Two Novel Immortal Pancreatic β-Cell Lines Expressing and Secreting Human Islet Amyloid Polypeptide Do Not Spontaneously Develop Islet Amyloid

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Type 2 diabetes is characterized by islet amyloid deposits, which are primarily composed of the amyloidogenic human form of islet amyloid polypeptide (IAPP, amylin). The mechanism of islet amyloido-genesis is not known, but other products (e.g., apolipoprotein E and perlecan) contained within islet amyloid may be necessary. Because rodent IAPP does not form islet amyloid, the currently available β-cell lines are not useful for studying processes involved in amyloid formation. To develop a suitable in vitro cell system for the study of islet amyloid formation, we generated two new β-cell lines that express the amyloidogenic human IAPP. We did this by crossbreeding human IAPP transgenic mice with RIP-Tag mice that develop islet tumors and then culturing one of these islet tumors from two separate offspring of this cross. The resultant 2350-2C0 and 2511 cell lines produce human as well as mouse IAPP-like immunoreactivity (IAPP-LI) and immunoreactive insulin (IRI). Incubation of both these cell lines with 16.7 mmol/l glucose resulted in a two- to fourfold increase in human IAPP-LI, mouse IAPP-LI, and IRI secretion compared with 1.67 mmol/l glucose and the combination of 16.7 mmol/l glucose and 10 mmol/l arginine, 0.1 mmol/l 3-isobutyl-1-methylxanthine (IBMX), and 5 μmol/l carbachol induced a >50-fold increase in the release of these peptides. The omission of calcium from the above secretagogue cocktail reduced secretion of all three peptides to only two- to sixfold higher than the 16.7 mmol/l glucose condition. Perifusion with 16.7 mmol/l glucose plus 0.1 mmol/l IBMX caused a biphasic secretion of human IAPP-LI and mouse IAPP-LI, as well as IRI, in both cell lines, with the peak of the first phase being five- to sixfold higher than the prestimulated 1.67 mmol/l glucose condition. Immunoelectron microscopic inspection of both 2350-2C0 and 2511 cells after 7 days of culture did not reveal the presence of amyloid fibrils, suggesting the need for other critical components. We conclude that we have established two novel β-cell lines that produce and secrete human IAPP in a regulated manner. These cell lines will be a useful tool to investigate the secretion of human IAPP as well as the necessity of other components for islet amyloid formation. *Diabetes* 48:1962–1970, 1999

Islet amyloid polypeptide (IAPP), also known as amylin, is a normal secretory product of the pancreatic β-cell and is the primary constituent of islet amyloid (1,2). Islet amyloid is a characteristic feature of the pancreatic pathology in patients with type 2 diabetes (3,4), where it replaces β-cell mass, resulting in reduced insulin secretion and likely contributing to increasing plasma glucose levels. Amyloid is also a feature of insulinomas (1,5). The mechanism of islet amyloid formation in type 2 diabetes and insulinomas is not known. The presence of an amyloidogenic sequence in IAPP as exists in the human, primate, and cat peptides, but not the rodent peptide, is a prerequisite (6), although it is not the sole factor responsible for IAPP deposition as islet amyloid (7). Other components, such as apolipoprotein E and the heparan sulfate proteoglycan perlecan, are present in amyloid and may be critical in the formation of islet amyloid and the amyloid associated with Alzheimer's disease (8–11). In fact, addition of perlecan to IAPP in vitro enhances amyloid fibril formation (12).

It has been suggested that impaired processing and/or secretion of IAPP by the islet β-cell may be a factor contributing to islet amyloidogenesis (13). Secretion of β-cell hormones (e.g., insulin, IAPP) occurs via two pathways: *1*) the regulated pathway, from which secretory granules are released in response to a stimulus (e.g., glucose) and for which calcium is essential for the exocytotic machinery, and *2*) the constitutive pathway, from which secretory vesicles are released by bulk flow relying on continued protein synthesis and which is calcium independent. Secretory granules in the regulated pathway contain endopeptidases that are responsible for prohormone cleavage and the secretion of mature peptides (14–16). Our understanding of β-cell peptide processing and secretion has been greatly aided by the devel-

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BSA, bovine serum albumin; CV, coefficient of variation; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HPLC, high-performance liquid chromatography; IAPP, islet amyloid polypeptide; IAPP-LI, islet amyloid polypeptide–like immunoreactivity; IBMX, 3-isobutyl-1-methylxanthine; IRI, immunoreactive insulin; KRBB, Kreb's Ringer bicarbonate buffer; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; TBS, Tris-buffered saline.

opment of β-cell lines that provide ample material that is relatively free of non-β endocrine cells and that can be readily studied under a variety of conditions.

The expression of the large T-antigen gene of the simian-40 virus has resulted in the development of a number of transformed β-cell lines that have been used to study the regulation of prohormone processing and release (17–19). For example, βTC-3 cells have been used to study processing and secretion of both insulin and mouse IAPP (20,21). The βTC and HIT cell lines were also important in the demonstration that prolonged culture in high glucose was associated with glucose desensitization and a loss of glucose-induced insulin secretion (22–24).

However, these cell lines are not ideal for studying the mechanism of islet amyloid formation because they produce and secrete the rodent form of IAPP, which does not form fibrils, and do not release the human form of the peptide, which is capable of forming amyloid fibrils. Thus, we endeavored to develop immortal β-cell lines that produce and secrete the amyloidogenic human form of IAPP. We did this by crossbreeding RIP-Tag mice that express the large T-antigen gene of the simian-40 virus and develop islet tumors (25) with our human IAPP transgenic mice that develop islet amyloid when fed a high-fat diet for 12–16 months (26). From this cross, we report here the establishment of two novel cell lines (2350-2C0 and 2511) that produce and secrete human IAPP in response to glucose and nonglucose secretagogues, an effect that is inhibited by the omission of calcium. Thus, these new cell lines that have the capacity for regulated secretion of human IAPP should provide us with a new tool for the study of human IAPP processing and/or secretion. Furthermore, these novel cell lines will allow us to examine the potential role of other components of amyloid (apolipoprotein E and perlecan) in the process of islet amyloidogenesis.

