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Roles of CTPL/Sfxn3 and Sfxn Family Members in Pancreatic Islet

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Abstract Pancreatic AR42J cells have the feature of pluripotency of the precursor cells of the gut endoderm. Betacellulin (BTC) and activin A (Act) convert them into insulin-secreting cells. Using mRNA differential display techniques, we have identified a novel mitochondrial transporter, which is highly expressed during the course of differentiation, and have designated it citrate transporter protein-like protein (CTPL). Recently sideroflexin 1 (Sfxn1) was shown to be a susceptible gene of *flexed-tail* (*f/f*) mice, and CTPL has turned out to be a rat orthologous protein of Sfxn3, a member of sideroflexin family. CTPL/Sfxn3 was targeted to mitochondrial membrane like Sfxn1. The expression levels of CTPL/Sfxn3, Sfxn2, and Sfxn5 were upregulated in the early phase of differentiation into insulin-secreting cells but the expression levels of Sfxn1 and Sfxn3 did not change. All Sfxn family members were expressed in rat pancreatic islet. The expression levels of CTPL/Sfxn3, Sfxn2, and Sfxn5 were also upregulated in islets of streptozotocin-induced diabetic rats compared to normal rats. The downregulation of CTPL/Sfxn3 in a rat insulinoma cell line, INS-1, with the antisense oligonucleotide did not affect the insulin secretion. Taken together, CTPL/Sfxn3 and some other family members might be important in the differentiation of pancreatic β-cells as a channel or a carrier molecule and be related to the regeneration of pancreatic endocrine cells. J. Cell. Biochem. 95: 1157–1168, 2005.

Key words: AR42J cells; islet; CTPL; Sfxn; mitochondria

Both endocrine and exocrine cells of the pancreas arise from epithelial cells in the pancreatic duct [Pictet et al., 1972; Teitelman and Lee, 1987]. Recent genetic studies indicate that pancreatic development depends on an integrated network of distinct transcription factors operating at various levels. A mouse homeobox protein, insulin promoter factor-1 (IPF-1/PDX-1), is required for the development of the pancreas [Jonsson et al., 1994]. Islet-1,

Abbreviations used: CTPL, citrate transporter protein-like protein; Sfxn, sideroflexin; Act, activin A; BTC, betacellulin; STZ, streptozotocin; IBMX, 3-isobutyl-1-methylxanthine; PGP9.5, protein gene product 9.5; E, embryonic day.

The nucleotide sequence reported in this article has been deposited in the GenBank database under GenBank accession number AF276997.

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Beta-2/NeuroD, Pax4, and Pax6 are necessary for the development and generation of mature islet cells [Ahlgren et al., 1997; Naya et al., 1997; Sosa-Pineda et al., 1997; St-Onge et al., 1997]. The bHLH protein PTF1-p48 is essential for the formation of the exocrine and the correct spatial organization of the endocrine pancreas [Krapp et al., 1998]. Protein gene product 9.5 (PGP9.5), known as a neuroendocrine cell marker [Thompson et al., 1983], has been reported to be a pancreatic endocrine progenitor cell marker [Rooman et al., 2000; Yokoyama-Hayashi et al., 2002]. Embryonic stem cells have been shown to differentiate into insulin-secreting structures similar to pancreatic islet [Lumelsky et al., 2001]. Rajagopal et al. [2003] claimed that the insulin secretion of the cells resulted from an uptake of insulin from the cultured media, but some recent studies support the results, showing an increase of insulin secretion after cell differentiation [Vaca et al., 2003; Segev et al., 2004].

Rat AR42J cells were derived from a chemically-induced pancreatic tumor and have the feature of pluripotency of the common precursor cells of the pancreas [Rosewicz et al., 1992; Ohnishi et al., 1995]. When exposed to dexamethasone, they become more acinar-like cells [Logsdon et al., 1985]. As we have shown, when these cells were treated with activin A (Act) and betacellulin (BTC) or hepatocyte growth factor, these cells differentiate into insulin-producing cells [Mashima et al., 1996a,b]. In this way, AR42J cells resemble the common precursor cells in the developing pancreas and provide an excellent in vitro model system to study the differentiation of both endocrine and exocrine cells. To clarify the molecular mechanisms of differentiation, we used the method of mRNA differential display and identified several genes that were up- or downregulated during the differentiation [Mashima et al., 1999]. Among them, we have focused on one gene that was highly upregulated when treated with Act + BTC. This gene could be a novel mitochondrial transporter and was homologous to citrate transporter protein [Azzi et al., 1993]. We have designated it citrate transporter protein-like protein (CTPL). Recently sideroflexin 1 (Sfxn1) was shown to be a responsible gene of *flexed-tail* (*f*/*f*) mice [Fleming et al., 2001], and CTPL has turned out to be a rat orthologous protein of Sfxn3, a member of sideroflexin family. In this study, we examined the roles of CTPL/Sfxn3 and the other Sfxn family members in pancreatic endocrine cells.

MATERIALS AND METHODS

Materials

Recombinant human Act was provided by Dr. Y. Eto of Central Research Laboratory, Ajinomoto, Inc. (Kawasaki, Japan). Recombinant human BTC [Seno et al., 1996] was generously provided by Dr. M. Seno of Okayama University (Okayama, Japan).

Cell Culture

INS-1 cells were cultured in RPMI 1640 medium supplemented with 10 mM HEPES, 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol as previously reported [Asfari et al., 1992]. To stain cells, cells were grown on non-coated cover slips.

