Cloning and Expression of Secretagogin, a Novel Neuroendocrine- and Pancreatic Islet of Langerhans-specific Ca\(^{2+}\)-binding Protein*

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We have cloned a novel pancreatic beta cell and neuroendocrine cell-specific calcium-binding protein termed secretagogin. The cDNA obtained by immunoscreening a human pancreatic cDNA library using the recently described murine monoclonal antibody D24 contains an open reading frame of 828 base pairs. This codes for a cytoplasmic protein with six putative EF finger hand calcium-binding motifs. The gene could be localized to chromosome 6 by alignment with GenBank genomic sequence data. Northern blot analysis demonstrated abundant expression of this protein in the pancreas and to a lesser extent in the thyroid, adrenal medulla, and cortex. In addition it was expressed in scant quantity in the gastrointestinal tract (stomach, small intestine, and colon). Thyroid tissue expression of secretagogin was restricted to C-cells. Using a sandwich capture enzyme-linked immunosorbenent assay with a detection limit of 6.5 pg/ml, considerable amounts of constitutively secreted protein could be measured in tissue culture supernatants of stably transfected RIN-5F and dog insulinoma (INS-H1) cell clones; however, in stably transfected Jurkat cells, the protein was only secreted upon CD3 stimulation. Functional analysis of transfected cell lines expressing secretagogin revealed an influence on calcium flux and cell proliferation. In RIN-5F cells, the antiproliferative effect is possibly due to secretagogin-triggered down-regulation of substance P transcription.

Ca\(^{2+}\)-binding proteins represent a diverse family of proteins expressed ubiquitously in mammalian tissues (1). Several subfamilies are found in different tissue types. The tissue distribution of individual Ca\(^{2+}\)-binding proteins is often specific; the anatomic localization of calbindin D-28K is restricted to various loci within the nervous system (2, 3), and the closely related calretinin is localized to central and peripheral neurons (4, 5). The biological role of these cytoplasmic Ca\(^{2+}\)-binding proteins is poorly understood (6). These proteins share a unique tandem repeat of a Ca\(^{2+}\)-binding loop flanked by two a helices known as the “EF-hand” Ca\(^{2+}\)-binding site (1). Calbindin D-28k (7) and calretinin (8) possess six such EF-hand Ca\(^{2+}\)-binding sites, which vary somewhat in Ca\(^{2+}\) binding affinity (9). In humans, calbindin D-28k and calretinin have been localized to chromosomes 8 and 16, respectively (10). The genomic structures of both proteins, which are highly evolutionarily conserved, have been elucidated and comprise 11 exons interrupted by 10 introns (11–13).

Ca\(^{2+}\) is known to play a central role in cell activation. A variety of cellular functions is triggered by cell surface receptor-ligand interactions followed by Ca\(^{2+}\) mobilization from Ca\(^{2+}\) storage pools within the endoplasmic reticulum. Cytosolic free Ca\(^{2+}\) binds to cytosolic molecules, as exemplified by calmodulin (14), which undergoes a conformational alteration in response to Ca\(^{2+}\) chelation (15) and then interacts with its specific target (16, 17).

Pancreatic islet beta cells deliver insulin packaged in granules in a Ca\(^{2+}\)-dependent manner (18–21) in response to elevated glucose concentration. An intracellular Ca\(^{2+}\) flux is followed by insulin release in response to elevated glucose (22–25), and intracellular Ca\(^{2+}\)-binding proteins are thought to play a central role in this process (26–30). Using a polyclonal antiserum to calbindin, this protein has been localized within the chicken pancreatic islet of Langerhans (31). When stable transfectants of RIN-5F insulinoma cells were generated with neuronal D-28k calbindin, a higher rate of insulin transcription and insulin secretion could be measured (32). Furthermore, granins (granular packaging proteins) interact with adaptin in immature granules in a Ca\(^{2+}\)-dependent fashion and thus calcium-binding proteins might play a more general role in secretory granule formation (33).

We have generated a murine monoclonal antibody (mAb) D24 against a human insulinoma (34), which binds to virtually all cells present in the pancreatic islets of Langerhans and to all insulinomas tested. In addition, mAb D24 stains tumors originating from the gastrointestinal neuroendocrine system including vipomas, gastrinomas, and carcinoid tumors. In contrast, this mAb does not stain the exocrine pancreatic tissue (34). Using this mAb we have screened a human pancreatic beta cell cDNA library and found three plaques reacting specifically with this antibody. Sequencing of all three gene inserts has revealed a unique mRNA sequence encoding a new Ca\(^{2+}\)-binding protein related to calbindin D-28k and calretinin. In

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The nucleotide sequence(s) reported in this paper has been submitted to GenBank™/EBI Data Bank with the accession number(s) Y16752.

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1 The abbreviations used are: mAb, monoclonal antibody; GST, glutathione S-transferase; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; bp, base pair(s); ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; RT, reverse transcription; RIA, radioimmunoassay; PAP, peroxidase anti-peroxidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
this study we also demonstrate that this protein is rather specifically expressed in the pancreas (islet of Langerhans) and to a very minor degree in the small intestine, colon, stomach, and (only very faintly) in thyroid and adrenal gland, organs known to contain neuroendocrine elements. In addition, we demonstrate certain functional properties of secretagogin including its influence on KCl-stimulated calcium flux in transfected RIN-5F cell clones, influence on gene expression in RIN-5F cell clones, and an inhibitory influence on the growth rate of Jurkat and RIN-5F transfectants.

**EXPERIMENTAL PROCEDURES**

The human pancreatic beta cell line was generously provided by Dr. M. Alan Permutt (Islet Transplantation Center, Washington University, St. Louis, MO).

**Cloning of Human Secretagogin**

A murine mAb (D24) was generated by using a human insulinoma as immunogen and selected by immunohistochemical staining of post-mortem human pancreatic tissue (34). This mAb was then used to immunoscreen the pancreatic beta cell library. Approximately 10^6 Agt11 phage were plated and incubated until the plaques were nearly confluent. The lawn was then treated with isopropyl-1-thio-β-D-galactopyranoside-soaked nitrocellulose (Hybond-C extra nitrocellulose membrane; Amersham Pharmacia Biotech, Amersham, United Kingdom) for 3.5 h. The filters were blocked with 10% skim milk, stained with a 1:3000 dilution of a 1:2000 dilution of D24, and finally washed, loaded horseradish peroxidase complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Three specifically recognized plaques were identified and plaque-purified. The inserts were PCR-amplified using a set of Agt11 flanking primers (4 min of denaturation at 94 °C, 30 cycles at 94 °C denaturation for 60 s, annealing at 55 °C for 60 s, and extension at 72 °C for 60 s). The PCR product was subcloned into pCRII vector (Invitrogen, San Diego, CA) and sequenced by an automated sequencing system. All three plaques contained the same insert. The resultant sequence was compared with the GenBank database. All three plaques contained the same insert. The resultant sequence was compared with the GenBank database.

