

Silencing of Secretin Receptor Function by Dimerization with a Misspliced Variant Secretin Receptor in Ductal Pancreatic Adenocarcinoma¹

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ABSTRACT

Secretin receptors that are key for regulation of healthy pancreatic ductal epithelial cells have been reported to be functionally absent on ductal pancreatic adenocarcinomas. Here, we examine the possible presence and function of molecular forms of the secretin receptor in pancreatic cancer cell lines and in primary tumors. Surprisingly, reverse transcription-PCR and sequencing demonstrated wild-type secretin receptor mRNA in each of four cell lines and three primary tumors. Lack of biological response to nanomolar concentrations of secretin was best explained by the demonstrated coexpression of a second and predominant transcript in each of the cell lines and tumors. This represented a variant of the secretin receptor in which the third exon was spliced out to eliminate residues 44–79 from the NH₂-terminal tail. This spliceoform has only recently been recognized in a rare gastrinoma, where it was incapable of binding secretin or signaling, and possessed dominant-negative activity to suppress hormone action at the wild-type secretin receptor (1). Overexpression of wild-type secretin receptor in Panc-1 cells driven by transfection of fully processed cDNA resulted in normal responsiveness to low concentrations of secretin, establishing the ability of these cells to produce a receptor capable of normal biosynthesis, trafficking, and signaling. Bioluminescence resonance energy transfer demonstrated that the variant receptor could form a heterodimer with wild-type receptor, providing a molecular mechanism for its dominant-negative activity. This suggests that missplicing is responsible for expression of a secretin receptor variant having the ability to suppress the function of wild-type receptor by a direct interaction. In 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays in receptor-bearing Chinese hamster ovary cells, the secretin receptor was shown to have growth-inhibitory effects. Suppression of this activity in pancreatic carcinoma might, therefore, facilitate tumor growth and progression of this aggressive neoplasm.

INTRODUCTION

Ductal pancreatic adenocarcinoma is one of the most lethal tumors, representing the fourteenth most frequent type of cancer, yet representing the fourth most common cause of cancer death in the United States. The molecular basis of the aggressive behavior of this tumor is not well understood. Among the candidates to contribute to this are hormone-receptor systems capable of regulating the growth of these cells. In this work, we have focused on secretin-responsive receptors that have been described as affecting pancreatic cancer cell growth (2–6). Indeed, secretin was shown previously to be an effective cocarcinogen for the development of pancreatic carcinoma in an animal model (7).

However, elucidation of the effects of this hormone on normal pancreas and pancreatic neoplasms have been complicated by the existence of two distinct class B G-protein-coupled receptors that are responsive to secretin, yet may have divergent and offsetting biological effects. Pancreatic ductal epithelial cells express both the secretin

receptor and a receptor for VIP³ (VPAC₁ receptor; Ref. 8). Secretin represents a potent agonist of the secretin receptor, being responsive to nanomolar concentrations of hormone, whereas it is a weak agonist of the VPAC₁ receptor, requiring 100-fold higher concentrations of hormone (8). The VPAC₁ receptor has been described as mediating stimulation of pancreatic cancer cell growth (6), whereas there is a preliminary report for the secretin receptor to inhibit such growth (5). This is true although both receptors are coupled to heterotrimeric Gs and stimulate adenylate cyclase. Of particular interest, ductal pancreatic adenocarcinomas have been characterized as only expressing a functional VPAC₁ receptor (3, 6, 9). This could theoretically shift the biological response to this hormone from balanced stimulation and inhibition of growth to net stimulation of growth and could represent an important determinant for tumor behavior. The molecular forms of the secretin receptor in pancreatic carcinoma have not been characterized previously.

In this work, we have for the first time directly demonstrated the presence of secretin receptors on pancreatic cancer cell lines and on tumors and surrounding normal pancreas from patients with pancreatic carcinoma. Tumor cell expression was unexpected, given the observations of absence of biological responses in these tumors and cell lines to nanomolar concentrations of secretin (3, 6, 9). The molecular explanation for this became apparent when, in addition to finding mRNA encoding wild-type secretin receptor, we identified mRNA encoding a variant spliceoform of the secretin receptor on the pancreatic cancer cells that had been demonstrated previously only in the setting of a rare presentation of a gastrinoma with a false-negative provocative test (1). In that work, this variant spliceoform was shown to have no ability to bind secretin or to signal in response to secretin and to possess dominant-negative activity to suppress the function of secretin at coexpressed wild-type secretin receptor (1).

Here, in addition to demonstrating that this missplicing event is relevant to other tumors, we have extended these insights to gain an understanding of the molecular mechanism of this dominant-negative effect. BRET studies showed that this variant spliceoform could establish heterodimers with the wild-type secretin receptor on the cell surface, where it could inhibit secretin binding and biological activity. We further demonstrated that secretin action at the secretin receptor could inhibit cell growth. The missplicing of the secretin receptor mRNA in pancreatic carcinoma may thus provide a novel mechanism for the reduction or elimination of a potentially important growth-inhibitory influence on these tumors.

MATERIALS AND METHODS

Materials. TRIzol, SuperScript II, and Lipofectin were from Life Technologies, Inc. PCR II vector was from Invitrogen. QuickChange site-directed mutagenesis kit was from Stratagene. Anti-HA monoclonal antibody 12CA5 was from Roche Molecular Biochemicals. GFP and YFP constructs were from Clontech.