RESEARCH DESIGN AND METHODS

Generation of a new transgenic mouse line. RIP-Tag mice expressing the large T-antigen gene of the simian-40 virus under the control of the rat insulin II promoter (kindly provided by Dr. Douglas Hanahan, University of California San Francisco) (25) and a line of human IAPP transgenic mice generated at the University of Washington (27) were used as breeders. Animals were reared at the animal research facility of the Veterans Affairs Puget Sound Health Care System. Double transgenic mice were generated by mating heterozygous male RIP-Tag mice with heterozygous female human IAPP transgenic mice. The presence of both transgenes in the offspring was determined by the polymerase chain reaction (PCR) of tail DNA using transgene-specific primers (28).

Generation of new immortal β-cell lines expressing human IAPP. A number of transgenic mice with β-cell expression of both human IAPP and Tag were killed at 11 weeks of age. Pancreatic tumors were excised from each mouse, triturated with a plastic Pasteur pipette, and dispersed in Dulbecco's modified Eagle's medium (DMEM). After culture for ~48 h, cells were passaged by trypsinization, monitored for growth, and subsequently passaged as necessary. Consequently, a number of cell lines were established that expressed and secreted human, as well as mouse, IAPP. In this report, we describe the detailed characterization of two cell lines, designated 2350-2C0 and 2511, that differ from other immortal β-cell lines in that they express human IAPP as well as murine IAPP. These cells were routinely cultured in DMEM media containing 25 mmol/l glucose and 15% heat-inactivated fetal calf serum (FCS) and passaged weekly by trypsinization.

Assessment of cell proliferation. Cells from the 2350-2C0 and 2511 lines were plated at a density of 2×10^5 and maintained in static incubation for 7 days in DMEM media containing 25 mmol/l glucose and 15% FCS. At days 3, 5, and 7, cells were removed by trypsinization, counted, and stored at –20°C. Determination of DNA content was performed as previously described (29).

Northern blot analysis. Cells (6×10^5) were plated on day 1 and grown for 5 days in DMEM media containing 16.7 mmol/l glucose and 15% FCS, with fresh DMEM media containing 16.7 mmol/l glucose and 15% FCS added on day 3. On day 5, RNA was extracted, electrophoresed on a 1.4% formaldehyde gel, and transferred to a nylon membrane using standard methods (27). IAPP was detected using a PCR-generated probe incorporating ^{32}P-dCTP, which anneals to both the human and mouse form.

Hormone secretion studies

Static incubations. Cells (2×10^5) were plated on day 1 in DMEM media containing 25 mmol/l glucose and 15% FCS. On day 5 of culture, they were rinsed twice in Kreb's Ringer bicarbonate buffer (KRBB) containing 1.67 mmol/l glucose and 0.1% bovine serum albumin (BSA) and preincubated for 2 h in the same buffer solution. Calcium was omitted from the buffer solution when calcium-free conditions were required. Hormone secretion was assessed after a 2-h incubation period in buffer solution with the following conditions: *1*) 1.67 mmol/l glucose and 2.5 mmol/l calcium; *2*) 16.7 mmol/l glucose and 2.5 mmol/l calcium; *3*) 16.7 mmol/l glucose in the presence of 10 mmol/l arginine, 0.1 mmol/l 3-isobutyl-1-methylxanthine (IBMX), 5 μmol/l carbachol, and 2.5 mmol/l calcium; and *4*) 16.7 mmol/l glucose plus 10 mmol/l arginine, 0.1 mmol/l IBMX, and 5 μmol/l carbachol in the absence of calcium. At the end of the 2-h period, media was collected and briefly centrifuged, and the supernatant was stored at –20°C until assay. Cells were extracted with 95% ethanol/0.22 mol/l HCl at –20°C for 1 h, and the extracts were stored at –20°C until assay.

Perifusion experiments. Approximately 7×10^6 cells were mixed in 1.5 ml 100–200 mesh BioGel P-2 and overlaid onto 1.1 ml 200–270 mesh BioGel P-2 in a column that was perifused at a rate of 1 ml/min with KRBB. After a 50-min baseline perifusion period with 1.67 mmol/l glucose and 0.1% BSA in KRBB, secretion was stimulated by perifusion with either 16.7 mmol/l glucose or 16.7 mmol/l glucose plus 0.1 mmol/l IBMX in KRBB containing 0.1% BSA for a further 61 min. Perifusate medium contained 2.5 mmol/l calcium. Fractions were collected at the times indicated and stored at –20°C until assay.

Immunohistochemistry. Cells were grown in eight-well chamber slides for 5 days, then fixed with 4% paraformaldehyde/0.1 mol/l phosphate buffer (pH 7.4) and rinsed in 0.1 mol/l phosphate buffer (pH 7.4). The presence of insulin, human IAPP, somatostatin, and glucagon in these cells was detected by double staining immunofluorescence. The following primary antisera diluted in 0.1 mol/l phosphate buffer (pH 7.4)/0.5% BSA were used: monoclonal insulin antibody (BioGeneX, San Ramon, CA) at a dilution of 1:100; polyclonal human IAPP antibody (5436) at a dilution of 1:1,000; polyclonal somatostatin antibody (AS10; kind gift of Dr. John Ensinck) at a dilution of 1:2,000; and polyclonal glucagon antibody (14C; kind gift of Dr. Robert McEvoy) (30) at a dilution of 1:1,000. Binding of the insulin antibody was detected using rabbit anti-mouse IgG conjugated to fluorescein isothiocyanate (Jackson ImmunoResearch, West Grove, PA), which fluoresced green, whereas human IAPP, somatostatin, and glucagon polyclonal antibody binding were detected using a goat anti-rabbit IgG conjugated to Cy3 (Jackson ImmunoResearch), which fluoresced red.

