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Cellular expression of Noc2, a Rab effector protein, in endocrine and exocrine tissues in the mouse

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Abstract

Noc2 is a Rab effector which participates in regulated exocytosis. It is expressed abundantly in endocrine cells but at low levels in exocrine tissues. Noc2-deficient mice, however, exhibit marked accumulation of secretory granules in exocrine cells rather than endocrine cells. In the present study, we investigated localization of Noc2 immunohistochemically in various endocrine and exocrine tissues in normal mice. Western blotting detected a Noc2-immunoreactive band of 38 kDa in isolated pancreatic islets, the adrenal gland, pituitary gland, and thyroid gland. Immunostaining for Noc2 labeled endocrine cells in the adrenal medulla and adenohypophysis, pancreatic islet cells, thyroid parafollicular cells, and gut endocrine cells, supporting the notion that Noc2 is a Rab effector protein shared by amine/peptide-secreting endocrine cells. Besides endocrine tissues, granular ducts in salivary glands contained Noc2. Although immunostaining failed to detect Noc2 in acinar cells of all exocrine glands examined, reverse transcriptase-polymerase chain reaction analysis detected the mRNA expression in exocrine pancreas. Ultrastructurally, Noc2 immunoreactivity was associated with the limiting membrane of granules in both pancreatic endocrine and salivary duct exocrine cells. The cellular and subcellular localizations of Noc2 should yield key information on its functional significance as well as account for the phenotype in Noc2-deficient mice.

Keywords

Rab/ Noc2/ endocrine cell/ exocrine cell/ immunohistochemistry

Introduction

Regulated exocytosis requires specialized proteins that control the trafficking of granules or vesicles to release sites and the exocytotic fusion of them with plasma membrane. Rab proteins, a family of Ras-related small GTP-binding proteins, have emerged as major candidates for such regulatory proteins in recepto-secretory cells (Pfeffer 1999, 2001; Zerial and McBride 2001). Rab proteins are molecular switches that cycle between a GTP-bound, active state and a GDP-bound, inactive state. The two forms of Rab protein are converted from one to the other by the action of GDP/GTP exchange factors and GTPase-activating proteins. While many Rab proteins are ubiquitously expressed, the Rab3 family proteins (Rab3A, Rab3B, Rab3C, and Rab3D) are enriched in the brain and secretory tissues, and have proven to be associated with Ca^{2+}-regulated exocytosis in mammals (Lledo et al. 1993; Fischer von Mollard et al. 1994; Castillo et al. 1997; Chen et al. 2002). Rab3A, the best-characterized member of the Rab3 subfamily, is a predominant isotype present in neurons and amine/peptide-secreting endocrine cells, controlling calcium-dependent exocytosis as a positive or negative regulator (Holz et al. 1994; Lledo et al. 1994; Regazzi et al. 1996; Takai et al. 1996, Geppert and Sudhöf 1998; Yaekura et al. 2003).

The active GTP-bound Rab proteins exert their functions by binding to effector proteins associated with the membrane components of synaptic vesicles and dense-cored granules in neurons and secretory cells. The first such protein identified was rabphilin3, which forms a complex with the GTP-bound form of Rab3A. Rabphilin3 possesses the C_2 domains with a high sequence homology to synaptotagmin (Shirataki et al. 1993); these are shown to bind to membrane phospholipids in a Ca^{2+}-dependent manner. Rabphilin3 colocalizes with Rab3A, its counterpart on synaptic vesicles in axon terminals (Shirataki et al. 1994; Li et al. 1994). The

localization of rabphilin3 is thought to be dependent on Rab3A (Li et al. 1994) although recent studies suggest a more specific interaction of rabphilin3 with Rab27A (Fukuda 2003; Fukuda et al. 2004). Rab3A is shared by neurons and endocrine cells, while rabphilin3 is expressed exclusively in neurons and neuron-like cells (adrenal chromaffin cells), and is absent or present only at very low levels in endocrine cells (Inagaki et al. 1994; Regazzi et al. 1996). On the basis of its structural similarity to the Rab3A-binding domain of rabphilin3, Kotake et al. (1997) identified a novel Rab3A effector protein from a rat pancreatic β cell line, designated Noc2 (No C_2 domain). Noc2 consists of 302 amino acids having 40.7% amino acid identity and 77.9% similarity to the N-terminal Rab3A-binding region (amino acids 1-304) of rabphilin3. Northern blot analysis demonstrated the predominant expression of Noc2 mRNA in endocrine tissues and hormone-secreting cell lines, but very low levels of expression in the brain (Kotake et al. 1997), suggesting that Noc2 may be involved in regulated exocytosis in endocrine cells.