Human pancreatic cancer cell lines were acquired from the American Type

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³ The abbreviations used are: VIP, vasoactive intestinal polypeptide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; BRET, bioluminescence resonance energy transfer; HA, hemagglutinin; GFP, green fluorescent protein; YFP, yellow fluorescent protein; CHO, Chinese hamster ovary; Rlu, *Renilla* luciferase; cAMP, cyclic AMP; RT-PCR, reverse transcription-PCR.

Culture Collection. All clinical biospecimens were collected, stored, and used with the approval of the Mayo Clinic Institutional Review Board. Secretin receptor-bearing and VPAC₁ receptor-bearing Chinese hamster ovary cell lines have been established and characterized in our laboratory previously (10).

Cell Culture. Human pancreatic cancer cell lines Panc-1 (11), MIA PaCa-2 (12), Su.86.86 (13), and Capan-2 (14) were used in this study. Panc-1, MIA PaCa-2, and Su.86.86 cells were cultured in DMEM with 3.7 mg/ml sodium bicarbonate, 10% FCS, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Capan-2 cells were cultured in RPMI 1640 supplemented with 10% FCS, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Cells were grown in tissue culture plasticware at 37°C in a humidified environment containing 5% CO₂. They were passaged approximately two times/week.

Receptor Constructs. The cDNA constructs of wild-type, variant spliceoform, and HA-tagged secretin receptor constructs in eukaryotic expression vectors have been established and characterized previously (1). Rat cholecystokinin type A and human secretin receptor constructs incorporating Rlu or YFP at the COOH-terminal end of the mature protein were generated as described previously (15). A GFP-fused, wild-type secretin receptor cDNA construct was also constructed using two steps. In the first step, an AgeI restriction enzyme digestion site was introduced into the human secretin receptor cDNA at the position of 1431 bp (GenBank accession number, U28281), and the stop codon TGA was replaced with CGA, using the Quick-Change site-directed mutagenesis kit. This was achieved using the following primers: sense, 5'-CTG CAG GAC CAG CAT CAT CCG ACC GGT GGAGCA GGG TCA CCC ATG-3'; and antisense, 5'-CAT GGG TGA CCC TGC TCC ACC GGT CGG ATG ATG CTG GTC CTG CAG-3'. In the second step, the secretin receptor cDNA was cloned into the GFP-N1 vector at EcoRI/AgeI sites. The identity of all constructs was verified by direct DNA sequencing.

Transfections were performed using Lipofectin for Panc-1 cells and using DEAE-dextran (1) for COS cells. For coexpression experiments in COS cells, 2 μ g of each DNA construct were used. These included pairs of constructs encoding GFP-tagged wild-type secretin receptor with HA-tagged variant secretin receptor spliceoform or receptor constructs fused at their COOH terminus with Rlu and YFP.

cAMP Assay. cAMP responses to agonist stimulation of intact cells were studied using a competition-binding assay protocol described previously (Ref. 16; Diagnostic Products Corp., Los Angeles, CA). Some of these assays were performed on cells that were transiently expressing receptor constructs. For these assays, cells were studied 48 h after transfection. Cells were stimulated with varied concentrations of secretin, VIP, or forskolin at 37°C for 30 min in the presence of 1 mM 3-isobutyl-1-methylxanthine in Krebs-Ringer-HEPES (KRH) buffer [25 mM HEPES (pH 7.4), 104 mM NaCl, 5 mM KCl, 1 mM KH₂PO₄, 1.2 mM MgSO₄, 2 mM CaCl₂, 1 mM phenylmethylsulfonyl fluoride, 0.01% soybean trypsin inhibitor, and 0.2% BSA]. The reaction was interrupted by adding ice-cold perchloric acid. After adjusting the pH to 6 with KHCO₃, cell lysates were cleared by incubation at 4°C for 20 min, and the supernatants were used in the assay. Radioactivity was quantified by scintillation counting in a Beckman LS6000. The results were expressed as percentages of maximal cAMP responses.

Reverse Transcription-PCR. Total RNA was prepared from the pancreatic cancer cell lines and primary tumors and surrounding normal pancreas using TRIzol reagent. Reverse transcription was carried out at 42°C for 40 min in 20 μ l of a mixture containing 5 μ g of RNA, 200 units of SuperScript II, 40 units of RNase inhibitor, 0.5 mM of each deoxynucleotide triphosphate, and 200 ng of random oligonucleotide primers. Reactions were stopped at 70°C for 15 min. Expression of wild-type and variant forms of secretin receptor mRNA were determined using PCR (sense primer, representing 107–127 bp of human secretin receptor, GenBank accession number U28281, 5'-CCA TGC GTC CCC ACC TGT CGC-3'; antisense primer, representing 560–538 bp of the same sequence, 5'-CTG TAG CCC ACG GTG TAC ATG AC-3'). The reaction contained 2 μ l of cDNA, 0.2 μ M sense and antisense primers, 0.4 μ M deoxynucleotide triphosphate mix, 1.5 mM Mg²⁺, and 2.5 units of Taq polymerase in a 50- μ l volume. The cDNA was denatured at 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 52°C for 2 min, and 72°C for 3 min. Elongation was performed at 72°C for 12 min.