High-performance liquid chromatography analysis. The method of high-performance liquid chromatography (HPLC) analysis of IAPP has been previously described (31). Briefly, 2350-2C0 and 2511 cell extracts were dried by vacuum cen-

TABLE 1
Proliferation and DNA content of 2350-2C0 and 2511 cell lines

	2350-2C0 cell line		2511 cell line	
Days in culture	Cell number ($\times 10^5$)	DNA (μg)	Cell number ($\times 10^5$)	DNA (μg)
0	2.00 ± 0.00	3.61 ± 0.25	2.00 ± 0.00	3.56 ± 0.40
3	2.60 ± 0.14	4.68 ± 0.25	2.75 ± 0.15	4.54 ± 0.04
5	4.08 ± 0.35	6.73 ± 0.96	4.15 ± 0.46	6.40 ± 1.29
7	7.61 ± 1.07	13.6 ± 1.71	6.87 ± 0.51	12.6 ± 1.76

Data are means ± SE and are taken from three independent experiments.

trifugation and reconstituted in distilled water containing 0.05% trifluroacetic acid. Human and mouse IAPP were separated by reverse-phase HPLC on a C18 column (Phenomenex, Torrance, CA) with a flow rate of 1 ml/min and a gradient of 15–40% acetonitrile (0.1% trifluroacetic acid) over 15–40 min, with fractions collected every 30 s. The quantity of human and mouse IAPP in each fraction was determined using specific immunoassays as described below.

Immunoassays. Immunoreactive insulin (IRI) was measured by a modification of the double antibody radioimmunoassay method of Morgan and Lazarow (32), using rat insulin as the standard. Mouse IAPP was measured with a radioimmunoassay using a polyclonal IAPP antibody (8342), HPLC-purified ^{125}I-labeled rat IAPP, and rat IAPP as standard (Peninsula, Belmont, CA). The 8342 antibody recognizes mouse and rat IAPP, but not human IAPP. The assay has an intra-assay coefficient of variation (CV) of 10% and an interassay CV of 9.1%. Human IAPP was assayed using an enzyme immunoabsorbance assay, using F024 as the capture antibody and F002 as the detection antibody (kind gift of Amylin Pharmaceuticals, San Diego, CA) (33). F002 specifically recognizes human, and not mouse, IAPP, such that samples that do not contain human IAPP read zero in this assay. The CVs for this assay are <10 and <15% for intra- and interassay, respectively (33).

Electron microscopic analysis

Tissue preparation. Cells were plated at a density of 2×10^5 on Thermonox coverslips (Nunc, Naperville, IL) and maintained in static incubation for 7 days in DMEM media containing 15% FCS and 16.7 mmol/l glucose. The cells were briefly rinsed with 37°C phosphate-buffered saline (PBS) and fixed with fresh 4% paraformaldehyde/1% gluteraldehyde in 0.1 mol/l PO_4, pH 7.35, for 1 h at room temperature. They were then rinsed three times with PBS over 20 min and washed overnight at 4°C with 0.05 mol/l of a glycine/PBS mixture. Cells to be examined for morphology were postfixed with OsO_4 in 0.1 mol/l PO_4 and embedded in Eponate 12 (Ted Pella, Redding, CA) by routine procedures. Cells for immunolabeling were dehydrated through ascending series of ethanol to 80% and infiltrated with 1:80% ethanol/LR 12 White hydrophilic acrylic resin (Ted Pella), followed by two 1-h changes of undiluted LR White at room temperature. The infiltration was continued with fresh LR White overnight at 4°C, and after a final change to fresh resin, polymerization under vacuum at 55°C for 48 h completed the embedment. After separation of the Thermonox coverslip from the embedded cells, 80–100 nm cross-sections were cut and mounted on 150-mesh nickel grids supported with carbon-coated parlodion.

Immunoelectron microscopy. After blocking nonspecific reactive sites with filtered 10% normal goat serum/1% BSA/PBS, immunolocalization of human IAPP in the cell lines was performed by treating the LR White–prepared sections with a polyclonal antibody raised in rabbits against human IAPP (5436) at a 1:8,000 dilution in 0.1% BSA/Tris-buffered saline (TBS), pH 7.6, overnight at 4°C. Insulin was similarly immunolocalized using rabbit polyclonal IgG to insulin (BioGeneX) at a final dilution of 1:200. Secondary antibody specificity was controlled by omitting the primary antibody from the diluent in the staining protocol. Antibody binding was visualized with goat anti-rabbit IgG/10 nm colloidal gold conjugate (Sigma, St. Louis, MO) diluted 1:50 in 0.1% BSA/TBS for 1 h at room temperature and stabilized by fixation with 3% gluteraldehyde/PBS for 10 min. The sections were subsequently postfixed with 1% OsO_4 vapors for 15 min and stained for tissue contrast with 7% aqueous uranyl acetate and lead citrate (34). They were viewed in a JEM 1200EX II (JEOL, Tokyo) electron microscope. A minimum of