To determine the functional significance of Noc2, we generated and analyzed Noc2-deficient ($Noc2^{-/-}$) mice. $Noc2^{-/-}$ mice exhibit glucose intolerance with impaired insulin secretion when exposed to stress (Matsumoto et al. 2004). Under normal conditions, however, no significant morphological changes indicating the impaired secretion of secretory granules were found in insulin-secreting β cells or in other endocrine cells of the knockout mice. A marked accumulation of secretory granules throughout the cytoplasm was found in various exocrine gland cells of the $Noc2^{-/-}$ mice, suggesting defective exocytosis in exocrine secretion. In accord with the morphological changes, there was no amylase secretion from the exocrine pancreas in response to adequate stimuli (Matsumoto et al. 2004). Although conventional Northern blotting showed an endocrine tissue-specific expression of Noc2 mRNA

(Kotake et al. 1997), another northern blot analysis with longer exposure time found a further broad expression of Noc2 mRNA in non-endocrine tissues including the liver, lung, kidney, heart and testis (Manabe et al. 2004). The present study sought to determine the cellular localization of Noc2 by immunohistochemistry at light and electron microscopic levels in a variety of endocrine and exocrine cells of normal mice.

Materials and methods

Antibody

A polyclonal antibody to Noc2 was raised against the amino acid residues 248-302 of mouse Noc2 (Fig. 1). The polypeptides were expressed as glutathione S-transferase (GST) fusion proteins using the pGEX4T-2 plasmid vector (Amersham Biosciences, Uppsala, Sweden). The fusion protein was purified with glutathione-Sepharose 4B (Amersham Biosciences), emulsified with Freund's complete adjuvant (Difco, Detroit, MI, USA), and injected subcutaneously into female New Zealand White rabbits and Hartley guinea-pigs at intervals of two weeks. Two weeks after the sixth injection, affinity-purified antibodies were prepared, first using protein G-Sepharose (Amersham Biosciences) and then using antigen peptides coupled to cyanogens bromide-activated Sepharose 4B (Amersham Biosciences). For the preparation of affinity media, polypeptides free of GST were obtained by the elution of a cleaved polypeptide after the in-column thrombin digestion of fusion proteins bound to glutathione-Sepharose 4B.

Western blotting

Various tissues were collected from the pancreas, pituitary gland, thyroid gland, adrenal gland, salivary glands, stomach, duodenum, testis, and ovary of adult ddY mice (8-weeks old; Japan SLC, Shizuoka, Japan) for immunoblot analyses. About ten large pancreatic islets from each animal were collected from the fresh pancreas under a dissecting microscope. For a control, the mouse insulin-secreting cell line (MIN6) was used. The fresh tissues were snap-frozen in liquid nitrogen, and homogenized for 30 min in the ice-cold 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM ethylenediamine-tetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF),

and 1 % Triton X-100. The samples were centrifuged (10,000 ×*g*, 2 min), and the supernatant was collected. The solubilized proteins were subjected to 12 % sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions. The proteins were then transferred to a nitrocellulose membrane (Hypond-P; Amersham, Buckinghamshire, England) and incubated with the affinity-purified guinea pig antibody against Noc2 (4 μg/ml) diluted with 0.01 M phosphate-buffered saline (PBS, pH 7.2) containing 0.05% Tween 20 for 1 h. The bound antibody was visualized using peroxidase-labeled goat anti-guinea pig IgG (Dako, Glostrup, Denmark) and an enhanced chemiluminescence (ECL) system (Amersham) according to the manufacturer's instructions.