Complete Sequence Determinations for Secretin Receptor mRNA Constructs. Full-length sequences were cloned and sequenced from Panc-1 cells to be certain of their identities. For this process, a cDNA library was prepared,

starting with RNA isolated from membrane-bound polysomes (17). In brief, confluent Panc-1 cells in 175-mm flasks were harvested in PBS buffer. Cells were then pelleted and homogenized with 7-ml, tight-fitting Dounce glass homogenizer, and the nuclei were removed by centrifugation. Free ribosomes and rough endoplasmic reticulum (membrane-bound polysomes) were separated by centrifugation of the cellular extracts in a discontinuous sucrose density gradient. RNA was extracted from the membrane-bound fraction with phenol-chloroform, precipitated with isopropanol, and washed with ethanol. Poly(A)⁺ RNA was isolated using PolyATtract mRNA Isolation System IV (Promega), following the standard protocol. Five μ g of isolated mRNA were subjected to cDNA library construction with the ZAP Express cDNA Gigapack II kit (Stratagene). The resulting cDNA library had a titer of 2×10^{-9} pfu/ml, and sequencing of 46 randomly picked colonies established that this was markedly enriched for membrane proteins and secreted products and had an average insert size of 1250 bp.

PCR was used to identify full-length cDNAs encoding the secretin receptor. Three μ l of each of four independent aliquots of the cDNA library were amplified using primers covering the full coding region of the secretin receptor cDNA [sense primer (107–127 bp), 5'-CCA TGC GTC CCC ACC TGT CGC-3'; antisense primer (1434–1413 bp), 5'-CTC TCA GAT GAT GCT GGT CCT G-3']. PCR conditions were the same as described above, except that the annealing temperature was 58°C. Products of the PCR reactions were separated on 1% agarose gels, and relevant bands were excised, subcloned into PCR II vector (Invitrogen), and fully sequenced.

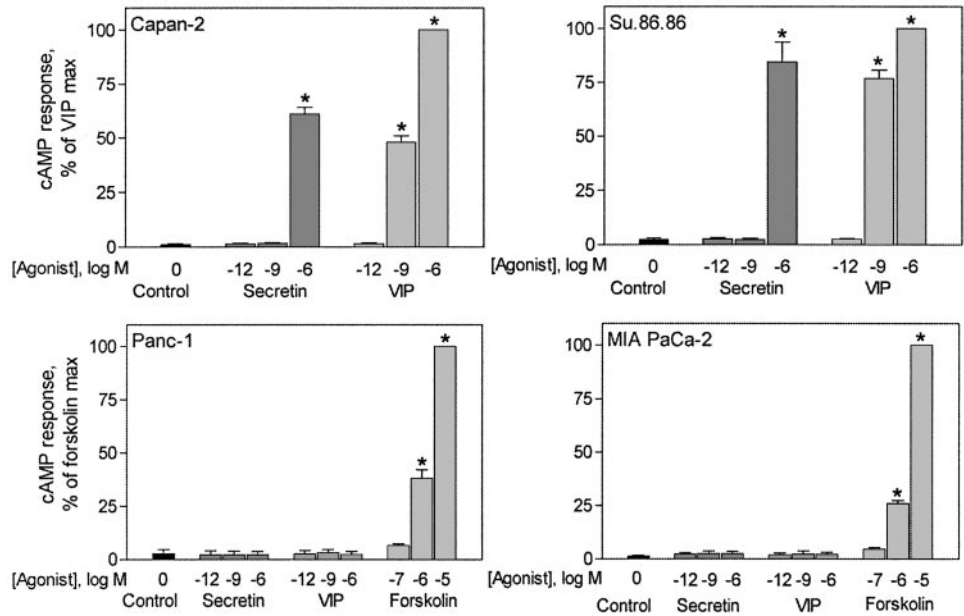
Immunohistochemistry and Confocal Microscopic Examination. Four μ g of cDNA constructs encoding HA epitope-tagged wild-type or variant secretin receptor spliceoform were transfected into Panc-1 cells in 24-well culture plates. Seventy-two h after transfection, cells were cooled for 20 min at 4°C in DMEM with supplements. Anti-HA epitope monoclonal antibody 12CA5 (1:500) was added, and the incubation was continued for another 60 min at 4°C. Cells were then washed three times with PBS at 4°C, fixed with 2% paraformaldehyde in PBS at room temperature for 30 min, and thoroughly washed again with PBS. After fixation and washing, cells were incubated at room temperature in blocking buffer (10% normal goat serum in PBS) for 30 min and for 30 min in goat antimouse IgG-rhodamine (1:200) diluted with blocking buffer. After washing with PBS, cells were mounted on slides with Vectashield mounting medium, sealed, and examined with a Zeiss 510 confocal microscope. A well-characterized fluorescent secretin analogue was also used for a morphological receptor binding assay in Panc-1 cells (18).