**RNA Analysis**

Multiple human tissue Northern blots were obtained from CLONTECH. Hybridization was performed using the 32P-labeled cDNA insert according to the manufacturer’s instructions. Blots were washed and exposed to x-ray film at −70 °C for 12 h.

**Production of Recombinant Protein**

The entire coding region of the human secretagogin gene was amplified by PCR using primers containing a BamHI site at the 5′-terminus and an EcoRI site at the 3′-terminus. Amplified cDNA was subsequently cut with EcoRI and BamHI and cloned into the pGEX-1T (Amersham Pharmacia Biotech) expression vector, which confers glutathione S-transferase (GST) and a thrombin cleavage site to the N-terminal of the inserted protein. The plasmid containing the PCR-generated insert was sequenced by an automated sequencing system to confirm the desired sequence of the fusion protein coding sequence. Recombinant Escherichia coli BL21 containing pGEX-1T with the desired protein gene was induced with isopropyl-1-thio-b-D-galactopyranoside (100 μM final concentration) for 2 h. The bacteria were pelleted, suspended in ice-cold PBS containing 0.1 mM phenylmethylsulfonyl fluoride, and sonicated until the cell suspension became translucent. After pelleting insoluble material, glutathione-Sepharose 4B slurry was added to the supernatant and mixed at room temperature for 5 min. The glutathione-Sepharose 4B was then pelleted and washed three times with PBS. Thrombin was added to the washed, loaded glutathione-Sepharose. The suspension was rotated at room temperature for 3 h, centrifuged, and the supernatant containing the cleaved secretagogin protein was harvested and frozen for further use.

**Expression of the Protein in Eukaryotic Cells**

The secretagogin cDNA coding sequence was cloned into the BamHI/EcoRI site of the mammalian expression vector pCDNA3 (+), which confers resistance to the antibiotic Zeocin. The ligated plasmid was used to transform E. coli INVaF to obtain adequate plasmid DNA for transfection. The plasmid was sequenced through the region of the cloning site to confirm its primary structure.

**Transient Expression in COS Cells**

COS cells were transiently transfected using a Lipofectin PerFect™ transfection kit (Invitrogen). DNA (10 μg) and Lipofectin (12 μl) were diluted in a combined volume of 2 ml and added to a monolayer of nearly confluent COS cells. The cells were incubated for 4 h with the transfection solution, which was then replaced with complete medium. After 24 h, the cells were washed in protein-free RPMI and incubated briefly with trypsin (0.25%) and EDTA (0.03%), and washed nonadherent cells were replated. At 72 h, the transfected cells were harvested by vigorous pipetting and loaded onto glass slides by cytocentrifuge for immunostaining.

**Stable Transfection**

**Jurkat Cells**—Logarithmically growing Jurkat cells were pelleted and resuspended in an electroporation cuvette (Gene Pulser Cuvettes; Bio-Rad) containing 800 μl of transfection buffer (21 mM Hepes, pH 7.05, 137 mM NaCl, 0.7 mM Na₂HPO₄, 5 mM KCl, 6 mM glucose) and the engineered vector pZeoSV2 or a sham-engineered control vector. Cells were transfected using the Gene Pulser (Bio-Rad) (280V, 960 μF) and then incubated for an additional 10 min at room temperature. The transfected cells were diluted and plated in 2 ml cultures and selected with Zeocin (500 μg/ml) after 24 h.

**RIN-5F Cells**—RIN-5F cells were obtained from ATCC (CRL-2058) and cultured in RPMI 1640 (10 mM glucose) supplemented with 10% (v/v) fetal calf serum, 2 ml L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin at 37 °C, and 5% CO₂. RIN-5F is a secondary clone of the rat islet tumor cell line RIN-m (ATCC CRL-2057). RIN-5F cells (1.5 × 10⁶) were seeded into 25-cm² tissue culture flasks and fed every 3 days. Cells were subcultured every 5–6 days using trypsin (0.25%) and EDTA (0.03%) in Hanks’ buffered saline solution without Ca²⁺ and Mg²⁺.

The insulinoma cell line INS-H1 was established from a dog pancreatic insulinoma (35) and cultured as indicated above for the rat insulinoma cell line RIN-5F.

**RIN-5F** and INS-H1 cells were transfected using a Lipofectin PerFect™ transfection kit (Invitrogen). DNA (10 μg) and Lipofectin (12 μl) were diluted to a combined volume of 2 ml and added to a monolayer of nearly confluent cells. The cells were incubated for 4 h with the transfection solution, which was then replaced with complete medium. After a 30-h incubation, selection was started with 170 μg/ml Zeocin. After 14 days of selection, colonies were hand-picked under microscopic observation using sterile conditions and propagated in 24-well dishes. Highly expressing clones were identified by performing a secretagogin ELISA of the RIN-5F cell supernatant as well as cell lysate and by immunocytochemistry of the cells using mAb D24.

**Insulin Secretion of RIN-5F**

RIN-5F cells (3 × 10⁶ cells/well) were seeded into 12-well cluster culture plates; after a 48-h period for cell adherence, the supernatant was replaced by 1 ml of fresh prewarmed culture medium with and without 20 mM KCl. After a 60-min incubation at 37 °C, 5% CO₂ in a 98% humidified incubator, the supernatant was harvested and centrifuged at 1000 × g for 5 min to pellet cells and cell fragments, and the resultant supernatant was then stored in Minisorb tubes at −20 °C for insulin and secretagogin analysis. Similar experiments were carried out over a 10-h period in 25-cm² tissue culture flasks containing 2.5 × 10⁶ cells and 5 ml of culture medium. All experiments were carried out in duplicate or triplicate samples.

**Immunohistochemistry**

Briefly, air-dried, acetone-fixed cytosin slides (see below) were sequentially incubated with mAb D24, rabbit anti-mouse immunoglobulin, alkaline phosphatase murine anti-alkaline phosphatase complex, and finally with Fast Red/naphthol ASBi phosphate as the chromogenic substrate. In the PAP method, horseradish peroxidase murine anti-peroxidase (PAP) was used for detection of the antigen. The cell layer was then counterstained in hematoxylin and a coverslip was applied using Glycergel as mountant (Dako, Glostrup, Denmark).