Streptozotocin (STZ) Treatment and Measurement of Plasma Glucose Levels

Male Wistar rats weighing approximately 300–325 g were injected ip with 70 mg STZ/kg. The STZ was dissolved in 0.2 ml of the citrate/phosphate buffer (31 mM citrate, 39 mM NaPi; pH 4.0–4.5). Control animals received injections of 0.2 ml of the citrate/phosphate buffer alone and were followed for the same periods of time as STZ-treated animals. Plasma glucose levels were determined and animals displaying plasma glucose levels greater than 250 mg/dl were considered to be diabetic. Pancreases were excised at 4 weeks after the injection of STZ. All rat experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Tokyo Institutional Animal Care and Use Committee.

Northern Blotting

Twenty microgram of total RNA extracted from AR42J cells was denatured and blotted onto a Hybond N^+ nylon membrane (Amersham, Arlington, IL). The blots were hybridized with ^{32}P-labeled cDNA probe and washed for 30 min in high stringency condition (0.1× standard saline citrate (SSC), 0.1% SDS) at 65°C before exposure to X-ray film.

Analysis of mRNA by Reverse Transcription-PCR

Rat pancreatic islet was prepared as described elsewhere [Lacy and Kostianovsky, 1967]. To eliminate the contamination of acinar cells, we picked up islet twice from the buffer. Total RNA was extracted from pancreatic islet by using Trizol Reagent (Invitrogen, Carlsbad, CA). For semi-quantitative RT-PCR, first-stranded cDNA was synthesized by using Superscript™ First-stranded Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instruction. Oligonucleotide primers and PCR cycles for semi-quantitative RT-PCR were listed in Table I.

Construction of CTPL/Sfxn3 Expression Vector

To construct the CTPL/Sfxn3 expression vector, we amplified a fragment of CTPL/Sfxn3 (nucleotides 228–1,198) by PCR using the sense primer (5′-ggaattcgcaagatgggcgacttgccc-3′) and the antisense primer (5′-ctcgagtcatcaaagccccttgttgtagggg-3′), followed by EcoRI and XhoI digestion. The fragment was subcloned into the EcoRI/XhoI sites of the vector, pcDNA3.1/His (Invitrogen), and was verified by sequencing. For transfection, LipofectAMINE PLUS Reagent (Invitrogen) was used according to the manufacturer's instruction. Forty-eight hours after the transfection, cells were fixed and stained.

Immunocytochemistry

For immunostaining, cells were grown on non-coated glass coverslips. Cells were fixed for 30 min in 3% paraformaldehyde in phosphate buffered saline (PBS), treated with 0.1% (vol/vol) Triton X-100 in PBS for 5 min, and incubated sequentially with Blocking Ace (Snow Brand, Tokyo, Japan), first antibody, and second antibody. Anti-Xpress Antibody (Invitrogen) was used to stain the exogenous CTPL/Sfxn3. Second antibody used in this study was FITC-conjugated anti-mouse IgG (Jackson Immuno Research Laboratories, West Grove, PA). Mito-Tracker Red CMXRos (Molecular Probes, Eugene, OR) was used to stain mitochondria before fixation. The cells were examined under a light microscope (Axiophoto; Carl Zeiss, Inc., Thornwood, NY).

Immuno-Electron Microscopy

A polyclonal antibody against CTPL/Sfxn3 was raised by immunizing rabbits with peptide (C)IQEPRWDQSTFLGRA (residues, 10–24) conjugated with keyhole limpect hemocyanine. Couping was done with m-Maleimidobenzoy-*N*-hydroxysuccinimide ester (Pierce) according to the manufacturer's instruction. The antiserum was purified with a PD10-gel column (Amersham Biosciences, Piscataway, NJ). The cells were fixed by immersion in a mixture of 0.2% glutaraldehyde, 2% paraformaldehyde, and 0.2% picric acid in 0.05M cacodylate buffer, pH 7.4, at 4°C for 2 h. After washes with the above buffer, the cells were collected and embedded in agarose. The agarose blocks were dehydrated through a graded ethanol series, and infiltrated with LR White resin (London Resin Co., Basingtoke, UK). Ultrathin sections were cut with a Reihert Ultracut-E microtome (Reichert-Jung, Vienna, Austria) equipped with a diamond knife and then mounted on nickel grids. Immunolabeling was performed by the one-face, double-labeling method with different gold particle sizes for CTPL/Sfxn3 and insulin. One side of the ultrathin section was preincubated with 1% BSA-PBS for 2 h, reacted with a mixture of rabbit anti-CTPL/Sfxn3 (1:500) and guinea pig anti-porcine insulin (1:2,000) overnight, rinsed with glycine-PBS, reacted with a

TABLE I. Sequences of PCR Primers Used in This Study and PCR Cycles Needed to Amplify the Products

Gene	Sense primer	Antisense primer	Size of product (bp)	GenBank accession number	PCR cycles	
					AR42J	Islet
Sfxn1	acgcagcaaagcaagccatcacgc	tctgaggaaacagagcacagcac	210	S70011	25	30
Sfxn2	tcccaatgatgcgacagcag	aagaggaggaagcagccacacag	256	XM_215265	30	30
CTPL/Sfxn3	actctggagaagaaagacttcc	tcccccagtctaaggttg	374	AF276997	25	30
Sfxn4	gaaaaccatcgaggaattgaggtg	aagctgactgcactggatctgtcc	297	XM_214736	30	35
Sfxn5	tcaggcacttcttggacatcatc	ggcgtcccgaaaggaatgtaac	232	NM_153298	30	30
PDX-1	acatctccccatacgaagtgcc	aagttgagcatcactgccagctcc	364	U04833		32
PGP9.5	ggctggaggaggagatcctg	agtgaattctctgcagaccttg	518	D10699		32
β-actin	tgagagggaaatcgtgcgtg	gatccacatctgctggaaggtg	460	V01217	18	25

mixture of 5-nm anti-rabbit IgG–gold complexes (BioCell, Res. Lab., Cardiff, UK) and 10-nm anti-guinea pig IgG–gold complexes (BioCell) for 1 h, rinsed with PBS and then washed with DW. After the immunolabeled section was stained with uranyl acetate, they were observed with a Hitachi H-7500 electron microscope at 80-kV.