BRET Analysis. The BRET assay was performed as we have described (15). In brief, 2 μ g of each cDNA construct of respective receptors fused to Rlu or YFP were cotransfected into COS cells. Forty-eight h after transfection, cells were detached mechanically, washed, and resuspended in KRH medium. Agonist stimulation was carried out at 37°C for 5 min. Subsequently, approximately one million transfected COS cells were transferred into a 1-ml cuvette, and the cell permeant substrate of Rlu, coelenterazine (Packard Bioscience), was added at a final concentration of 5 μ M. The bioluminescence emission was immediately monitored in the region of the spectrum between 400 and 600 nm using a SPEX Fluorolog spectrofluorometer (SPEX Industries, Edison, NJ). The BRET ratio was defined as [(emission at 510–590) – (emission at 440–500) \times Cf]/(emission at 440–500), where Cf represents (emission at 510–590)/(emission at 440–500) for the wild-type secretin receptor fused to Rlu (15).

Cell Growth Assays. Cell growth was assayed using the CellTiter 96 Aqueous ONE Solution Cell Proliferation Assay from Promega (Madison, WI). Approximately 6000 CHO cells expressing secretin receptor (10) or VPAC₁ receptor (10) were plated in each well of a 96-well tissue culture plate with 100 μ l of Ham's F-12 medium containing 5% fetal clone-2. After 24 h of growth, the medium was replaced with Ham's F-12 medium containing 0.5 or 5% fetal clone-2, and cells were stimulated with 1 μ M VIP or secretin. Fresh peptide was added every 24 h for 72 h. MTT assay procedures recommended by the manufacturer were used, and data were represented as percentages of values detected in cells cultured under the same conditions in the absence of peptide.

Statistical Analysis. All observations were repeated at least three times in independent experiments and were expressed as mean \pm SE. Differences between groups of data were assessed using the *t* test for unpaired values or the one-way ANOVA, using *P* < 0.05 as the level of statistical significance.

Fig. 1. Effects of secretin on intracellular cAMP levels in pancreatic cancer cell lines. Pancreatic cancer cell lines were stimulated with secretin and VIP for 30 min at 37°C. If there was no significant response to these secretagogues, cells were also stimulated with forskolin. Cellular cAMP levels are expressed as percentages of maximal levels achieved and represent means of duplicate determinations from three independent experiments; bars, SE. *, $P < 0.001$ relative to control values.



RESULTS

cAMP Levels in Pancreatic Cancer Cells. To examine whether functional secretin receptors were expressed on pancreatic cancer cells, four different human pancreatic cancer cell lines were subjected to secretin stimulation (Fig. 1). One nM secretin failed to stimulate an increase in cellular cAMP content in any of these cell lines. In contrast, 1 μ M secretin (1000-fold higher concentration that can stimulate the VPAC₁ receptor) stimulation elicited an increase in cAMP level in Capan-2 and Su86.86 cells but not in the other two cell lines studied. The cell lines responsive to high concentrations of secretin were also responsive to 1 nM VIP, supporting the likely mediation of these effects by VPAC₁ receptors (8). This is consistent with previous reports (6, 9).

Because both VIP and secretin were unable to stimulate cAMP responses in Panc-1 and MIA PaCa-2 cells, we tested whether the adenylate cyclase system was functional in those cells. This was achieved by stimulating them with forskolin to directly activate adenylate cyclase. As shown in Fig. 1, forskolin stimulated cAMP accumulation in each of these cell lines in a concentration-dependent manner with potency similar to that reported in other model systems (19, 20). This verifies the functional adenylate cyclase-cAMP signaling in these two cell lines. These results suggest that the defect explaining the failure of secretin to stimulate cAMP accumulation in

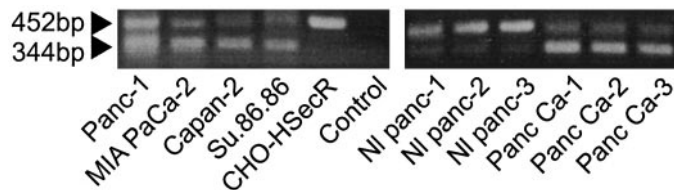


Fig. 2. RT-PCR evidence of secretin receptor expression in pancreatic cancer. Total RNA was isolated from four pancreatic cancer cell lines, the human secretin receptor-expressing CHO-HSecR cell line, and from tumors (*Panc Ca*) and surrounding normal pancreas (*NI panc*) in three patients with ductular pancreatic carcinoma. The negative control included no cellular RNA. Samples were analyzed by RT-PCR, as described in "Materials and Methods." Products were separated on 1% agarose gels, yielding two distinct cDNA bands in each of the pancreatic cancer samples, with only the larger of these observed in the wild-type control, and with no bands in the control lane. Images are representative of three similar experiments.