**Cytosin Preparation and Immunofluorescence Analysis**

Aliquots of approximately 2 × 10⁶ cells were loaded by cytocentrifuge onto glass slides. Air-dried (2 h), acetone-fixed (5 min) cytospin slides were blocked with 3% bovine serum in PBS and then incubated with mAb D24 (tissue culture supernatant) and rabbit polyclonal anti-insu-
lin IgG (Cappel 11187; reactive with human, pig, and rat insulin) (final concentration 1:150) for 60 min. After two washes in PBS for 10 min, slides were incubated for 60 min in fluorescein-conjugated donkey anti-rabbit IgG and rhodamine-conjugated donkey anti-mouse IgG (both diluted 1:2000; Jackson ImmunoResearch). Control slides containing non-transfected RIN-5F cells were incubated with non-immune rabbit serum, mAb D24, and secondary antibodies as above. After staining, slides were washed in PBS and a drop of 1% n-propyl gallate (Sigma) was used to reduce photobleaching. All specimens were examined by confocal imaging using a Leica DM IRBE microscope and the Leica TCS 4D confocal system.

**Cell Fractionation**

Gentle homogenization conditions were chosen to limit possible breakage of endosomes and other granular compartments such as neuroendocrine secretory granules. A 75% confluent cell layer was washed with PBS twice and then scraped into 2.5 ml of PBS containing protease inhibitors (10 μM l-aminopropion, 1 μM pepstatin, 10 μg/ml leupeptin, and 0.8 mM Pefabloc). The scraped cell suspension was then centrifuged at 1000 rpm at 4 °C. The cell pellet was resuspended in homogenization buffer (250 mM sucrose, 3 mM imidazole, pH 7.4, containing protease inhibitors as above). The cells were centrifuged again at 2500 rpm for 10 min at 4 °C. Cells were then resuspended in homogenization buffer as above containing 1 mM EDTA and doubled concentration of the protease inhibitors. The cell suspension was then drawn through a 22-gauge needle attached to a 1-ml syringe five times, then centrifuged at 3000 rpm for 10 min at 4 °C to pellet unbroken cells and nuclei. The post-nuclear supernatant was then centrifuged for 30 min at 55,000 rpm at 4 °C in a table-top Beckman centrifuge using the TL-100 rotor. The supernatant was collected as cytosol, and the pellet was resuspended in homogenization buffer. The protein content was determined in each fraction with a protein assay reagent (Pierce).

**Immunoblotting**

Endogenous and transfected secretagogin were analyzed by loading aliquots of each cell suspension or cell fraction derived from RIN-5F cells and transfected derivatives onto a 12% SDS-PAGE gel and electrophoretically transferring the protein to nitrocellulose. The blot was blocked with 10% skim milk and then incubated with rabbit polyclonal anti-secretagogin serum, followed by peroxidase-conjugated goat anti-rabbit immunoglobulin, and further developed by BM chemiluminescent reagent (Roche Molecular Biochemicals, Vienna, Austria). To detect recombinant secretagogin, aliquots of whole cell extract of transformed E. coli BL21, and purified protein cleaved by thrombin from loaded glutathione-Sephacel were loaded onto 10% SDS-PAGE gels, run under reducing conditions, and transferred to nitrocellulose. Filters were incubated in mAb D24 supernatant and developed as described above (see immunoelectrophoresing).

**Analysis of Tumor mRNA**

Fresh-frozen tissue samples stored in liquid nitrogen obtained from patients with pheochromocytoma (n = 1), adrenal adenoma (n = 1), carcinoid tumor (n = 4), neuroendocrine carcinoma (n = 2), insulinoma (n = 1), gastrinoma (n = 2), pancreatic tissue and Graves’ disease (n = 1) were homogenized and mRNA was extracted according to the method of Chomczynski and Sacchi (36) using RNAzol (Cinna Biotech, Houston, TX).

**Reverse Transcription-PCR**

First-strand cDNA was synthesized using Advantage™ RT-for-PCR kit (CLONTECH, Palo Alto, CA). cDNA (10 μl) was used as PCR template, and thermal cycling parameters were set to 30 cycles 94 °C (60 s) for denaturation, 55 °C (60 s) for annealing and 72 °C (60 s) for synthesis using gene-specific primers. The products were separated on a 1% agarose gel, and positive samples were cloned into pCRII vector (Invitrogen) and sequenced by an automated sequencing system.

**Northern Blotting**

Total RNA was isolated from homogenized tissue or in vitro grown cells with or without using RNAzol (Cinna Biotech). RNA was size-fractionated on a 1.2% agarose gel containing 0.7% formaldehyde, transferred to Hybond-N membranes (Amersham Pharmacia Biotech) using 10× SSC, and hybridized with a 32PdCTP Klenow-labeled random primed probe and the GAPDH probe as a control for RNA loading. RNA was fixed to the membrane by UV cross-linking (Stratalinker, Stratagene, La Jolla, CA). The membrane was hybridized and washed under conditions of high stringency (50% formamide, 10% dextran sulfate, 5× SSC, 1× Denhardt’s solution, 1% SDS, 100 μg/ml denatured herring sperm DNA, and 20 μg Tris at 42 °C). In some experiments, the 28 S ribosomal RNA oligonucleotide probe was used as a control for RNA loading (CLONTECH). The probe was labeled by the kinase reaction, and hybridization was conducted at 41 °C in 900 mMol/L NaCl, 500 mMol/L tris HCl, and 12 mMol/L EDTA overnight, followed by five washing steps (20 min each) at room temperature with 2× SSC, 0.1% SDS (w/v).

**Generation of Rabbit Polyclonal Antiserum**

Recombinant secretagogin was prepared by expressing a secretagogin-GST fusion protein in E. coli. The recombinant secretagogin was purified by glutathione-Sepharose affinity chromatography and thrombin cleavage to yield a single species of 32 kDa by SDS-PAGE. Purified recombinant secretagogin (540 μg) was emulsified in complete Freund’s adjuvant and injected subcutaneously into a 5-month-old male rabbit. Additional immunizations with 260 μg were administered on days 44 and 70 in incomplete Freund’s adjuvant. Serum collected 1 month after the third immunization contained high titer antibody activity against the recombinant protein when tested by ELISA.