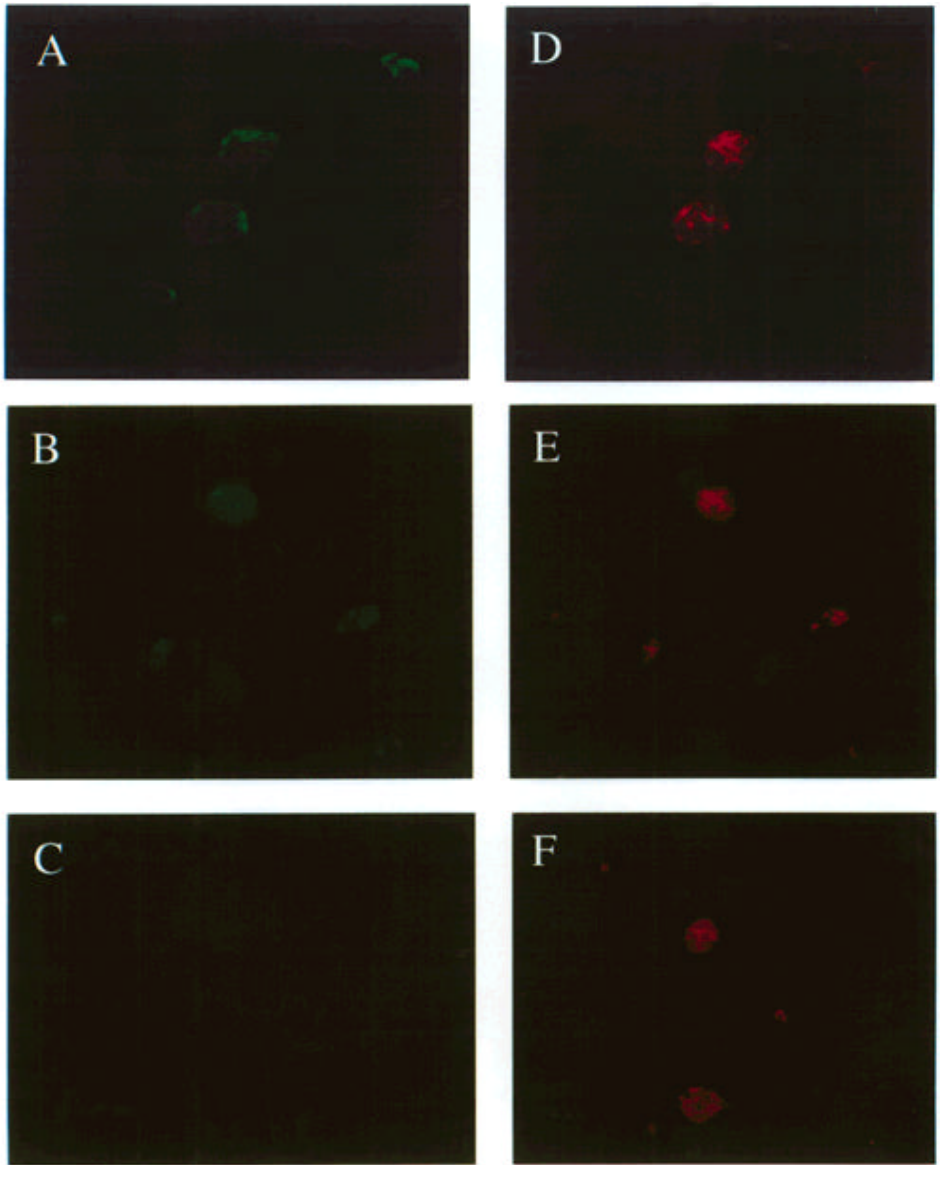

FIG. 1. Dual-label immunohistochemistry of insulin and IAPP, insulin and glucagon, and insulin and somatostatin in 2350-2C0 cells. *A–C*: insulin (green); *D*: IAPP; *E*: glucagon; *F*: somatostatin (red). Cells were grown for 5 days, fixed with 4% paraformaldehyde, and stained with the appropriate combination of antibodies.

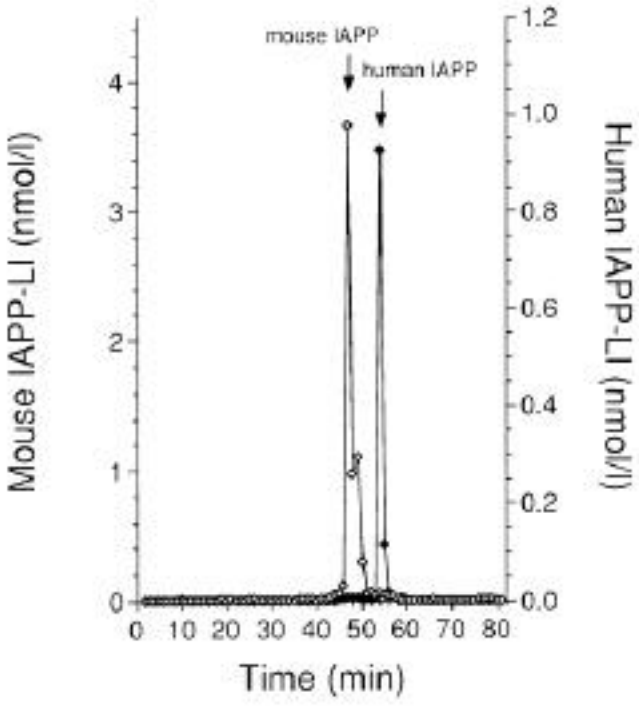

FIG. 2. HPLC analysis of an extract of 2511 cells demonstrating the presence of both mouse and human IAPP in the cells. Peptides were eluted on an acetonitrile gradient, and fractions were subsequently assayed using immunoassays specific for mouse and human IAPP. The elution times of synthetic mouse and human IAPP are illustrated.

40 islands of 5–10 cells each were examined for the presence of intra- and/or extracellular sites reactive to human IAPP or mouse insulin antibody.

Calculations and statistical analysis. Fractional release was calculated by dividing the amount of hormone measured in the media by the sum of the amount of hormone in the media plus that in cells.

Statistical analysis was performed using the Mann-Whitney *U* test. Data are presented as means ± SE of three independent experiments in which triplicate measurements were made. $P < 0.05$ was considered significant.

RESULTS

Proliferation and DNA content of 2350-2C0 and 2511 cells. Proliferation and DNA content of the two new cell lines was assessed by plating an equal number of cells at day 0 and determining DNA levels and the cells per dish at various intervals over a 7-day period. Table 1 shows that the number of cells and DNA content at 3, 5, and 7 days is comparable between the two human IAPP–expressing cell lines after plating at a density of 2×10^5 cells per well.

Northern blot analysis. Northern blot analysis of 2350-2C0 and 2511 cell lines for IAPP showed the presence of two specific bands, which correspond to the mouse and human forms, with the expected molecular size of ~1.0 and 1.4 kb, respectively (27).

Immunohistochemistry. 2350-2C0 cells were examined for the presence of insulin, human IAPP, glucagon, and somatostatin immunoreactivity by immunostaining. Insulin and human IAPP and insulin and glucagon localized to the same cells (Fig. 1), as previously described in other βTC cell lines (35). The majority of cells immunostained for insulin, with most of these insulin-positive cells also immunostaining for human IAPP. The number of glucagon-positive cells was much smaller than that of insulin-positive cells. Immunostaining for somatostatin was less common and was not found in cells containing insulin. A similar pattern of staining was found for the 2511 cell line (data not shown).

HPLC separation of mouse and human IAPP. Cell extracts from 2511 cells were subjected to HPLC analysis and confirmed the production of both mouse and human IAPP by this cell line (Fig. 2). Immunoreactivity for each peptide was observed in the expected fractions based on the known elution times of synthetic mouse and human IAPP. These findings are compatible with processing of the propeptide forms of IAPP to the intact 37–amino acid forms. A similar HPLC profile was obtained from 2350-2C0 cell extracts (data not shown).