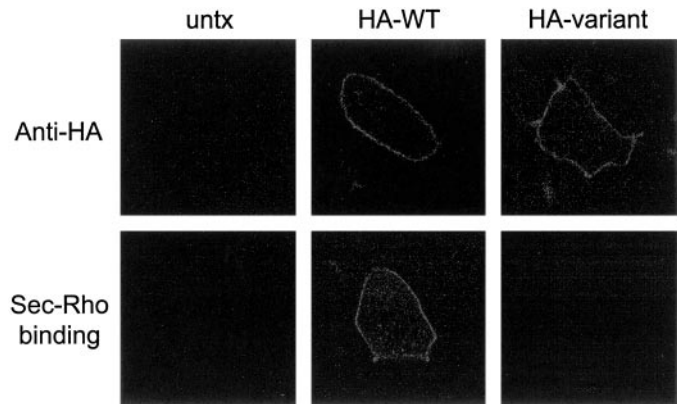


Fig. 3. Expression of wild-type and variant secretin receptors in Panc-1 cells. HA-tagged wild-type and variant secretin receptor constructs were transfected into Panc-1 cells using lipofectin. Seventy-two h after transfection, the cells were immunostained with anti-HA antibody and examined by confocal microscopy (*top row images*). Surface staining was observed for each of the transfected cells but not in controls. Aliquots of the same cells were also incubated with a well-characterized fluorescent secretin analogue (50 nM) at 4°C for 1 h, washed with PBS, fixed, and examined by confocal microscopy (*bottom row images*). Ligand binding was observed only in cells expressing wild-type secretin receptor.

pancreatic cancer cells is proximal to this enzyme, at the level of the receptor or its G-protein coupling.

Expression and Cloning of Secretin Receptors. RT-PCR was performed using specific primers to examine whether the secretin receptor is expressed in pancreatic carcinoma. A secretin receptor-expressing CHO cell line (21) was used as a positive control for RT-PCR. The oligonucleotide primers used in this assay spanned the only splice variant described to date for the secretin receptor (1). As described below, we also established the full-length sequences of the products amplified in this assay. Two distinct forms of secretin receptor transcripts were detected in all pancreatic cancer cell lines and in primary tumors from three patients with pancreatic carcinoma (Fig. 2). The shorter transcript was the predominant form expressed in the cancer cells and tissues. In contrast, tumor-free pancreas from the same patients expressed only the longer secretin receptor transcript. Direct DNA sequencing established that the 452-bp transcript represents wild-type secretin receptor, whereas the predominant shorter

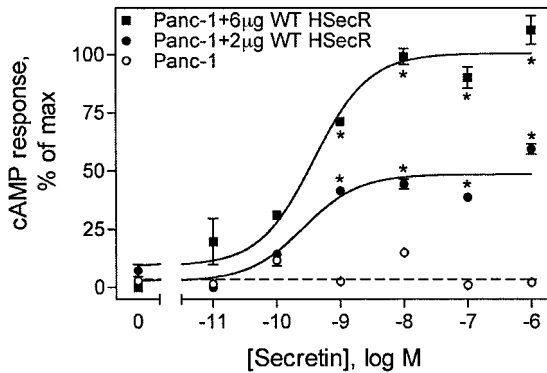


Fig. 4. Effects of secretin on cAMP in Panc-1 cells transfected with wild-type secretin receptor cDNA. Noted amounts of the wild-type secretin receptor cDNA construct were transfected into Panc-1 cells using lipofectin. Cells were subsequently stimulated with various concentrations of secretin for 30 min at 37°C. Cellular cAMP levels (means of duplicate determinations from three independent experiments; bars, SE) are expressed as percentages of maximal responses obtained from the cells transfected with 6 µg of cDNA. Conditions significantly different from basal levels are noted. *, $P < 0.001$.

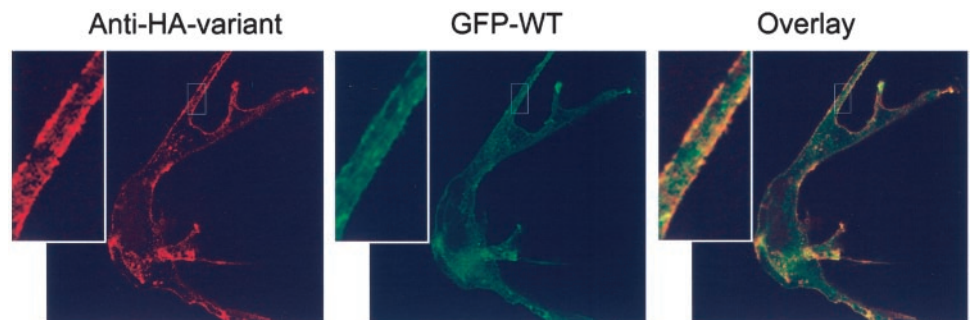
transcript (344-bp fragment) comes from a variant spliceform of the receptor. We observed this spliceform [HsecR(Δ 301–408)] previously in a gastrinoma, where it was shown to act as a dominant-negative inhibitor of wild-type secretin receptor (1).

To be certain that both wild-type and variant spliceform of the secretin receptor were present as full-length transcripts in pancreatic cancer cells, we screened a cDNA library that was constructed from Panc-1 cells using a well-established protocol to enrich for cDNAs encoding membrane proteins (17). Indeed, full-length variant spliceform and wild-type secretin receptor cDNAs were successfully amplified and sequenced from four independent aliquots of this library. No other receptor variants were identified in this very sensitive screening procedure.

To test whether these constructs could undergo normal biosynthesis and trafficking to the cell surface in pancreatic carcinoma cells, constructs encoding wild-type and variant spliceform of the secretin receptor were tagged with HA epitope and expressed in Panc-1 cells. Immunolocalization using confocal microscopy confirmed that both receptor forms were expressed on the cell surface (Fig. 3). However, only the overexpressed wild-type secretin receptor was able to bind ligand, as demonstrated morphologically by localization of a fluorescent secretin analogue (Ref. 18; Fig. 3). This is consistent with our previous observations of expression of these constructs in COS cells (1).