Western Blotting

Protein extracts from INS-1 cells and rat pancreatic islets were prepared for immunoblotting as described previously [Kanzaki et al., 1999]. For Western blotting, membranes were blocked by incubation for 1 h with 10% Blocking Ace (Snow Brand, Japan) in PBS. After blocking, the membranes were incubated with anti-CTPL/Sfxn3 antibody (1:100 dilution) for 1 h at room temperature, washed three times with PBS containing 0.1% Tween-20 for 10 min, and incubated for 30 min with horseradish peroxidase-conjugated goat anti-rabbit whole immunogloblin (Nordic, 1:500 in PBS, 0.1% Tween-20). After washing, the enhanced chemiluminescence Western blotting detection reagent (Amersham Biosciences) was added, and the reaction was allowed to proceed according to manufacturer's recommendations. Exposure to X-ray film was 1–3 min at room temperature. To remove the probe, membranes were incubated with the stripping buffer containing 62.5 mM Tris/HCl (pH 6.7) and 2% SDS for 30 min at 50°C. Anti-actin (1–19) antibody was purchased from Santa Cruz Biotechnology.

Static Incubation for Determination of Insulin Secretion

INS-1 cells (approx. 3×10^5 cells/well) were seeded in multiwell plates coated with poly-L-ornithine (Sigma) and cultured for 24 h before the transfection. For antisense oligonucleotide transfection, OligofectAMINE Reagent (Invitrogen) was used according to the manufacturer's instruction. Forty-eight hours after the transfection, cells were preincubated in modified Krebs–Ringer bicarbonate-HEPES buffer (KRBH) containing 3 mM glucose for 30 min, followed by the incubation in the same buffer containing test substances as indicated for 30 min at 37°C. Insulin secreted in the incubation buffer was measured by RIA using rat insulin as standard. Statistics results are presented as mean ± SEM from three independent experiments. Statistical significance was determined by unpaired Student's *t*-test. In case of multiple comparisons, data were evaluated by one-way ANOVA followed by post hoc analysis of Scheffe. Differences between experimental and control groups were considered "significant" at $P < 0.05$. Antisense oligonucleotides used in this study were AS1: 5′-ttgatatttaagggcaagtc-3′ (nucleotides 258–239), AS1′: 5′-tgttgatatttaagg-3′ (nucleotides 260–246), AS2: 5′-ttgtgcttggatctgag-3′ (nucleotides 1,156–1,140), Control1: 5′-aagtcagtggtgaagctatgac-3′, and Control2: 5′-tgggtttgtctcagactttg-3′.

RESULTS

Molecular Cloning of CTPL/Sfxn3

In mRNA differential display, we have identified several novel genes upregulated by more than tenfold when AR42J cells were incubated with Act + BTC [Mashima et al., 1999]. Those upregulations were also attenuated when we blocked the differentiation into insulin-secreting cells by PD098059, an inhibitor of MAP kinase cascade [Dudley et al., 1995; Mashima et al., 1999]. We picked up one gene (*AB18*), which was highly upregulated with Act + BTC and the size of mRNA was estimated to be about 3.2 kbp according to the size of Northern blot analysis [Fig. 1; Mashima et al., 1999]. From the rat islet cDNA library, which was a generous gift from Dr. Takeda, Institute of Molecular & Cellular Regulation, Gunma University, we obtained the 2,699-bp cDNA, which comprises an open reading frame of 963 bp, 232-bp 5′-UTR, and a 1,504-bp 3′-UTR with the poly(A) tail. There may be some additional regions of the 5′-UTR to be cloned; however, there is an in-frame stop codon in the 5′-UTR of the sequence. The open reading frame encodes a predicted polypeptide of 321 amino acids with a calculated molecular mass of 35.4 kDa. With the Blast search, this gene has 76% homology to rat mitochondrial tricarboxylate carrier [Azzi et al., 1993; GenBank accession no. S70011] and we designated this gene as CTPL (GenBank accession no. AF276997). However, another mitochondrial protein (GenBank accession no. L12016), which is a member of the mitochondrial carrier family (MCF), has been conclusively shown to transport tricarboxylic acids [Kaplan et al., 1993; Xu et al., 1995] and the function of CTPL may not be related to the carrier of tricarboxylate. Recently Sfxn1 was shown to be a susceptible gene of *flexed-tail* (*f/f*) mice [Fleming et al., 2001] and