**Secretagogin ELISA**

Aliquots of D24 culture supernatant (100 μl) were incubated for 16 h at 4 °C in flat-bottomed 96-well Reacti-Bind™ goat anti-mouse coated plates (Pierce). After a brief wash with PBS, 100 μl of biological fluid was added and incubated for 2 h at room temperature under constant shaking. Serial dilutions of human recombinant secretagogin were included as standards in each experiment. The plate was incubated with PBS containing 0.1% Tween 20 in an ELISA wash station (Dynatech, Denkendorf, Germany). Rabbit anti-human secretagogin antisemirum (dilution 1:300) was then incubated for 1.5 h under constant shaking with a Behring shaker (Behring AG, Marburg, Germany) at room temperature. After four washes, the plate was incubated with polyclonal horse-radish peroxidase-conjugated goat anti-rabbit immunoglobulin diluted 1:1000 (Kirkegaard & Perry, Gaithersburg, MD) for 1 h. The plate was washed with PBS containing 0.1% Tween 20 and developed using the TMB two-component peroxidase substrate solution (Kirkegaard & Perry). The reaction was stopped by adding 3 ml H3PO4, and quantitated by absorbance at 450 nm using a Dynatech ELISA reader.

**Rat Insulin RIA**

The insulin content was determined by a sensitive rat insulin RIA kit (Linco Research, Inc.) according to the manufacturer’s directions or in some experiments using the Pharmacia insulin RIA (Amersham Pharmacia Biotech) using a rat insulin standard. Insulin secretion experiments were carried out in triplicate at least five times using multiple passages (passages 3–7) of 10 different clones.

**Functional Analysis**

Atlas cDNA Expression Array: Purification of Total RNA—Total RNA was extracted from clone RIN-5Fb23, a stably secretagogin-transfected clone and clone RIN-5Fb6, a sham transfectant. In brief, RNA isolation was performed using an Atlas pure total RNA isolation kit (CLONTECH). After DNase I treatment, RNA was further purified and precipitated and then the probe was prepared essentially as described in the manufacturer’s instruction manual. cDNA synthesis was performed at 48 °C including [α-32P]dATP reaction buffer and Moloney murine leukemia virus reverse transcriptase. A column chromatography step was included to eliminate nonincorporated radioactivity. Hybridization of the atlas array was performed overnight at 68 °C. After stringent washes with 2× SSC, 1% SDS followed by 0.1× SSC, 0.5% SDS, membranes were exposed for autoradiography to Kodak XAR5-Omat films at ~80 °C.

**Measurement of Cell Growth in Stable Transfected RIN-5F and Jurkat Cells**—RIN-5F cell clones were seeded at 2 × 105 cells/Petri dish in triplicate. Cells were harvested by trypsin-EDTA treatment and viable cells counted by hemocytometer using trypsin blue after 72 and 120 h of incubation. Jurkat cells growing in suspension were seeded at 5 × 105 cells/ml in Falcon culture flasks in triplicate.

**Calcium Flux Measurement**—Stably expressing secretagogin-transfected RIN-5F cells or wild type and sham transfectant controls (105/ml) were suspended in RPMI containing 1% fetal calf serum and 5.0 μM acetoxyethyl ester of Fura-2 (Molecular Probes, Eugene, OR) for 30 min at 37 °C in the dark. The tissue culture flask adherent cells were detached using trypsin-EDTA as indicated above. Detached Fura-2-
Identification of a Novel Calcium-binding Protein

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RESULTS

Isolation of the cDNA Clones for Human Secretagogin

Using a human insulinoma as an immunogen, we generated a pancreatic beta cell-specific mouse monoclonal antibody (34). This mAb was utilized to screen a human pancreatic beta cell cDNA library constructed in agt11. Three independent positive clones were plaque-purified. All three clones had the identical insert of 1437 bp based on sequence analysis; one was used for further analysis.

In Fig. 1 the composite nucleotide sequence of the cDNA and the deduced amino acid sequence is presented. The 5'-untranslated nucleotide sequence contains 156 bp and the 3'-untranslated region 450 bp. The polyadenylation signal was found at bases 1412–1417, which is 20 bp upstream from the end of the identified cDNA. A poly(A) tail could not be found in any of the three clones. The coding region specifies a protein of 276 amino acids and a calculated molecular mass of 32 kDa. Sequence analysis suggests that the protein has six tandem repeats of the EF-hand Ca2+-binding domain. Secondary structure analysis predicts a loop at all six putative Ca2+-binding sites. Five 12-amino acid EF-hand sequences were identified in the genomic clone. We have analyzed the amino acid sequence in the 10 exons of this genomic clone (HS501N12) are spread over more than 33 kilobases, which is larger than the size predicted by the cDNA sequence (34). This suggests that one or more carboxyl-terminal exons was lacking in the genomic clone HS501N12. As demonstrated in Fig. 3, the 10 exons from the known genomic secretagogin clone (HS501N12) are spread over more than 33 kilobases and one or more terminal exons are nonidentified at the 3' terminus (Fig. 3).

Secretagogin Expression in Human Tissue

Normal Tissue—To further investigate the tissue expression of secretagogin mRNA, multiple tissue Northern blots from human endocrine organs were analyzed with a cDNA probe specific for secretagogin, or as a control for RNA loading, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). When normalized for differences in RNA loading, the intensity of the pancreatic secretagogin mRNA signal is over 4 times greater (432%) than the GAPDH mRNA signal (Fig. 4). Immunohistochemical analysis of expressed protein has shown that secretagogin expression is restricted to the islet of Langerhans (38). This is of interest that two mRNA size variants are present in the genome.

Chromosomal Localization

A comparison of the secretagogin cDNA sequence with GenBank® data identified a single genomic clone derived from human chromosome 6 (Fig. 3). The predicted amino acid sequence of secretagogin matched the first 10 exons identified in clone HS501N12 derived from chromosome 6p22.1–22.3. Although the amino acid sequence in the 10 exons of this genomic clone was identical to secretagogin, the 42 carboxyl-terminal amino acids were not identified in the genomic clone. We have previously identified secretagogin in insulinomas at the protein level and have confirmed that it is expressed as a 32-kDa species, which matches the size predicted by the cDNA sequence (34). This suggests that one or more carboxyl-terminal exons was lacking in the genomic clone HS501N12. As demonstrated in Fig. 3, the 10 exons from the known genomic secretagogin clone (HS501N12) are spread over more than 33 kilobase pairs and one or more terminal exons are nonidentified at the 3' terminus (Fig. 3).