Transfection of Panc-1 cells with the human secretin receptor cDNA construct (2 or 6 µg per 100-mm dish) established responsiveness to even low concentrations of secretin (1 nM), as demonstrated by the increase in cAMP after stimulation (3–4-fold increase above basal levels at maximal response; Fig. 4). In Panc-1 cells transfected with variant spliceform cDNA, no cAMP response was detected in response to secretin concentrations as high as 1 µM (data not shown).

Fig. 5. Colocalization of wild-type and variant secretin receptors on cell surfaces. COS cells were cotransfected with HA-tagged variant secretin receptor and GFP-fused wild-type secretin receptor constructs (2 µg of each). The cells were fixed 72 h after transfection, at which time they were examined by confocal microscopy. In the left panel is shown a typical cell immunostained with anti-HA antibody. In the middle panel, the GFP fluorescence from the same cell is shown. In the right panel is shown the composite of both fluorophores. Also shown are enlarged views of this cell in each panel.



Colocalization and Dimerization of Wild-Type and Variant Secretin Receptors. We have reported previously the dominant-negative effects of this variant spliceform of the secretin receptor on wild-type secretin receptor function (1). However, the molecular basis for this negative effect was unknown. In the current work, we have studied the possible interactions of wild-type and variant secretin receptors in COS cells, where the endogenous secretin receptor was absent. GFP-fused wild-type secretin receptor and HA-tagged variant spliceform of the secretin receptor were expressed in these cells. Cotransfection of these two constructs resulted in coexpression of the two forms of the secretin receptor on the cell surface with similar expression efficiency (Fig. 5). This indicates that these two receptors are colocalized and may directly interact with each other on the cell membrane, thereby inhibiting secretin receptor function.

In our laboratory, we have recently established BRET techniques to study protein-protein interactions of cell surface receptors (15). This technique was used in the present study to understand whether the variant receptor actually forms a complex with the wild-type receptor on the cell surface. Each of the tagged receptor constructs used for these studies was also directly demonstrated to express the binding and signaling properties of the analogous untagged receptor (data not shown). Fig. 6 shows clear BRET signals that were detected in the coexpression system (BRET ratio 0.15 for HsecR-Rlu:HsecR-YFP, 0.12 for HsecR-Rlu:HsecR(Δ 301–408)-YFP, and 0.09 for HsecR(Δ 301–408)-Rlu:HsecR(Δ 301–408)-YFP). No BRET signal was detected for the cells expressing the Rlu-tagged wild-type or variant secretin receptor alone. As an additional control, we also showed that in cells coexpressing Rlu-tagged secretin receptor and YFP-fused to the structurally unrelated cholecystokinin receptor, the BRET signal was extremely low. This is the range of signal that we reported previously for coexpression of a Rlu- or YFP-tagged soluble protein with the opposite tag present on a membrane receptor (15).

The data from the BRET experiments thus indicate that secretin receptors do form homodimers and can form heterodimers with the variant spliceform of the secretin receptor. This secretin receptor variant can also form homodimers with itself, although the level of apparent association may be less than that of the wild-type receptor. This may indicate a role of the NH₂-terminal domain in the dimerization of secretin receptors on the cell surface. Of note, agonist occupation did not affect the status of secretin receptor dimerization in any of these conditions (Fig. 6). This is consistent with phenomena that have been described among some other G protein-coupled receptors (22–24).

Growth Effects of Secretin-responsive Receptors. Existing literature clearly supports a growth-stimulatory effect of VIP acting through the VPAC₁ receptor (6) but is only suggestive of a growth-inhibitory effect of secretin acting through the secretin receptor, based on a single abstract published 5 years ago (5). We, therefore, directly examined the growth effects of these receptors expressed in the identical cellular environment of receptor-bearing CHO cell lines

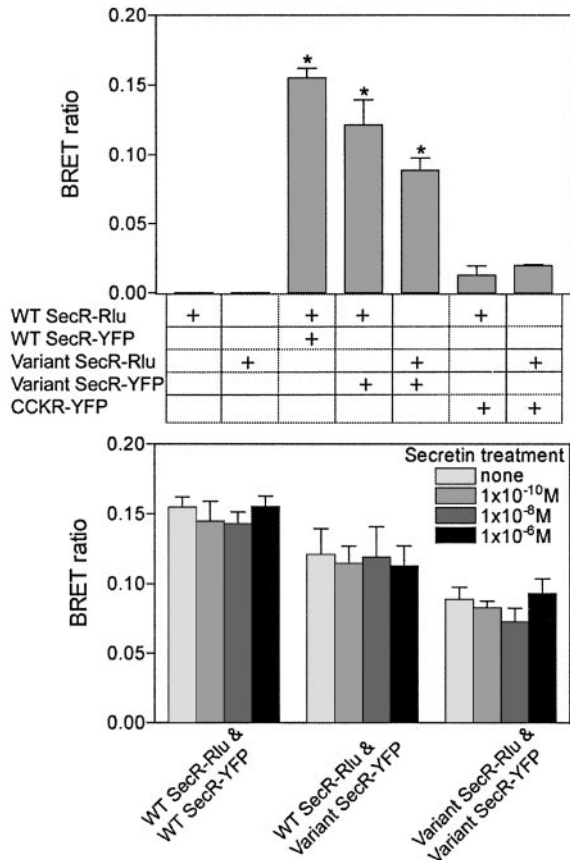
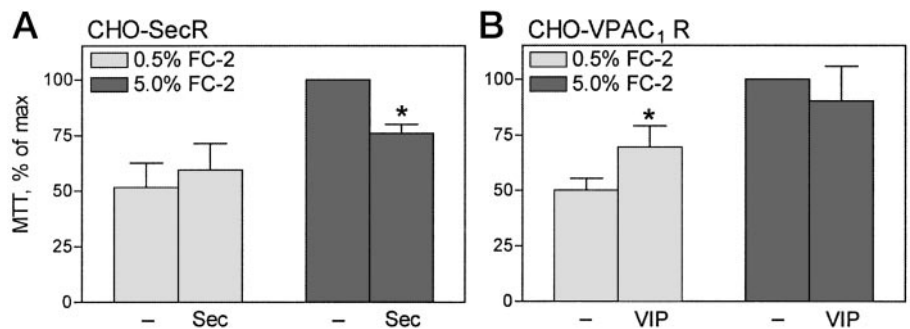