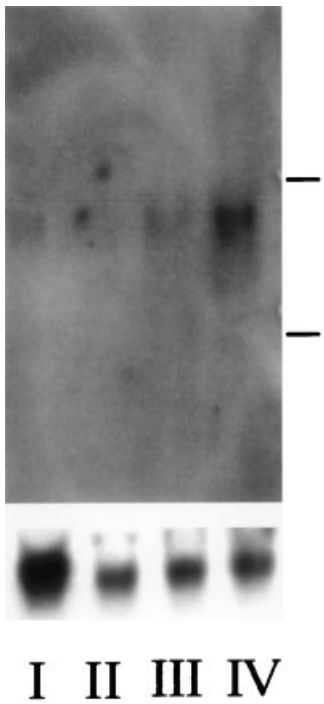

Fig. 1. Northern blot analysis of CTPL/Sfxn3 expression in AR42J cells. Total RNA (20 μg) isolated from naïve (I), Dexamethasone-treated (II), Activin A-treated (III), and Activin A + Betacellulin-treated (IV) cells was blotted onto a nylon membrane and probed with ^{32}P-labeled CTPL/Sfxn3 cDNA. The bars indicate the relative positions of 28s and 18s ribosomal RNA. The blot was reprobed by ^{32}P-labeled rat G3PDH cDNA (**lower panel**).

the orthologous rat protein was proved to be the mitochondrial tricarboxylate carrier [Azzi et al., 1993; GenBank accession no. S70011]. Mouse Sfxn forms a family, Sfxn1 to Sfxn5, from the examination of mammalian EST and genomic database searches [Fleming et al., 2001]. CTPL had 98.7% identity and 99.4% similarity with mouse Sfxn3 in the amino acid level and was considered to be a rat orthologous protein of Sfxn3.

Expression of Sfxn Family Members in Rat Pancreatic Islet

As *CTPL/Sfxn3* gene was cloned from islet cDNA library and mouse Sfxn3 was ubiquitously expressed [Fleming et al., 2001], CTPL/Sfxn3 was expected to be expressed in pancreatic islet. We confirmed the expression of CTPL/Sfxn3 in islet by RT-PCR and examined the expressions of the other Sfxn members; Sfxn1, Sfxn2, Sfxn4, and Sfxn5. As shown in Figure 2, all Sfxn family members were expressed in rat pancreatic islet.

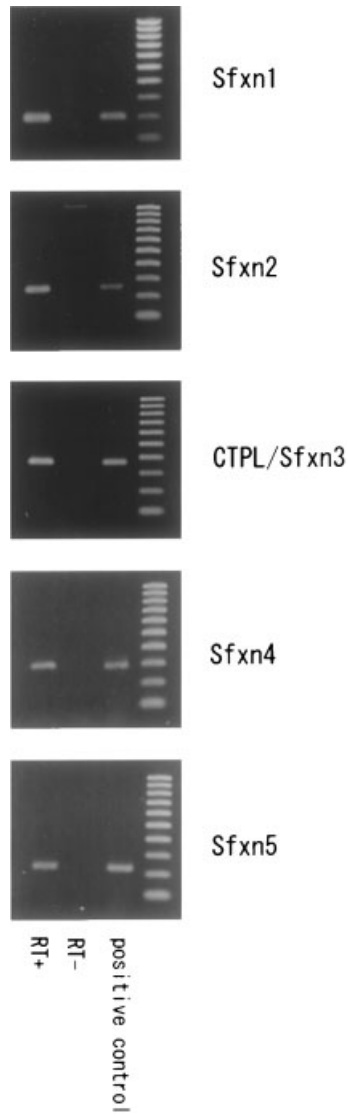

Fig. 2. RT-PCR of Sfxn family members in rat pancreatic islet. RT-PCR was performed using rat islet total RNA as a template. Samples with (RT+) or without (RT−) reverse transcriptase were loaded. **Third lane** was the positive control of rat liver cDNA for Sfxn1 and Sfxn2 and rat brain cDNA for CTPL/Sfxn3, Sfxn4, and Sfxn5. The **right lane** is a 100-bp ladder size marker.

Changes of the Expression Levels of Sfxn Family Members in AR42J Cells During the Differentiation Into Insulin-Secreting Cells

The expression of CTPL/Sfxn3 was highly upregulated in AR42J cells during the differentiation into insulin-secreting cells [Mashima et al., 1999]. Next we examined the changes of expression levels of the other Sfxn family members in AR42J cells by Northern blot analysis (Fig. 3A). As we showed previously, the expression level of CTPL/Sfxn3 was rapidly