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Since Northern blot analysis demonstrated secretagogin expression, we assayed the ability of secretagogin to block agonist-induced calcium mobilization in RIN-5F cells. Three independent positive clones were plaque-purified. All three clones had the identical insert of 1437 bp based on sequence analysis; one was used for further analysis. In Fig. 1 the composite nucleotide sequence of the cDNA and the deduced amino acid sequence is presented. The 5'-untranslated nucleotide sequence contains 156 bp and the 3'-untranslated region 450 bp. The polyadenylation signal was found at bases 1412–1417, which is 20 bp upstream from the end of the identified cDNA. A poly(A) tail could not be found in any of the three clones. The coding region specifies a protein of 276 amino acids and a calculated molecular mass of 32 kDa. Sequence analysis suggests that the protein has six tandem repeats of the EF-hand Ca2+-binding domain. Secondary structure analysis predicts a loop at all six putative Ca2+-binding sites. Five 12-amino acid EF-hand sequences were identified in the genomic clone. We have analyzed the amino acid sequence in the 10 exons of this genomic clone (HS501N12) are spread over more than 33 kilobases, which is larger than the size predicted by the cDNA sequence (34). This suggests that one or more carboxyl-terminal exons was lacking in the genomic clone HS501N12. As demonstrated in Fig. 3, the 10 exons from the known genomic secretagogin clone (HS501N12) are spread over more than 33 kilobase pairs and one or more terminal exons are nonidentified at the 3' terminus (Fig. 3).

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Since Northern blot analysis demonstrated secretagogin expression, we assayed the ability of secretagogin to block agonist-induced calcium mobilization in RIN-5F cells. Three independent positive clones were plaque-purified. All three clones had the identical insert of 1437 bp based on sequence analysis; one was used for further analysis. In Fig. 1 the composite nucleotide sequence of the cDNA and the deduced amino acid sequence is presented. The 5'-untranslated nucleotide sequence contains 156 bp and the 3'-untranslated region 450 bp. The polyadenylation signal was found at bases 1412–1417, which is 20 bp upstream from the end of the identified cDNA. A poly(A) tail could not be found in any of the three clones. The coding region specifies a protein of 276 amino acids and a calculated molecular mass of 32 kDa. Sequence analysis suggests that the protein has six tandem repeats of the EF-hand Ca2+-binding domain. Secondary structure analysis predicts a loop at all six putative Ca2+-binding sites. Five of these binding sites were 12 amino acids in length and possessed an E at position 12; however, site 4 had only 8 residues, which contrasts with the 12-amino acid EF-hand sequences found in the troponin/calmodulin/S100 superfamily (38). This might represent a nonfunctional site. The predicted amino acid sequence of secretagogin was highly homologous to human and murine calbindin and calretinin (Fig. 2).
mRNA in thyroid tissue, we analyzed thyroid tissue at the immunohistochemical level to identify secretagogin expression and its tissue distribution pattern. We used thyroid tissue containing C-cell hyperplasia. As demonstrated in Fig. 5A, cells with typical C-cell morphology and tissue distribution reacted with mAb D24. Fig. 5A shows nodular C-cell hyperplasia (33) and 3 C-cells (32) in juxtaposition to a thyroid follicle. These cells were confirmed to be C-cells, based on their staining for calcitonin (data not shown). Very weak staining of colonic and gastric mucosal cells was also observed. This result is not surprising, since several other neuroendocrine and beta cell-specific proteins such as STF-1 (39) are also expressed in mucosal cells.

Neuroendocrine Tumors—The expression of secretagogin by neoplastic neuroendocrine cells was analyzed in 12 clinical specimens. The tumor type was identified by immunohistochemical criteria and typical clinical symptoms of inappropriate hormone secretion as well as laboratory features of typical marker species in the patients' serum (carcinoid (n = 4), gastrinoma (n = 2), insulinoma (n = 1), neuroendocrine carcinoma (n = 2), adrenal adenoma (n = 1), pheochromocytoma (n = 1), and hyperfunctional thyroid adenoma (n = 1)). Several of these neoplasms (Fig. 6) contained substantial quantities of secretagogin mRNA; carcinoid tumors, gastrinoma, and insulinoma contained 103 ± 54%, 61 ± 44%, and 22% respectively, of loaded 28 S rRNA. In contrast, secretagogin mRNA could not be detected by Northern analysis in adrenal adenoma, pheochromocytoma, hyperfunctional thyroid adenoma (data not shown), and, interestingly, in neuroendocrine carcinoma (Fig. 6). However, scant quantities of secretagogin could be detected by RT-PCR in pheochromocytoma and adrenal adenoma (data not shown).

Intracellular Localization of Secretagogin

RIN-5FB23 rat insulinoma cells stably transfected with secretagogin were disrupted by shearing and separated into crude nuclei, cytoplasm, and membrane fractions. Insulin was nearly entirely recovered in the membrane fraction (93%), which contains the dense intracellular granules and rough endoplasmic reticulum, with only 7% detected by RIA in the cytosolic fraction. In contrast, secretagogin was present in the cytosolic fraction (90%) (data not shown). Quantitation of insulin and secretagogin in the transfected RIN-5F when studied by immunoblotting were similar to that observed in the secretagogin-transfected dog insulinoma cell line INS-H1 (Fig. 7). The compartmentalization of secretagogin and insulin was confirmed by confocal microscopy. Immunofluorescence staining of the stable secretagogin transfectant RIN-5FB23 (Fig. 8) for insulin (green) using a rabbit anti-insulin antibody and secretagogin (red) using mAb D24 demonstrated that these two proteins were only rarely colocalizing (yellow) within individual granules.

The confocal images suggested that some secretagogin may be present within nuclei (Fig. 8). Immunoblotting of the nuclear fraction proteins following SDS-PAGE (Fig. 7) confirmed the presence of secretagogin in the nuclear fraction, but some of
the observed immunoblot signal may be due to the presence of rare intact cells or adherent cytoplasm to nuclei within the fraction.

Purification of Recombinant Human Protein

The GST fusion protein expressed in E. coli was detected by SDS-PAGE and immunoblotting as a prominent band of ~49 kDa. The fusion protein (Fig. 9A, lane 1) was purified from the E. coli lysates by glutathione-Sepharose affinity chromatography and eluted with an excess of glutathione. Secretagogin, liberated from the GST fusion protein by incubating the glutathione-Sepharose GST-protein slurry with thrombin as described above, had a molecular mass of 32 kDa (Fig. 9A, lanes 2 and 3). The homogeneity of the purified preparation was indicated by a single band when analyzed by SDS-PAGE.