Fig. 6. BRET assays. The top panel illustrates quantitative BRET data from a series of experiments with COS cells expressing the different combinations of cDNA constructs illustrated in the chart below. Values represent means of data from three independent experiments; bars, SE. *, $P < 0.001$ relative to the background levels in each of the controls. The bottom panel illustrates the effects of secretin stimulation on the receptor BRET signals. COS cells expressing the noted combinations of cDNA constructs were incubated with various concentrations of secretin for 5 min at 37°C. BRET ratios were determined. Values represent means of data from three independent experiments (no condition was statistically different from its own unstimulated control); bars, SE.

(Fig. 7). Treatment of the secretin receptor-bearing cells with 1 μM secretin for 72 h reduced the MTT level by 24% in cells that had been cultured in 5% fetal clone-2-containing medium but had no effects on cell growth when cultured in 0.5% fetal clone 2-containing medium. This suggests that secretin inhibits growth factor-stimulated cell proliferation. On the other hand, VIP treatment enhanced the MTT level in cells that had been cultured in 0.5% fetal clone 2-containing medium by 39% but had no effects on cell growth when cultured in 5% fetal clone 2-containing medium. This indicates that VIP has a net stimulatory effect on the growth of these cells, confirming the growth effects of this receptor that were reported previously (6).

Fig. 7. Cell growth assays. Secretin receptor-bearing or VPAC₁ receptor-bearing CHO cells were stimulated with 1 μM secretin or VIP for 72 h in the presence of 0.5 or 5% fetal clone 2 in Ham's F-12 medium. Cell proliferation was assayed using the Promega CellTiter 96 Aqueous ONE Solution Cell Proliferation Assay. MTT level was expressed as the percentage of the level detected in cells cultured under identical conditions in the absence of added peptide (means; bars, SE; $n = 6-9$). A, the effects of secretin on MTT level in secretin receptor-bearing CHO cells. B, effects of VIP on MTT levels in VPAC₁ receptor-bearing CHO cells. *, $P < 0.05$ compared with control cells for each series of experiments. Sec, secretin.



DISCUSSION

Secretin is a classical regulator of the exocrine pancreas, acting via the class B G protein-coupled secretin receptor to stimulate pancreatic ductular cells to secrete bicarbonate and water (25). Among structurally related receptors, the VPAC₁ receptor is also known to bind and to be stimulated by secretin, although it is at least 100-fold less sensitive to this hormone than the secretin receptor (8). Both the secretin receptor and the VPAC₁ receptor are believed to signal similarly, mediated by coupling with G_s, leading to the activation of adenylate cyclase.

However, these receptors may have opposite effects on the growth of ductular pancreatic cells (3, 5, 6). The VPAC₁ receptor stimulates the growth of these cells, whereas a preliminary report suggests that the secretin receptor inhibits growth. Perhaps consistent with the latter is an older reference for reduced rate of DNA synthesis early after treatment of Syrian golden hamsters with secretin (26). The presence of both of these receptors having offsetting effects may normally provide balanced impact on cell growth. However, in pancreatic cancer, the VPAC₁ receptor continues to be expressed and to be quite active, whereas the typical secretin receptor function has not been identified (3, 6, 9). This could result in growth stimulation that is not offset by the possible normal inhibitory action of the secretin receptor. Such a net effect to stimulate growth could have profound importance in the progression and aggressive behavior of this neoplasm.

We now confirm these growth effects for secretin-responsive receptors. VIP acting through the VPAC₁ receptor stimulated growth, whereas secretin acting through the secretin receptor inhibited cell growth. This further supports the theoretical importance of the functional loss of secretin action in pancreatic carcinoma cells. However, evidence for the absence of the secretin receptor in pancreatic cancer cells has been limited to cell physiological studies. These have demonstrated the requirement for high concentrations of secretin to stimulate cellular adenylate cyclase (3, 6, 9). Such a concentration-response is most consistent with mediation by the VPAC₁ receptor that is often present on these cells. Indeed, we now confirm that none of the four pancreatic cancer cell lines that were studied had any cAMP response to 1 nM secretin, whereas two of four lines responded to 1 μM secretin. Cell lines responsive to high concentrations of secretin were also responsive to low concentrations of VIP.