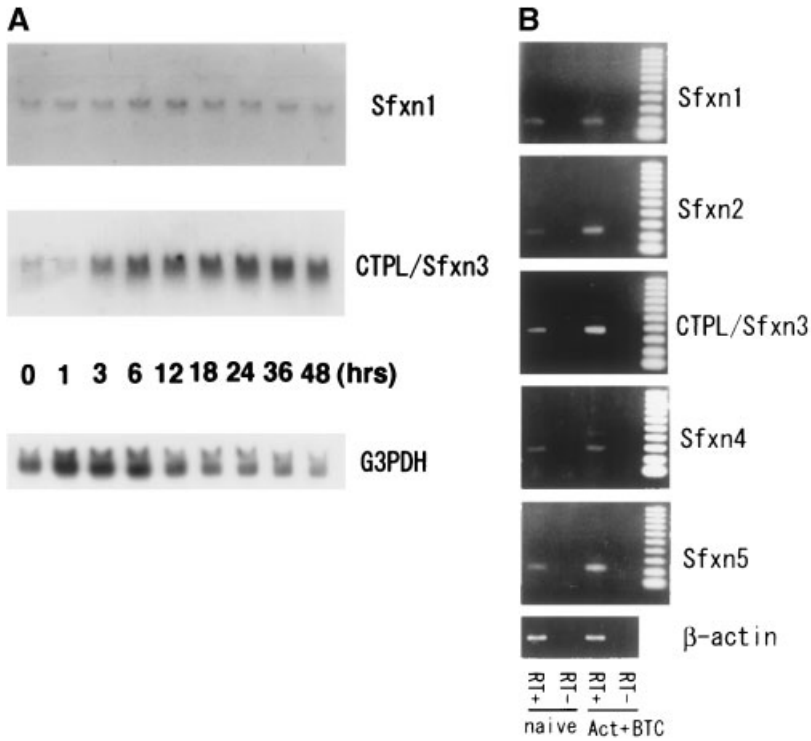

Fig. 3. Changes of expression levels of Sfxn family members in AR42J Cells. **A**: Total RNA (20 μg) was isolated from ACT + BTC-treated cells at 0, 1, 3, 6, 12, 18, 24, 36, and 48 h, blotted onto a nylon membrane and probed with ^{32}P-labeled cDNA probe. The blot was reprobed by ^{32}P-labeled rat G3PDH cDNA (**lower panel**). **B**: mRNA of Sfxn family members were compared in naïve AR42J cells and Activin A + Betacellulin-treated cells (48 h) by semi-quantitative RT-PCR. Samples with (RT+) or without (RT−) reverse transcriptase treatment were loaded. The **right lane** is a 100-bp ladder size marker.

increased in response to Act + BTC within 3 h. On the other hand, the expression of Sfxn1 did not change during the differentiation and the expressions of Sfxn2, -4, and -5 could not be detected. In the next set of experiments, we compared the expression levels of Sfxn family members by semi-quantitative RT-PCR. As shown in Figure 3B, CTPL/Sfxn3, Sfxn2, and Sfxn5 were upregulated in Act + BTC-treated AR42J cells and the expression levels of Sfxn1 and Sfxn4 did not change. In Sfxn2, Sfxn4, and Sfxn5, we needed five more PCR cycles to compare the expression levels than in Sfxn1 and CTPL/Sfxn3 (Table I). The difference of PCR cycles needed to amplify the products may reflect the signal intensities in Northern blot analysis (Fig. 3A).

Subcellular Localization of CTPL/Sfxn3 Protein in INS-1 Cells

Sfxn1 had no canonical mitochondrial targeting signal but it was targeted to mitochondria [Fleming et al., 2001]. To investigate the subcellular localization of CTPL/Sfxn3, we ectopically expressed CTPL/Sfxn3 protein using the expression vector CTPL/Sfxn3-pcDNA3.1/His. We could see a punctate pattern in the cytoplasm of INS-1 cells, a rat insulinoma cell line, consistent with CTPL/Sfxn3 protein being present in intracellular organelles (Fig. 4A). To confirm that CTPL/Sfxn3-positive cytoplasmic organelles were mitochondrial, we treated INS-1 cells with mitochondrial-specific fluorescent dye MitoTracker Red CMXRos (Molecular Probes) to label mitochondria before fixation. As shown in Figure 4B,C, CTPL/Sfxn3 protein was targeted to the mitochondrial membrane. Ultrastructural examination of the β-cells has suggested that the mitochondria are often in close proximity to the secretory insulin granules [Wollheim, 2000]. The electron microscopical study in INS-1 cell showed that endogenous CTPL/Sfxn3 (small gold particle) was present in mitochondria (Fig. 5a) and many secretory insulin granules (large gold particle) were located near mitochondria (Fig. 5b).

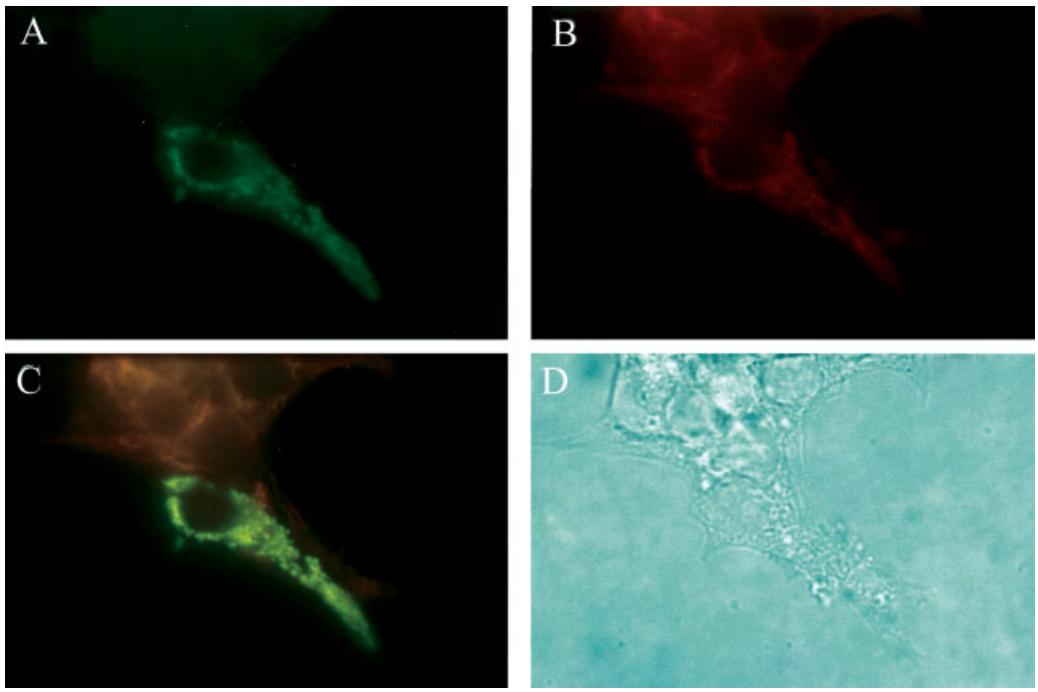

Fig. 4. Subcellular localization of CTPL/Sfxn3 in INS-1. INS-1 cells were transfected with CTPL/rSfxn3-pcDNA3.1/His (**A**) and pulsed with Mitotracker Red CMXRos before fixation (**B**). Colocalization of the His-tagged protein and mitochondria is seen as yellow fluorescence in the merged image (**C**). **D**: Phase-contrast image of the same field.