Rat Insulinoma Cells Express Endogenous Secretagogin

Although the murine mAb D24 was specific for human secretagogin, when polyclonal rabbit anti-recombintant human secretagogin antiserum was used in immunoblotting of transfected RIN-5F cells, a doublet band was present (Fig. 9B) in contrast to the single band observed using wild type or sham-transfected RIN-5F. The transfected human moiety had a slightly higher relative mass than the endogenous rat homologue. It is important to note that the wild type RIN-5F and sham transfectants express substantial quantities of endogenous rat secretagogin. Thus, functional differences between secretagogin transfectants and control wild type or sham-transfected RIN-5F cells must reflect quantitative differences in secretagogin expression, rather than qualitative effects attributable to a gain-of-function phenotype in the transfected cells. In the transfected dog insulinoma cell clone INS-H1, only the human moiety could be detected by the rabbit polyclonal antibody.

Secretagogin Expression in Mammalian Cells in Vitro

As demonstrated in Fig. 5B, the cytoplasmic staining pattern of pZeoSV2 transiently transfected COS cells had a reticular pattern (n = 3). The strongest staining was seen 72 h after transfection. Stably transfected Jurkat cells were constructed by transfection with pZeoSV2 and selected with Zeocin as described above. After 2 weeks of Zeocin selection, colonies were handpicked under microscopic observation and propagated in 48-well plates.

RIN-5F rat and INS-H1 dog insulinoma cells were similarly transfected using the Lipofectin method, and after Zeocin selection outgrowing colonies were hand-picked and expanded. The best expressing clones were identified by immunostaining using mAb D24 and by secretagogin ELISA using tissue culture supernatant or cell lysate from individual clones. Eight RIN-5F clones and two Jurkat clones were selected and chosen for further experiments.
Secretagogin Is Secreted in Vitro

We developed a sandwich ELISA using mAb D24 and polyclonal rabbit anti-recombinant secretagogin to study the level of secretagogin in tissue culture cell supernatant. This sensitive assay had a threshold of detection of 6.5 pg/ml. We have been able to quantitate constitutively secreted human secretagogin from transfected RIN-5F cell and dog insulinoma INS-H1 clones (INS-H1tr1, INS-H1tr2, RIN-5FG, RIN-5FE, RIN-5FL, RIN-5FB22, RIN-5FB23, RIN-5FN, RIN-5PJ, and RIN-5FF: 10–82 pg/ml/48 h/1 × 10^6 cells). Although no secretagogin was detected in culture supernatants of stable Jurkat transfectants containing secretagogin cDNA, brief exposure of these cells (but not wild type or sham transfectants) to mAb OKT3 (2 μg/ml, 2 min, 37 °C) triggered brisk secretion. We have detected secretagogin levels of 105 ± 19 pg/ml/1.5 × 10^7 cells (n = 3) when measured 2 min after CD3 ligation.

Functional Analysis

Effect of Secretagogin on Calcium Flux—Calcium flux experiments using KCl to induce a calcium flux in secretagogin-transfected RIN-5F cells revealed a significant difference between secretagogin-expressing clones and wild type or sham-transfected RIN cells. Secretagogin transfectants expressing greater than 30 pg/ml/10^6 cells exhibited a greater calcium flux in response to KCl than the vector-transfected controls (p < 0.002, n = 6 for each clone) (Fig. 10, A and B). This effect was reduced but still detectable when only 12 pg were expressed (clone E). Fig. 11 displays KCl-induced calcium flux, as determined by single-cell microscopic analysis. In each experiment, 16–24 Fura-3-loaded RIN-5F cells were gated and monitored by measuring the calcium-flux every second. Bars represent the mean ± S.D. of KCl-induced calcium flux in four to six experiments. A higher Ca²⁺ flux was observed in the secretagogin transfectants (Fig. 11). The difference was significant when compared with sham transfectants (p < 0.008) and wild type (p < 0.004).

Effect of Secretagogin on Insulin Secretion and mRNA Expression—When the basal level of insulin secretion of control (wild type and sham-transfected) and secretagogin-transfected RIN-5F cells were analyzed (n = 10 clones), the quantity of insulin secreted by secretagogin-transfected cells was approximately 3–4-fold greater than that secreted by the controls (Fig. 10C). This effect was observed in secretagogin transfectant clones expressing 10–70 pg/10^6 cells and was highly significant (p < 0.004) when compared with both the controls. The level of secreted insulin did not vary substantially in a given clonal line despite the presence of elevated glucose (15 or 20 mM), although this is not surprising since insulin secretion by the wild type cell line was not sensitive to glucose concentration (data not shown). Following KCl-induced Ca²⁺-flux, insulin secretion by the secretagogin-transfected cell line was increased by about 30%. Insulin secretion experiments were carried out in passages 3–6 of RIN-5F cell transfectants; the level of insulin did not vary using cells harvested after three or six passages.

When insulin mRNA expression was measured under normal tissue culture conditions, a 5–10-fold higher basal level of insulin mRNA transcription was measured by Northern analysis in human secretagogin-expressing RIN-5F clones when compared with sham transfectant and wild type cells (Fig. 13). This increase in insulin transcription was not observed in those RIN-5F secretagogin transfectants (n = 3) expressing more than 200 pg/10^3 cells.

Effect of Secretagogin on RIN-5F Cell Growth Rate—Significant cell growth retardation was seen in the RIN-5F insulinoma cell clones (RIN-5FB23, RIN-5FL and RIN-5FF) secreting 50 pg/ml/48 h/10 × 10^6 cells when compared with sham transfectants and wild type cells (Fig. 12). A similar growth retardation (50% after 48 h) was also observed in secretagogin-transfected Jurkat clones (n = 2) data not shown.

To further analyze this effect, a rat gene array was screened using RNA obtained from the secretagogin-overexpressing clone RIN-5FB23 and compared with a sham transfectant clone. Differences in the level of cDNA specific for several genes were observed. Most strikingly, a down-regulation of substance P (4.5-fold) and rac-α serine/threonine kinase (RAC-PK-C) (3-fold) expression was seen in the human secretagogin-expressing clone. In contrast, a 1.9-fold up-regulation of secretogranin V and 1.6-fold up-regulation of chromogranin C were observed. Enhanced expression of these neuroendocrine-specific gene products suggests that expression of human secretagogin results in reduction of growth and a shift in the cell synthetic program toward endocrine differentiation or cell maturation.
Influence of Secretagogin on Substance P Expression

The dramatic difference in substance P mRNA levels observed in the gene array studies was explored by performing Northern blot analysis on multiple secretagogin-transfected cells. Samples of mRNA from eight secretagogin-transfected RIN-5F clones, sham transfectants, and wild type cells were studied. In all the secretagogin transfectants (n = 8 clones), we found a 5–15-fold reduction of substance P mRNA when compared with sham transfectants and to wild type cells (Fig. 13), and this was independent of the number of cell passages in vitro. Interestingly, the changes in substance P mRNA expression did not correlate with insulin mRNA transcription or insulin protein synthesis (data not shown), which confirms data of other investigators who stress that substance P and insulin expression are regulated separately (40). However, all cells with low level expression of substance P mRNA had lower growth rates than high substance P-expressing clones.