However, wild-type secretin receptor mRNA was present in each pancreatic cancer cell line and primary ductal pancreatic adenocarcinoma studied. Normal full-length wild-type secretin receptor mRNA was present in a cDNA library prepared from Panc-1 cells, although this represented one of the cell lines in which there was no cAMP stimulation in response to 1 μM secretin. Perhaps adding to this mystery was the demonstration that when this cell line was transfected with a wild-type secretin receptor construct, it became responsive to 1 nM secretin. This ensured the ability of this cell to normally produce,

process, and deliver this receptor to the cell surface, where it could normally couple with its G protein and stimulate adenylate cyclase.

The best current explanation for the unresponsiveness of the secretin receptor in these tumors relates to the misspliced form of the receptor mRNA now identified. This variant spliceoform has only been found previously in the rare clinical setting of gastrinoma with a false-negative secretin provocative test (1). This spliceoform was shown to possess no intrinsic ability to bind secretin or to signal in response to secretin, while possessing dominant-negative activity to suppress the action of secretin on the wild-type secretin receptor in model cell systems (1). However, this has not yet been directly demonstrated in pancreatic carcinoma at levels of endogenous receptor expression. The effect was structurally specific, with secretin receptor variant not inhibiting the function of coexpressed VPAC₁ receptor (1). The dominant-negative action was also shown to be concentration dependent, related to the stoichiometric ratio of variant: wild-type receptor (1). Indeed, in the present report, the variant spliceoform of the secretin receptor mRNA predominated over wild-type secretin receptor mRNA in each pancreatic cancer cell line. This also explains why the shift in stoichiometric expression effected by transfection of these cells with wild-type receptor resulted in the onset of responsiveness to nanomolar concentrations of secretin.

Receptors often are down-regulated in cultured cells. Of note, secretin receptor function is rapidly lost when pancreatic ductal epithelial cells are cultured *in vitro* (27). It is, therefore, quite important that each of the primary ductal pancreatic adenocarcinomas studied in this report also expressed the same variant spliceoform of the secretin receptor mRNA observed in the pancreatic cell lines. Additionally, this variant was present in similar stoichiometric excess over the wild-type secretin receptor mRNA in the primary tumors as was observed in the cell lines. This was true despite finding that the surrounding normal pancreatic tissue in these patients expressed only wild-type secretin receptor mRNA. It is interesting that the G protein-coupled type B cholecystokinin/gastrin receptor is also often misspliced in pancreatic cancer (28).

The high frequency of this specific secretin receptor missplicing event in the cell lines and primary tumors studied in this work suggests that this may represent a fundamental abnormality in pancreatic cancer. The presence of the same splicing abnormality in another tumor [the gastrinoma previously studied (1)] may suggest that this may even be more fundamental to the processes of carcinogenesis. Indeed, mRNA splicing abnormalities and abnormalities in splicing factor activity have been described in several tumors (29–32).

Although missplicing events can go unnoticed if there is no important phenotypic impact, the missplicing of the secretin receptor described in this report has the potential to be quite important. This relates to its ability to inhibit the action of what may represent a normal growth inhibitor, the wild-type secretin receptor (5). This alternative splicing event results in the splicing out of the third exon, encoding residues 44–79 of the mature secretin receptor (1). This in-frame deletion disrupts the NH₂-terminal domain of the secretin receptor, known to be extremely important for secretin binding and biological activity (33–37). Indeed, in the first report of this spliceoform, it was demonstrated that this construct could be delivered to the surface of a COS cell, where it was not able to bind secretin or to have secretin stimulation, and led to an increase in cAMP (1). In the current work, we show that this is also true when expressed on the surface of a pancreatic cancer cell line.

The molecular mechanism for the dominant-negative effect of the secretin receptor spliceoform has not been established previously. There has been recent interest in the possible dimerization or oligomerization of G protein-coupled receptors (22–24). Resonance energy transfer studies have demonstrated that this can occur in living

cells for a small number of receptors in this superfamily. Here, we have used BRET to demonstrate that the wild-type secretin receptor can associate with itself on the cell surface. Additionally, the variant spliceoform of the secretin receptor was also shown to be capable of association with itself on the cell surface. Of interest, agonist binding had no effect on the BRET signal from these receptors. This is consistent with a subset of the other G protein-coupled receptors that have been studied similarly, where oligomerization has been shown to increase (38, 39), decrease (15, 40), or remain unchanged (41, 42) in response to agonist stimulation.

It is noteworthy that BRET studies also showed that this variant spliceoform could associate on the cell surface with the wild-type secretin receptor, providing a potential molecular mechanism for its dominant-negative activity. Although the precise molecular mechanism remains undefined, this appears to represent a direct molecular interaction that can occur independent of the highly characteristic disulfide-bonded NH₂-terminal domain (43). This also provides a molecular mechanism for silencing a normal receptor in a setting in which a mutation would only be expected to affect a single allele and likely have no apparent functional impact. Thus, receptor missplicing to create a dominant-negative isoform has potentially great significance.

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