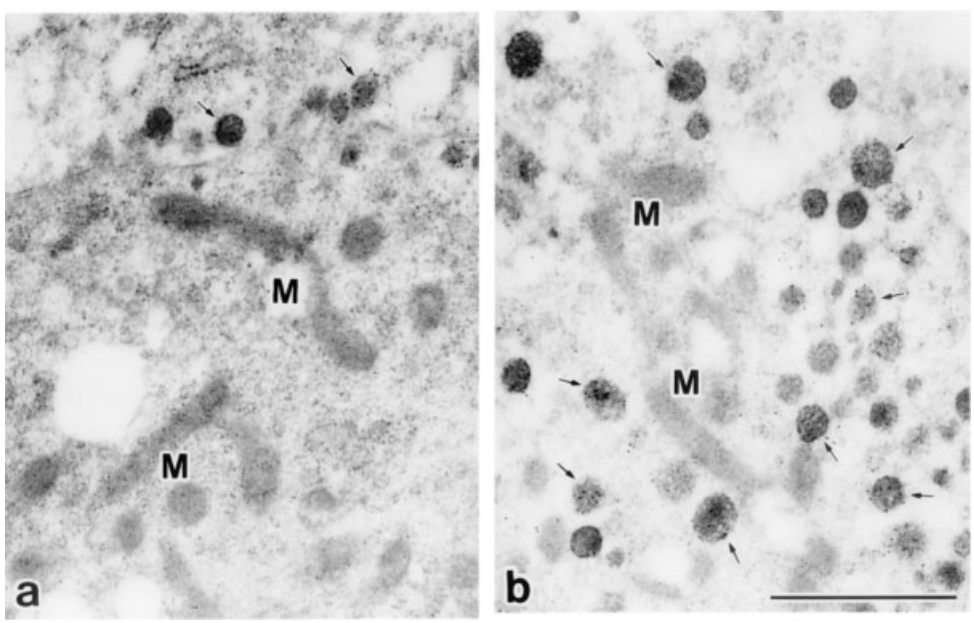

Fig. 5. Electron micrographs of INS-1 cell. INS-1 cell was double-immunolabeled for CTPL/Sfxn3 (small gold particles) and insulin (large gold particles). CTPL/Sfxn3 was targeted to mitochondria (**a**) and many secretory insulin granules were located near mitochondria (**b**). M: mitochondria, arrows: secretory granules. Bar = 1 μm.

Comparison of the Expression Level of CTPL/Sfxn3 and the Other Family Members in Normal and STZ-Induced Rat Pancreatic Islet

In the next set of experiments, we compared the expression levels of CTPL/Sfxn3 and the other Sfxn family members between normal rats and STZ-induced diabetic rats. As shown in Figure 6B, when we used islets from mild diabetic rats (plasma glucose level >250 mg/dl), the expressions of PDX-1 and PGP9.5 were increased in STZ-induced rats, meaning that islet regeneration was stimulated. In that condition, the expression levels of CTPL/Sfxn3, Sfxn2, and Sfxn5 were upregulated and the expression levels of Sfxn1 and Sfxn4 did not change. These changes were all the same to those in Act + BTC-treated AR42J cells.

Influence of CTPL/Sfxn3 on Insulin Secretion

As CTPL/Sfxn3 is present in mitochondria which are often located near the secretory insulin granules, and the expression of CTPL/Sfxn3 is increased in STZ-induced diabetic rats, we next studied whether the expression level of CTPL/Sfxn3 might affect the insulin secretion. As shown in Figure 7A–C, the expression level of CTPL/Sfxn3 was markedly decreased in mRNA level and protein level with the transfection of antisense oligonucleotide (AS1′) compared to the control. However, the release of immunoreactive insulin did not change in the AS1′-transfected cells with the stimulation of the elevation of ambient glucose, 3-isobutyl-1-methylxanthine (IBMX) and a depolarizing concentration of potassium.

DISCUSSION

In the present study, we have identified a novel cDNA, CTPL/Sfxn3, encoding a protein with 321 amino acids belonging to a mitochondrial transporter family. Mitochondrial anion transporters, such as citrate carrier,