Peptide content of 2350-2C0 and 2511 cells. Total cellular content of IRI and human and mouse IAPP-like immunoreactivity (IAPP-LI) for the two cell lines under static incubation conditions is shown in Table 2. The concentration of IRI is similar in the 2350-2C0 and 2511 cells. In contrast, mouse IAPP is sixfold and human IAPP-LI is twofold higher in 2511 compared with 2350-2C0 cells. Despite this increase in human IAPP expression, the rate of proliferation was similar between the two cell lines (Table 1).

Hormone secretion during static incubations. IRI secretion from 2350-2C0 cells after 2 h of incubation increased

TABLE 2
Cellular content of IRI and mouse and human IAPP-LI in 2350-2C0 and 2511 cells in static incubation conditions

Condition	IRI (nmol/l)	Mouse IAPP-LI (nmol/l)	Human IAPP-LI (nmol/l)
2350-2C0			
G1.67	120 ± 16	0.93 ± 0.28	0.35 ± 0.04
G16.7	123 ± 11	0.65 ± 0.20	0.21 ± 0.02
G16.7 + C	133 ± 8	0.65 ± 0.11	0.19 ± 0.02
G16.7 + C – Ca^{2+}	136 ± 6	0.55 ± 0.15	0.18 ± 0.04
2511			
G1.67	147 ± 52	4.79 ± 0.69	0.57 ± 0.15
G16.7	154 ± 37	4.14 ± 1.02	0.55 ± 0.12
G16.7 + C	130 ± 32	3.93 ± 0.47	0.47 ± 0.12
G16.7 + C – Ca^{2+}	134 ± 8	3.62 ± 0.20	0.48 ± 0.05

Data are means ± SE and are taken from three independent experiments. G1.67, 1.67 mmol/l glucose; G16.7, 16.7 mmol/l glucose; G16.7 + C, 16.7 mmol/l glucose plus a cocktail of 10 mmol/l arginine, 0.1 mmol/l IBMX, and 5 μmol/l carbachol; G16.7 + C – Ca^{2+}, 16.7 mmol/l glucose plus a cocktail of 10 mmol/l arginine, 0.1 mmol/l IBMX, and 5 μmol/l carbachol, in the absence of calcium.

TABLE 3
Percent fractional release by 2350-2C0 and 2511 cells of IRI and mouse and human IAPP-LI over a 2-h period in static incubation conditions

Condition	IRI secretion	Mouse IAPP-LI secretion	Human IAPP-LI secretion
2350-2C0			
G1.67	0.23 ± 0.06	0.57 ± 0.60	0.22 ± 0.01
G16.7	0.40 ± 0.03*	1.00 ± 0.80	0.80 ± 0.04*
G16.7 + C	16.5 ± 3.02*†	31.0 ± 13.9*†	18.6 ± 4.02*†
G16.7 + C – Ca^{2+}	0.72 ± 0.09‡	4.04 ± 2.70‡	0.81 ± 0.21‡
2511			
G1.67	0.30 ± 0.06	0.49 ± 0.19	0.52 ± 0.21
G16.7	0.43 ± 0.03*	1.24 ± 0.13*	0.80 ± 0.21*
G16.7 + C	26.4 ± 1.00*†	25.2 ± 4.11*†	25.9 ± 0.77*†
G16.7 + C – Ca^{2+}	2.65 ± 0.09‡	7.28 ± 0.56‡	2.98 ± 0.20‡

Data are means ± SE and are taken from three independent experiments. G1.67, 1.67 mmol/l glucose; G16.7, 16.7 mmol/l glucose; G16.7 + C, 16.7 mmol/l glucose plus a cocktail of 10 mmol/l arginine, 0.1 mmol/l IBMX, and 5 µmol/l carbachol; G16.7 + C – Ca^{2+}, 16.7 mmol/l glucose plus a cocktail of 10 mmol/l arginine, 0.1 mmol/l IBMX, and 5 µmol/l carbachol, in the absence of calcium. *$P < 0.05$ vs. G1.67; †$P < 0.01$ vs. G16.7; ‡$P < 0.01$ vs. G16.7 + C.

1.7-fold with high (16.7 mmol/l) glucose and 72-fold when high glucose was combined with the cocktail of secretagogues (IBMX, arginine, and carbachol) (Table 3). The omission of calcium from media containing 16.7 mmol/l glucose plus secretagogue cocktail resulted in a marked decrease in insulin secretion, to a level only 1.8-fold higher than that seen in the 16.7 mmol/l glucose plus calcium condition. Similarly, IRI secretion from 2511 cells was increased by 1.4-fold in response to 16.7 mmol/l glucose, the combination of high glucose and secretagogues eliciting an 88-fold increase, whereas the omission of calcium from the high glucose and secretagogue cocktail reduced IRI secretion to a level 6.2-fold higher than that seen in the 1.67 mmol/l glucose plus calcium condition (Table 3).

Mouse IAPP-LI release paralleled that of IRI in 2350-2C0 cells, with 16.7 mmol/l glucose concentration causing a 1.7-fold increase compared with low glucose. In the presence of 16.7 mmol/l glucose plus the secretagogue cocktail, release increased 54-fold compared with 1.67 mmol/l glucose, whereas the omission of calcium was associated with a marked decrease in mouse IAPP-LI secretion, but it was still fourfold higher than that seen in 16.7 mmol/l glucose alone. Likewise, 16.7 mmol/l glucose elicited a 2.5-fold increase in mouse IAPP-LI secretion compared with 1.67 mmol/l glucose from the 2511 cell line. Addition of secretagogue cocktail to 16.7 mmol/l glucose increased mouse IAPP-LI secretion by 51-fold compared with 1.67 mmol/l glucose, whereas the omission of calcium reduced secretion to 5.9-fold compared with the 16.7 mmol/l glucose condition (Table 3).