DISCUSSION

We have successfully cloned and expressed the antigen recognized by the islet cell- and neuroendocrine cell-specific mAb D24 (34). Sequence analysis reveals that the expressed protein, which we have termed secretagogin, has six Ca$$^{2+}$$-binding loops with typical EF-hand tandem repeats and has marked homology to the well known calcium-binding proteins calbindin and calretinin, which also have six EF-hand motifs, although conservative amino acid substitutions are present in some of the secretagogin motifs (Fig. 2). The predicted loops in the secretagogin EF-hand sequence are homologous to those in calbindin and calretinin, although some are shorter than the 12-residue loops present in members of the troponin/calmodulin/S100 superfamily. The functionality and avidity for calcium of the fourth EF-hand sequence may be reduced in comparison to the longer sequences terminating in glutamic acid. Crystal structures of other close relatives such as calmodulin, troponin C, and intestinal calcium-binding protein demonstrate that the amino acid Glu (at position 12) contributes both side-chain...
Identification of a Novel Calcium-binding Protein

Fig. 8. Two-color immunofluorescence confocal microscopy of insulin and secretagogin in RIN-5FB23 rat insulinoma cells. A cytopsin preparation of trypsinized RIN-5FB23 after fixation in acetone was stained with rabbit polyclonal anti-insulin antibody and D24 mAb. Insulin staining is green, secretagogin staining is red, and colocalization of insulin and secretagogin is yellow. Secretagogin staining was higher in cells appearing larger but was present in all cells, although to a varying amount. Staining for secretagogin was also observed within the nucleus. Colocalization of both proteins was seen (yellow granules) but was not very frequent, confirming the cell fractionation data. The microscopic image was recorded by Normaski optics. Shown is a representative experiment for clone RIN-5FB23 (n = 3); similar results were seen for clone RIN-5FF (n = 2).

Fig. 9. A, generation of human secretagogin: immunoblotting of E. coli lysate transformed with secretagogin coding region in the pGEX vector. Lane 1, mAb D24 immunoblot of secretagogin expressed as a GST fusion protein; lane 2, secretagogin after cleavage with thrombin; lane 3, purified thrombin-cleaved secretagogin stained by Coomassie Blue. Molecular size markers are indicated at the left. Shown is a representative experiment out of two. B, identification of rat endogenous secretagogin. The cell extracts prepared from the human-secretagogin stable transfected RIN-5FB23, wild type, and sham transfected were loaded onto a 12% SDS-PAGE gel in order to obtain better resolution. Immunoblotting was performed with rabbit polyclonal anti-secretagogin antisera. The upper band represents transfected human secretagogin, whereas the slightly lower band represents the endogenous rat secretagogin (n = 4).

oxygen atoms to the ion coordination, although it is positioned in the neighboring helix (38).

Northern blot analysis demonstrated secretagogin mRNA within the pancreas and to a very low level in the thyroid, adrenal medulla and cortex, small intestine, stomach, and colon, largely within probable neuroendocrine cells residing in juxtaepithelial locations. Immunohistochemical staining demonstrated considerable quantities of secretagogin within the pancreatic islet of Langerhans (34) and the thyroid, especially in a patient with C-cell hyperplasia. Although secretagogin represents an acidic protein like chromogranins, it does not appear to be packaged in typical mature presecretory, neuroendocrine granules. However, there is clear evidence that the protein is constitutively secreted from transfected RIN-5F cells and can be secreted from transfected Jurkat cells following antibody ligation of CD3.

Insulin localization within distinct beta cell compartments during post-translational processing may provide insights into the compartmentalization of secretagogin. Insulin and other regulated secretory proteins are packaged in clathrin-coated granules which emerge from the trans-most cisternae of the Golgi (41). In contrast, non-regulated proteins enter a constitutive pathway and exit the Golgi in low density vesicles, which fuse readily with the plasma membrane. However, even regulated proteins participate in a constitutive-like pathway (42). Prolactin and its intermediate cleavage forms are stored in immature, less dense granules that are distinct from the dense secretory granules, which contain mature insulin (43). Under conditions (e.g., hypo- or euglycemia) where regulated insulin secretion from mature granules is suppressed, there is limited secretion of insulin and peptide C from the immature secretory granules (42).

The structural basis for calcium-dependent regulated insulin secretion may be based in part on the presence of calcium-regulated proteins in dense granules. For example, the calcium-dependent interactions of granin family proteins with adaptins play an important role in the formation and trafficking of dense secretory granules (33). Moreover, the C isoform of calmodulin-dependent protein kinase II is present within insulin-containing secretory granules and is likely to be involved in insulin secretion (44). Since distinct members of the EF-hand superfamily are known to act by modulating the activity of specific protein kinases (45), it is possible that secretagogin regulates as yet undefined protein kinases or other effector molecules within neuroendocrine cells.

The rather heterogeneous staining observed by confocal microscopy and cell fractionation studies suggests that secretagogin is localized within a distinct intracellular compartment different from mature insulin. The OKT3 mAb-induced secretion data obtained from transfected Jurkat cells indicate that secretagogin is present in some form of secretory granule. However, the cell fractionation experiments (Fig. 7) demonstrate that most secretagogin was contained within the cytosol. Perhaps loosely packaged granules are not sedimenting in the membrane fraction, or the secretagogin-containing compartment is disrupted by the cell fractionation process. Alternatively, cytosolic secretagogin may be associated with granular membrane proteins and released in the process of granule secretion.