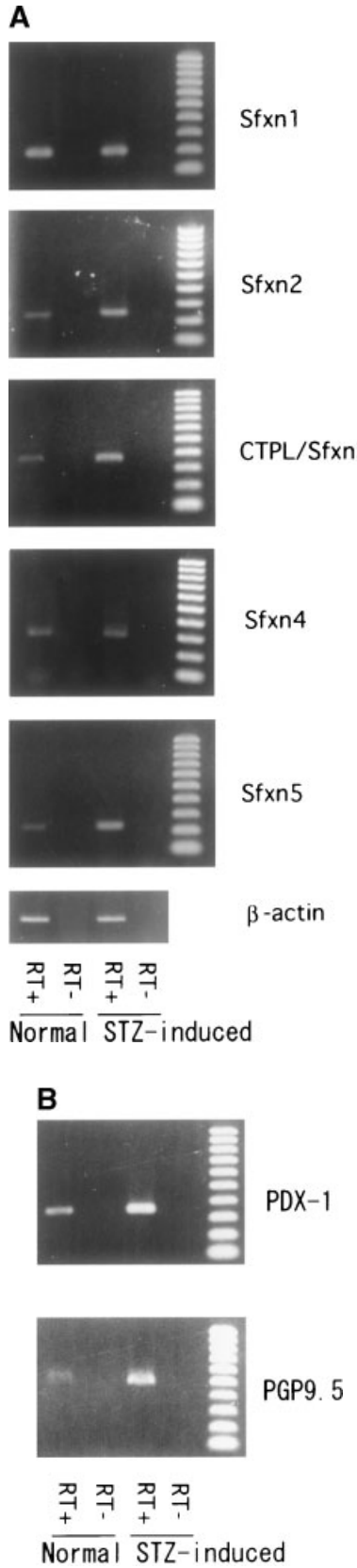

Fig. 6. Comparison of the expression levels of Sfxn family members, PDX-1, and PGP9.5 in normal and STZ-induced rat pancreatic islet. mRNA of Sfxn family members (**A**), PDX-1, and PGP9.5 (**B**) were compared in normal and STZ-induced rat pancreatic islet by semi-quantitative RT-PCR. Samples with (RT+) or without (RT−) reverse transcriptase treatment were loaded. The **right lane** is a 100-bp ladder size marker. The experiments were repeated three times independently and the representative figures are shown.

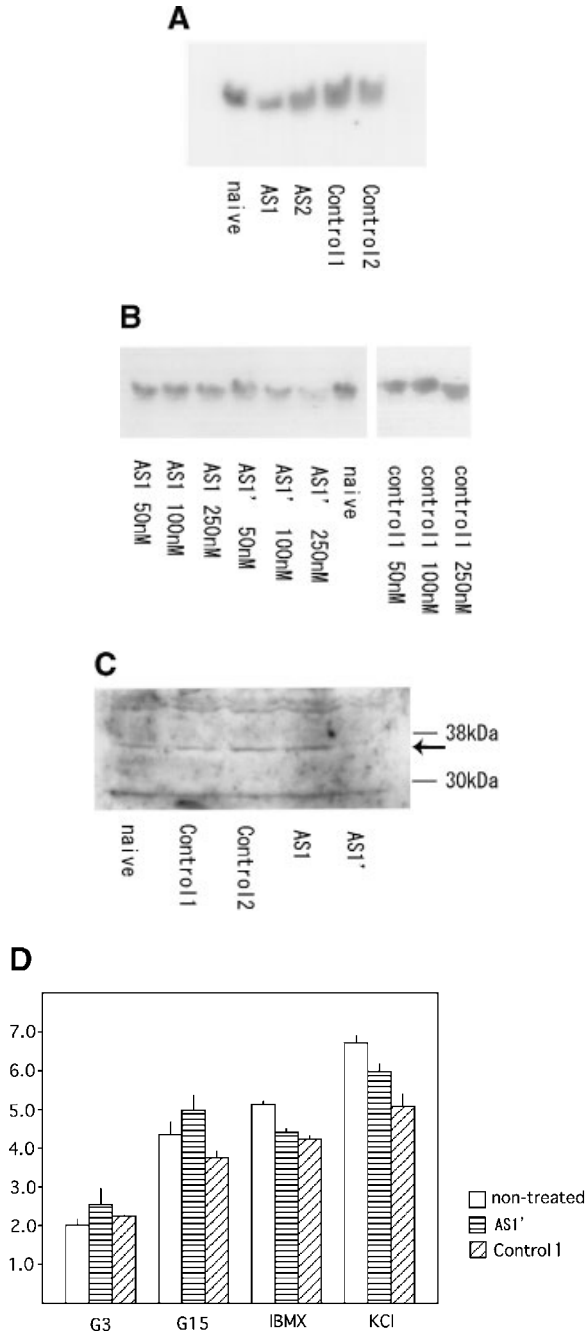

Fig. 7. Insulin secretion in INS-1 cells. **A**: INS-1 cells were transfected with antisense oligonucleotide or control oligonucleotide (250 nM) and incubated for 48 h. mRNA level was compared by Northern blot analysis. **B**: AS1′, which is consisted of the short core fragment of AS1, blocked the expression of CTPL/Sfxn3 in a dose-dependent manner. **C**: CTPL/Sfxn3 protein level was compared by Western blot analysis. The concentration of antisense oligonucleotide used was 250 nM. The arrow indicates the position of the protein. **D**: For the measurement of insulin secretion, INS-1 cells were incubated for 60 min in KRB buffer containing 3 mM glucose, 15 mM glucose, 40 mM potassium, or 100 μM IBMX and insulin secretion was measured. Statistics results are presented as mean ± SEM from three independent experiments.

pyruvate carrier, phosphate carrier, and dicarboxylate carrier, usually have six transmembrane domains and have a tripartite structure, made up of related sequences about 100 amino acids in length [Palmieri, 1994]. Each repetitive element contains two hydrophobic stretches separated by an extensive hydrophilic region [Palmieri, 1994]. Sideroflexin family members are predicted to have five transmembrane domains according to Kyte and Doolittle algorithm. Microcytic and hypochromatic anemia is a fully penetrant feature of *flexed tail* (*f/f*) mice and iron deposits are seen in mitochondria of erythrocyte in the fetal and neonatal stage [Chui et al., 1977]. Sfxn1 bears no canonical mitochondrial targeting signal but it was targeted to mitochondrial membrane [Fleming et al., 2001]. These features suggest that Sfxn family members might be channels or carrier molecules in mitochondria, which either supply substrate or remove product.

