTP	EX (++)
10A	Sporadic gastrinoma	EX (+++); tumor (+)
12A	MEN1 gastrinoma[b]	tumor (+)[c]
13A	Sporadic somatostatinoma	tumor (+/++)[c]
14B	Sporadic insulinoma	EX (+++); tumor (+)
19B	Sporadic insulinoma	tumor (+)[c]
21A	Sporadic insulinoma	tumor (++)[c]
15A	MEN1 gastrinoma	tumor (−)

NTP, Nontumoral pancreas; EX, exocrine cells; ISL, islet cells.

[a] Menin +++ in PP-positive cells.

[b] Although clinical and family history were consistent with MEN1 syndrome, no germ line mutation was found in the coding portion of MEN1.

[c] No normal exocrine cells in sample.

more, the quantitative aspect of this approach provides a means of investigating possible correlations between menin expression and the biological/clinical properties of different tumors. Our findings thus provide the first description of the *in situ* pattern of expression of menin in human pancreas and provide a basis for further studies aimed at defining the pattern of menin expression in other tissues.

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