In 2350-2C0 cells, 16.7 mmol/l glucose concentration produced a 3.6-fold increase in human IAPP-LI release above that in 1.67 mmol/l glucose; 16.7 mmol/l glucose plus the secretagogue cocktail produced an 84-fold increase compared with 1.67 mmol/l glucose and, when calcium was omitted from the medium fractional release of human IAPP-LI, decreased to levels similar to those observed with 16.7 mmol/l glucose in 2350-2C0 cells (Table 3). In 2511 cells, 16.7 mmol/l glucose induced a 1.5-fold increase, and the addition of secretagogue cocktail to high glucose concentration a 50-fold increase, in human IAPP secretion compared with 1.67 mmol/l glucose (Table 3). The omission of calcium from the 16.7 mmol/l glucose plus secretagogues condition reduced human IAPP secretion from 2511 cells to 5.7-fold that seen with 1.67 mmol/l glucose plus calcium (Table 3).

Hormone secretion during dynamic perifusion. Perifusion of 2350-2C0 and 2511 cells with the combination of 16.7 mmol/l glucose and 0.1 mmol/l IBMX resulted in a biphasic stimulation of IAPP-LI and IRI release. In 2350-2C0 cells, peak early-phase secretion increased 4.7-fold for IRI, 6.2-fold for mouse IAPP-LI, and 4.8-fold for human IAPP-LI over the basal prestimulated condition (Fig. 3). Similarly, perifusion of 2511 cells with 16.7 mmol/l glucose plus 0.1 mmol/l IBMX increased peak early-phase secretion of IRI by 7-fold, mouse IAPP-LI by 5.2-fold, and human IAPP-LI by 7.6-fold. The typical early-phase response was followed by a sustained later-phase response, with the peak in IRI secretion corresponding to the peak(s) in IAPP-LI secretion. As with the mRNA and cellular content results, the secretion of the three hormones was higher from the 2511 compared with the 2350-2C0 cells.

Perifusion with 16.7 mmol/l glucose alone had a small impact on IRI and IAPP-LI secretion from both 2350-2C0 and 2511 cells (Fig. 3).

The ratio of IAPP to IRI remained constant in both cell lines and under both conditions, suggesting corelease of these peptides (Fig. 4). The percentage of IAPP to insulin released by the 2350-2C0 cell line differed for the IAPP peptides: ~5% for mouse IAPP-LI/IRI and 0.6% for human IAPP-LI/IRI. In the 2511 cell line, the findings were similar, with ratios of 10% for mouse IAPP-LI/IRI and 0.4% for human IAPP-LI/IRI.

Electron microscopic examination. 2350-2C0 and 2511 cells were grown at either 1.67 or 16.7 mmol/l glucose for 7 days, and three wells from each condition were examined for the presence of amyloid fibrils by electron microscopy. As illustrated in Fig. 5*A*, the transformed cells exhibited typical morphological characteristics, including the presence of secretory granules. Careful examination of cells at either glucose concentration failed to identify typical amyloid fibrils either intra- or extracellularly in cells prepared for either morphology or immunoelectron microscopy. Use of anti-human IAPP antibody, which had previously demonstrated

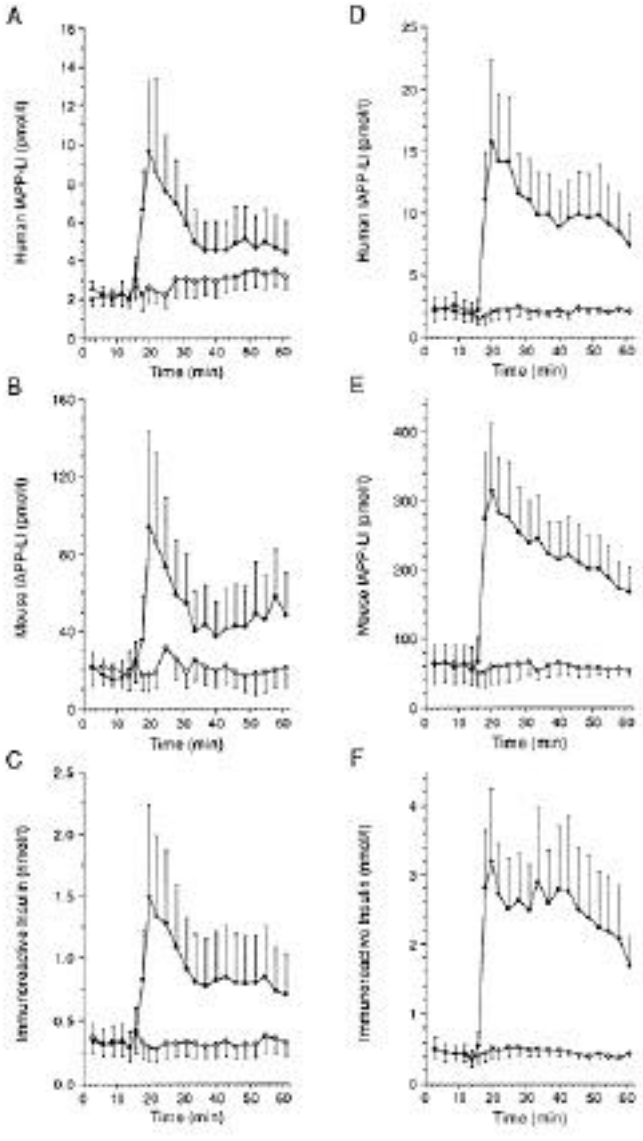

FIG. 3. Dynamic secretion of human IAPP-LI (*A* and *D*), mouse IAPP-LI (*B* and *E*), and IRI (*C* and *F*) from 2350-2C0 (*A–C*) and 2511 (*D–F*) cells in response to perifusion with medium containing 16.7 mmol/l glucose (◇) and 16.7 mmol/l glucose plus 0.1 mmol/l IBMX (◆). Cells were perifused with 1.67 mmol/l glucose before stimulation.