Immunohistochemical staining of colonic, small intestine, and stomach mucosal tissue demonstrated stained cells in close juxtaposition to the mucosal cell layers where neuroendocrine cells are typically located. The observation that mucosal cells were also stained to some degree raises several questions. It is possible that mAb D24 recognizes a distinct calcium-binding protein in mucosal cells that is homologous to secretagogin. The other possibility is that bona fide secretagogin is indeed found...
FIG. 10. A, intracellular Ca\(^{2+}\) mobilization in RIN-5F cells stimulated with KCl (cell suspension). Calcium mobilization in Fura-2-AM-loaded RIN-5F cells (wild type, sham, and secretagogin transfectant) was measured following the application of KCl (20 mM final concentration). Shown are three representative secretagogin-transfectant clones out of eight analyzed. Clones RIN-5FB22, RIN-5FB23, RIN-5FN, RIN-5FL, and RIN-5FF expressed in excess of 30 pg/3 \times 10^5 cells and RIN-5FE, RIN-5FD, and RIN-5FJ expressed lesser quantities (see clone E). Values (mean \pm S.D.) represent the increase of the intracellular calcium \([Ca]\) after addition of agonist (n = 6 for each clone). The peak calcium mobilization \([Ca]\) in secretagogin-transfected cells, which express more than 30 pg/3 \times 10^5 cells secretagogin \((p < 0.002, \text{Student's} \, t \, \text{test})\) when compared with sham transfectants and to wild type cells. B, intracellular secretagogin expression. Data shown represent means \pm S.D. of intracellular secretagogin expression in 3 \times 10^5 transfected RIN-5F cell clones (RIN-5FB22, RIN-5FB23, and RIN-5FE). Each experiment was carried out by quantitative ELISA of cell lysate obtained from 3 \times 10^5 RIN-5F cells, which were homogenized in 1 ml of lysis buffer containing protease inhibitors \((n = 3)\). Statistically significant greater quantities of secretagogin was expressed intracellularly in clones RIN-5FB22 \((p < 0.0001)\) and RIN-5FB23 \((p < 0.004)\) when compared with RIN-5FE. As expected, the wild type (WT) and sham-transfected (ST) clones did not express human secretagogin detectable by capture ELISA. C, insulin protein secretion measured by an insulin-specific RIA. Stable secretagogin RIN-5F transfectants (see clones B22, B23, and E) secrete greater quantities of insulin following stimulation with a secretagogue such as KCl (20 mM) compared with wild type (WT) and sham transfectant (ST) RIN-5F cells. The experiment shown was carried out in triplicate \((\text{mean} \pm \text{S.D.})\) over 1 h and is representative of five experiments on different days. Values of secretagogin transfectants are significantly higher \((p < 0.004)\) than wild type and sham-transfected clones. Secretagogin secretion was only detected in secretagogin-transfected cells (data not shown).
in small quantities since other beta cell and islet of Langerhans-specific proteins such as somatostatin transactivation factor (STF-1) (39) are also expressed in the intestinal mucosa. Although the physiological role of secretagogin is not completely clear at this time, the markedly reduced growth rate in RIN cells and Jurkat cells stably transfected with secretagogin suggests that the protein plays a role in growth control and differentiation, or that the expression of secretagogin may inhibit cell replication indirectly by modulating intracellular Ca\(^{2+}\) storage pools. For example, when the closely related protein calbindin was overexpressed in neurons, its ability to chelate Ca\(^{2+}\) dampened the toxic effects of exocytotoxic neurotransmitters (46) and it has been suggested that changes in Ca\(^{2+}\)-binding proteins play an important role in neurodegenerative diseases (47). Additional members of the EF-hand Ca\(^{2+}\)-binding family have recently been identified, which possess novel regulatory mechanisms. In contrast to the stimulatory effects of S100B on Ndr kinase (45), calbrain, a novel two-EF-hand domain protein expressed exclusively in brain loci, suppresses calmodulin-dependent protein kinase II activity, which is instrumental in a variety of neuronal functions (48). A four-EF-hand domain protein, DREAM, was identified, which acts as a Ca\(^{2+}\)-sensitive transcriptional repressor. It binds directly to a downstream regulatory element in the prodynorphin and c-fos genes in the absence of Ca\(^{2+}\), but in the presence of Ca\(^{2+}\) its repressor function is lost (49). Thus, secretagogin may exert an effect on the physiology of neuroendocrine cells through one or more distinct mechanisms.

We have demonstrated down-regulation of substance P in RIN-5F cells following overexpression of secretagogin. Since the expression of substance P (a putative beta cell trophic factor) is largely restricted to rapidly proliferating minimally differentiated fetal and neonatal islet cells as well as pancreatic beta cell lines (40), it is possible that the secretagogin-induced suppression of substance P transcription is responsible for the observed reduction in secretagogin-transfected cell growth rates. The seven-transmembrane-spanning receptor for substance P (NK-R1) was also shown to be expressed on the RIN cells by gene array screen (data not shown). This raises the possibility that substance P acts as an autocrine growth, differentiation, or trophic factor for these cells. Some insights regarding the effect of calcium-binding proteins on hormone secretion have been reported (32). These investigators established stable transfectants of rat calbindin in the insulin-secreting rat insulinoma cell line RIN. In this model system, overexpression of calbindin, which is highly homologous to secretagogin, induced a much higher rate of insulin transcription and secretion in comparison with wild type RIN cells. No observations were reported by these authors regarding the growth rates of calbindin-transfected clones. We observed a similar augmentation of basal insulin mRNA levels in secretagogin-transfected RIN cells but must stress that it is not necessarily a direct effect of secretagogin. It is possible that indirect effects of secretagogin on differentiation and growth rate might be responsible for the observed increase in insulin transcription.

**FIG. 11.** Calcium flux in individual RIN-5F cells. A total of 15–20 adherent cells of each population were monitored individually using an inverted fluorescence microscope with recording of fluorescence ratio data at 1-s intervals. Adherent RIN-5F clones were monitored at 37 °C in flux buffer, and after reaching stable fluorescence (typically 180 s), KCl was added (20 mM final concentration) resulting in a brisk increase in calcium flux. Values (mean ± S.D.) represent KCl agonist induced calcium flux (Fura-2 fluorescence F340/F380 derived from gated cells or cell clusters). Secretagogin transfecants (B23) showed a significantly higher calcium flux when compared with wild type (WT) and sham transfectants (ST) (p < 0.0076, n = 4–6).

**FIG. 12.** Growth rate of stably transfected RIN-5F cells. Secretagogin transfection lowers the growth rate of RIN-5F cells (data represent means of triplicate values ± S.D.). When analyzed (n = 3) at 5 days, the difference in growth rate between the secretagogin and sham transfectants and between secretagogin transfecants and wild type cells was significant (Student’s t test, p < 0.04).
In conclusion, we describe a novel calcium-binding protein termed secretagogin, which was cloned from a human pancreatic beta cell library and is expressed by neuroendocrine cells of the islet of Langerhans and in tumors of neuroendocrine origin, including gastrinoma, carcinoid, and insulinoma. It is also found in scant quantity in the thyroid (C-cells), in adrenal gland, and in the stomach and intestine. This protein has been successfully produced in E. coli. We present evidence that this protein can regulate cell growth and cell maturation perhaps by down-regulating substance P in insulinoma cells, and may be useful as a marker of a subtype of granular secretion from neuroendocrine tumor cells.

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