AR42J cells were originally derived from a rat pancreatic acinar cell tumor and they possess both exocrine and neuroendocrine properties [Rosewicz et al., 1992]. When these cells were incubated with Act, a member of TGF-β superfamily, and BTC, a member of EGF superfamily, or hepatocyte growth factor, they differentiate into insulin-secreting cells [Mashima et al., 1996a,b]. CTPL/Sfxn3 was identified as one of molecules whose expression levels were upregulated with the incubation of Act + BTC more than tenfold than naive cells and the upregulation was attenuated by PD058059 [Mashima et al., 1999]. The expression of CTPL/Sfxn3 was rapidly increased in response to Act + BTC after 3 h [Fig. 3; Mashima et al., 1999], suggesting that the protein encoded by CTPL/Sfxn3 exert the function at an early phase in the differentiation of AR42J cells.

Mitochondrial carriers are encoded by nuclear genes and have to be imported into the mitochondrial membranes. Like Sfxn1 and most mitochondrial carriers except phosphate and citrate carriers in mammals [Runswick et al., 1987, 1990], CTPL/Sfxn3 has no obvious amino-terminal mitochondrial import sequence, but localization studies clearly showed that CTPL/Sfxn3 is expressed in mitochondria (Figs. 4 and 5). Ultrastructural examination of the β-cells has suggested that the mitochondria are often in close proximity to the secretory insulin granules [Fig. 5; Wollheim, 2000]. In pancreatic β-cells, ATP and other mitochondrial factors

accomplish the coupling of glucose metabolism to insulin secretion. The mitochondrial anion transporters play important roles in metabolic regulation. In STZ-induced diabetic rats, the activities of the pyruvate and dicarboxylate transporters were increased and the activity of the citrate transporter was progressively decreased with time [Kaplan et al., 1990]. Daily supplementation of STZ-induced diabetic rats with insulin reversed the alterations of the activities of mitochondrial anion transporters [Kaplan et al., 1991]. The activity of the ADP/ATP transporter was decreased in diabetic status [Lerner et al., 1972]. The expression levels of CTPL/Sfxn3, Sfxn2, and Sfxn5 were upregulated in STZ-induced diabetic rats compared to normal rats, and the changes were all the same to those seen in the differentiation of AR42J cells into insulin-secreting cells (Figs. 3 and 6A). In STZ-induced experimental diabetes, islet regeneration is stimulated to compensate for the loss of functional β-cells. PDX-1 is a homeobox transcription factor, which binds and transactivates the insulin promoter and is crucial for pancreatic organogenesis [Jonsson et al., 1994]. During pancreatic development, differentiated cell types arise from ductal progenitor cells expressing PDX-1 [Guz et al., 1995]. In fetal pancreas, a cluster of cells expressed PGP9.5, a known neuroendocrine marker [Thompson et al., 1983], among initial epithelial bud at embryonic day 11.5 (E11.5) and PGP9.5 is considered to be a marker for the progenitor of pancreatic endocrine cells [Rooman et al., 2000; Yokoyama-Hayashi et al., 2002]. As shown in Figure 6B, PDX-1 and PGP9.5 were upregulated in STZ-induced pancreatic islets from mild diabetic rats, showing the stimulation of islet regeneration. The upregulations of CTPL/Sfxn3, Sfxn2, and Sfxn5 in diabetic islets and in Act + BTC-treated AR42J cells suggest that these molecules have some functions related to the regeneration of pancreatic endocrine cells.

In the case of Sfxn1, the expression pattern in the embryo varies with the stage of development and very high levels of mRNA are present in the liver during the period of embryonic hepatic erythropoiesis [Fleming et al., 2001]. The anemia in *flexed-tail* (*f/f*) mice is transient, most severe at E15, persists through the second week of postnatal life and ameliorates. This cessation coincides with the physiologic disappearance of erythropoietic activity on the liver. Sfxn1 is widely expressed but present in the highest levels in adult kidney and liver and Sfxn2 is also present mainly in liver and kidney. The anemia in *flexed-tail* (*f/f*) mice is considered to be due to the erythropoietic activity in the liver during the embryonic and neonatal stages, but it could be possible that the disappearance of anemia is due to the compensation of Sfxn2 for the impaired Sfxn1 function, though the expression level of Sfxn2 in fetal and neonatal stages is unknown.

Sfxn4 is mainly expressed in kidney, Sfxn5 is in brain, and CTPL/Sfxn3 is ubiquitously expressed [Fleming et al., 2001]. All Sfxn family members were detected by RT-PCR in pancreatic islet. Antisense oligonucleotide study of CTPL/Sfxn3 did not show any changes in insulin secretion, but other Sfxn family members might compensate for the function of CTPL/Sfxn3.

CTPL/Sfxn3 was highly expressed in the early phase of differentiation of AR42J cells into insulin-secreting cells and was upregulated in diabetic rats. This molecule might be important in the differentiation of pancreatic β-cells as a channel or a carrier molecule present in mitochondria. Sfxn2 and Sfxn5 may also be related to the regeneration of pancreatic endocrine cells. Further studies, including intense observations of expressions of each molecule in fetal and neonatal stages are needed to confirm our speculations.

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