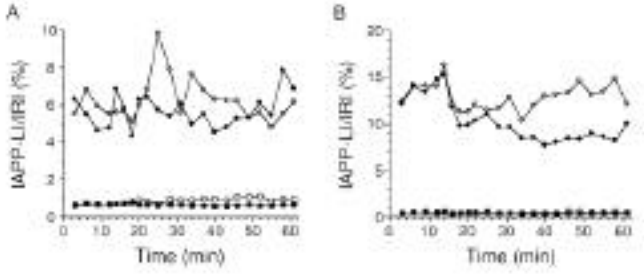

FIG. 4. Ratio of IAPP-LI to IRI secreted from 2350-2C0 (*A*) and 2511 (*B*) cells in response to 16.7 mmol/l glucose (□, ◇) and 16.7 mmol/l glucose plus 0.1 mmol/l IBMX (■, ◆). The IAPP-LI/IRI ratios illustrated are for human IAPP-LI/IRI (□, ■) and mouse IAPP-LI/IRI (◇, ◆).

reactivity in fibrils extracellular to β-cells in sections of transgenic mouse pancreas (26), was not successful in localizing areas of even small collections of typical fibrillar material intra- or extracellularly in either cell line. However, human IAPP antibody–positive material was identified intracellularly in secretory granules (Fig. 5*B*), as was insulin antibody–positive material (Fig. 5*C*).

DISCUSSION

Immortal β-cell lines have been used extensively for studying peptide synthesis and secretion, but currently available cell lines (e.g., βTC, HIT, INS) express rodent IAPP that is not capable of fibrillogenesis (6). Thus, to study cellular events involved in amyloid fibril formation, it was imperative to create a cell line that expresses an amyloidogenic form of IAPP such as human IAPP. We report here the establishment of two novel immortal β-cell lines that express, produce, and secrete the human form of IAPP. The expression and production of human IAPP in a cell line that is capable of processing the peptide and secreting it in a regulated manner will now allow for the study of these functions, which have been hypothesized to be involved in islet amyloidogenesis (13), and for the evaluation of the potential critical importance of other components of islet amyloid in fibril formation (11).

The content of human IAPP-LI and mouse IAPP-LI in 2511 cells was two- and sixfold higher, respectively, than that seen in 2350-2C0 cells, whereas IRI content was similar. The cell lines that we have established and those reported in the literature are heterogeneous rather than clonal populations of cells, β-cells being notoriously difficult to clone (18). Thus, the 2511 cell line probably contains a greater proportion of clones that express and produce human and mouse IAPP-LI than the 2350-2C0 cell line. Despite this difference in human IAPP-LI content, both cell lines have similar growth rates and hormone secretion patterns, suggesting that the clones that are present have not been selected for their resistance to human IAPP and that human IAPP is unlikely to be a major regulator of peptide release in these cell lines.

Both the 2350-2C0 and 2511 cell lines are capable of processing both mouse and human IAPP to the mature 37–amino acid peptide, as evidenced by HPLC analysis. Furthermore, human IAPP-LI, mouse IAPP-LI, and IRI secretion were stimulated by glucose and nonglucose secretagogues in both cell lines. In the 2350-2C0 and 2511 cell lines, secretion of IRI as well as mouse and human IAPP-LI in response to 16.7 mmol/l glucose was approximately two- to fourfold greater than that to 1.67 mmol/l glucose, which is consistent with other cell lines, such as βTC-3, βTC-6, HIT-T15, and INS-1, in which secretion has been observed to be two- to threefold higher (17,36–38).

Stimulation of granule exocytosis by incubation in a cocktail of secretagogues (glucose, arginine, carbachol, and IBMX), resulted in the release of ~20–30% of total cell content for IRI and human and mouse IAPP-LI in 2350-C0 and 2511 cells. These high percentages reflect the potency of the combined effects of arginine, carbachol, and IBMX in the presence of high glucose. The omission of calcium from this cocktail dramatically reduced secretion of all three peptides to a level two to six times higher than stimulation with 16.7 mmol/l glucose alone. These findings indicate that IAPP-LI and IRI are being secreted via the regulated pathway, which is calcium dependent. Collectively, these results suggest that the 2350-2C0 and 2511 cell lines represent physiologically appropriate

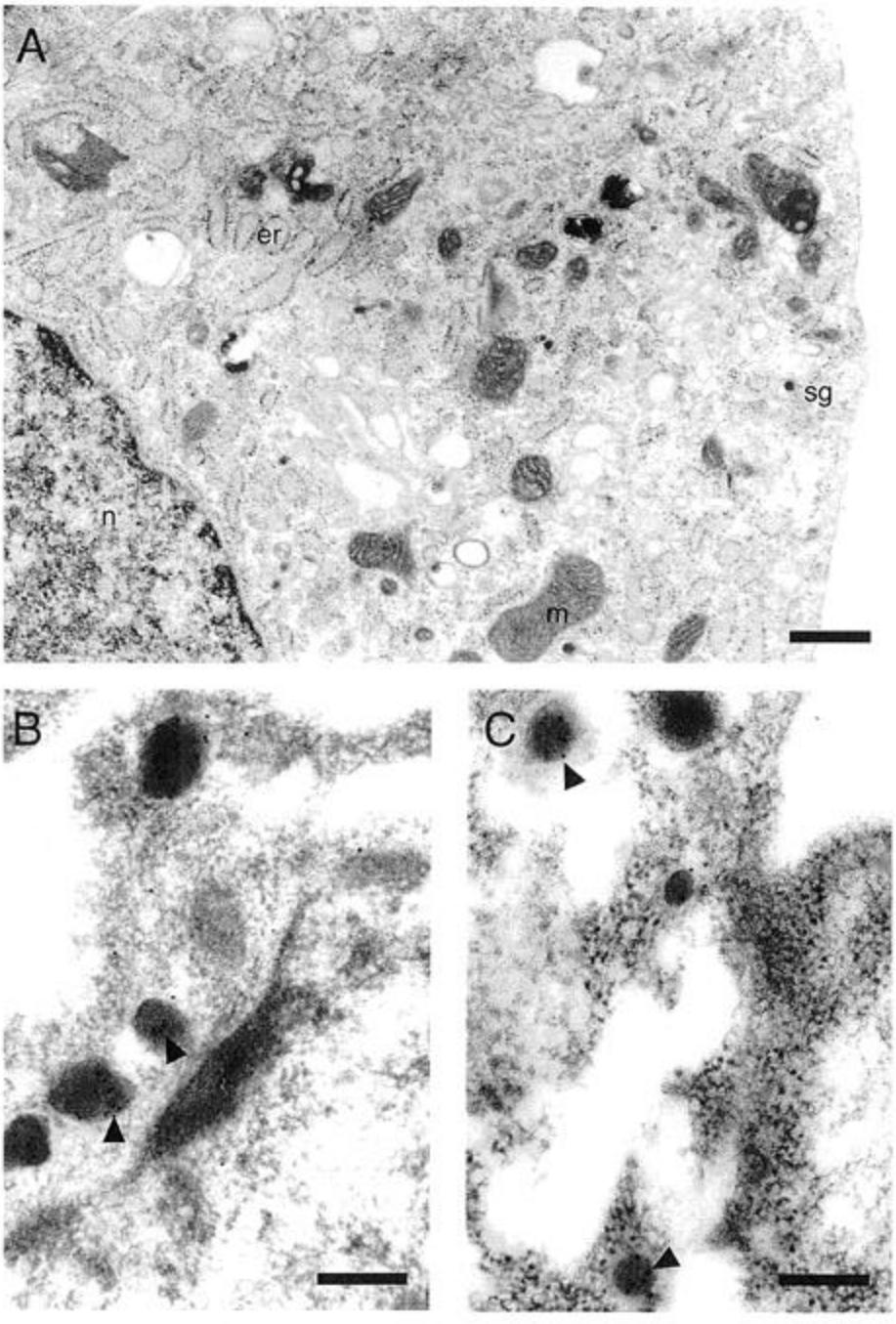

FIG. 5. Electron micrographs of the 2511 cell line illustrating features observed in sections of both cell lines. *A*: Section of typical morphology of cells illustrating nucleus (n), mitochondria (m), rough endoplasmic reticulum (er), and secretory granules (sg). The edge of the culture is illustrated on the right. *B*: Nonosmicated section immunogold labeled for human IAPP. Secretory granules contain human IAPP, as demonstrated by the presence of immunogold particles (arrowheads). *C*: Nonosmicated section immunogold labeled for insulin. Secretory granules contain insulin, as demonstrated by the presence of immunogold particles (arrowheads). Bars = 500 nm in *A* and 200 nm in *B* and *C*.

systems for the study of hormone production, processing, and secretion, especially as they relate to human IAPP.

Perifusion of both 2350-2C0 and 2511 cells with 16.7 mmol/l glucose and 0.1 mmol/l IBMX elicited a biphasic secretion of both IAPP-LI and IRI, reminiscent of that seen in islet cultures. Biphasic release of IRI has also been observed in other β-cell lines, such as βTC-6 and HIT-T15 (35). Cosecretion of IAPP-LI and IRI from the same secretory granules is strongly suggested by the fact that the peak responses for these two peptides coincided in both cell lines, and that the IAPP-LI–to–IRI ratio did not change over the course of the perifusion.

The omission of calcium, while markedly impairing secretion, did not completely suppress stimulated hormone secretion to basal levels. It has been shown that a rise in cAMP levels can result in insulin secretion without a rise in intracellular calcium levels (39). The IBMX in the secretagogue cocktail would be predicted to markedly impair the degradation of cAMP, and may explain the sustained hormone secretion observed under this condition. Another possibility may be that even though the cells were rinsed and preincubated for 2 h with calcium-free medium, there is still sufficient intracellular calcium that can be mobilized to result in significant hormone release.

The ratio of mouse IAPP-LI to IRI secretion in the 2350-2C0 and 2511 cell lines is similar to that observed from rat islets, and represents in large part regulated release (40). Perfusion of rat pancreas with secretagogues plus calcium after treatment of rats with streptozotocin (41) or dexamethasone (42) or when the pancreas is perfused with a ramped glucose

infusion (43) has also been associated with disproportionately increased IAPP release from the regulated pathway. On the other hand, disproportionately increased secretion of IAPP has previously been described in neonatal rat islet monolayers due to increased secretion of IAPP via the constitutive pathway (44). Similarly, βTC-3 cells have been shown to secrete a significant proportion of their content via a constitutive pathway (20,21). The form of the peptide released by the βTC-3 cells was largely unprocessed, comprising proIAPP and proinsulin. The differences in processing within the secretory pathways, and thus the products released, may be important factors in islet amyloid formation, based on the observation that the NH_2-terminal portion of proIAPP is present in islet amyloid deposits in human type 2 diabetes (45). Changing conditions under which these two cell lines are cultured, e.g., in the presence of different fatty acids (46,47), will allow for the study of many of these processes.

The absence of the typical amyloid fibrils in cultures of both 2350-2C0 and 2511 cells is consistent with the hypothesis that simple expression and production of an amyloidogenic form of the peptide under the conditions used in the present study is insufficient for formation (7). A previous approach to creating an in vitro cell system involved expressing human IAPP in COS-1 cells (48). In these transfected cells, it was observed that cells expressing human IAPP contained intracellular fibrils and that most cells were degenerating and dying 96 h after transfection. These findings suggested that marked overproduction of IAPP may be involved in producing cell death under these experimental conditions. This is something we have not observed in our cell lines, as shown by the similar doubling times for the 2350-2C0 and 2511 cells, which is compatible with observations in other cell lines derived from RIP-Tag mice (18). While it could be argued that our failure to observe the typical amyloid fibrils intracellularly may simply be due to insufficient human IAPP production, we believe that the explanation is not this simple and that these findings are consistent with observations in transgenic human IAPP mice and in pancreas samples from subjects with type 2 diabetes. The amyloid that develops after 12–16 months on a high-fat diet in transgenic mice and islet amyloid in human type 2 diabetes is not associated with intracellular IAPP fibrils, but both consistently show amyloid fibrils extracellularly (26,49,50), compatible with the need for release of these peptides into the extracellular space and the possible requirement for other amyloidogenic factors if there is not massive overproduction of IAPP (11). Furthermore, the 2350-2C0 and 2511 cells are models of pancreatic β-cells in that they are capable of sorting proIAPP to granules, processing it to the 37–amino acid form of the peptide and releasing IAPP in response to typical β-cell secretagogues. These processes, which do not occur in COS-1 cells, may be important factors involved in preventing or enhancing islet amyloid formation in disease states.

In conclusion, we have established two novel immortal β-cell lines that produce and secrete human IAPP-LI, mouse IAPP-LI, and IRI from the regulated secretory pathway. These cell lines should prove useful in investigating human IAPP production, processing, and secretion and whether other factors are important in the pathogenesis of amyloid fibril formation.

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