Immunohistochemistry

Ten adult male and 10 female ddY mice (8-weeks old; Japan SLC) were used in the immunohistochemical study. Noc2-deficient mice (Matsumoto et al. 2004) were used to confirm the specificity of the immunoreaction with the Noc2 antibody. After 24 h starvation the animals were deeply anesthetized by an intraperitoneal injection of pentobarbital sodium, then perfused through the left ventricle of the heart with physiological saline and subsequently with 4% paraformaldehyde in a 0.1 M phosphate buffer, pH 7.4. Tissue samples were collected from the various endocrine tissues (pituitary gland, pineal body, thyroid gland, adrenal gland) and the gastrointestinal tract (pancreas, salivary glands, stomach, duodenum, jejunum, proximal colon); all were immersed in the same fixative for an additional 6 h. All experiments using animals were performed under protocols following the Guidelines for Animal Experimentation, Graduate School of Medicine, Hokkaido University.

Fixed tissues were dipped in a 30% sucrose solution overnight at 4°C, embedded in

Tissue-Tek OCT compound (Sakura FineTechnical, Tokyo, Japan), and quickly frozen in liquid nitrogen. Frozen sections, about 10 μm in thickness, were mounted on poly-L-lysine-coated glass slides and stained by the avidin-biotin complex (ABC) method. They were pretreated both with 0.3% Triton X-100-containing PBS (pH 7.2) to enhance the penetration of antibodies and with 0.03% H_2O_2 in methanol to block the endogeneous peroxidase activity. After the preincubation with the normal goat serum, the sections were incubated overnight with a rabbit anti-mouse Noc2 antibody (2 μg/ml). They were then incubated with biotinylated goat anti-rabbit immunoglobulins (Nichirei, Tokyo, Japan) followed by incubation with the streptavidin-peroxidase complex (Nichirei), each for 1h. The antigen-antibody reaction was visualized by incubation in a 0.05 M Tris-HCl buffer (pH 7.6) containing 0.01% 3,3'-diaminobenzidine and 0.001% H_2O_2.

Double immunostaining

Frozen sections from fixed samples were incubated with the guinea pig or rabbit antibodies against Noc2 at concentrations of 8 μg/ml and 2 μg/ml, respectively, followed by either Cy3-labeled donkey anti-guinea pig IgG (1: 200, Jackson ImmunoResearch, West Grove, PA, USA) or Cy3-labeled donkey anti-rabbit IgG (1:200, Jackson ImmunoResearch). After rinsing, the same sections were incubated with one of the antisera against insulin (raised in a guinea pig: Zymed, South San Francisco, CA, USA), glucagon (in a guinea pig: Progen Immuno-Diagnostica, Heidelberg, Germany), ghrelin (in a rabbit: donated by Yanaihara Institute, Fujinomiya, Japan), histidine decarboxylase (HDC) (in a guinea pig: American Research Products, Inc., Belmont, MA, USA), rat chromogranin A 296-316 (in a rabbit: Y090, Yanaihara Institute), human α-calcitonin gene-related peptide (CGRP) (in a guinea pig: Peninsula Laboratories, San

Carlos, CA, USA), and rat epidermal growth factor (EGF) (in a rabbit: Y231, Yanaihara Institute). All stainings were followed by incubation with the fluorescein isothiocyanate (FITC)-labeled secondary antibodies and were observed under a confocal laser scanning microscope (Fluoview; Olympus, Tokyo, Japan).

Immunoelectron microscopy

For pre-embedding immunoelectron microscopy, frozen sections (15 μm in thickness) were prepared from the paraformaldehyde-fixed tissues and processed for the silver-intensified immunogold method. The sections on glass slides were incubated with the rabbit Noc2 antibody at 2 μg/ml and subsequently reacted with goat anti-rabbit IgG covalently linked to 1-nm gold particles (1: 200; BBInternational, Golden Gate, UK). Following silver enhancement using HQ silver (Nanoprobes, Stony Brook, NY, USA), the sections were osmificated, dehydrated, and directly embedded in Epon (Nisshin EM, Tokyo, Japan). Ultrathin sections were prepared and stained with an aqueous solution of uranyl acetate and lead citrate for observation under an electron microscope (H-7100, Hitachi, Tokyo, Japan).

For post-embedding immunoelectron microscopy, mice were perfusion-fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), and dissected pancreas was immersed in the same fixative for additional 2 h. After post-fixation in 1% tannic acid, the tissues were embedded in LR White resin monomer, as described previously (Sakai et al. 2005). Ultrathin sections were directly incubated with 5% normal goat serum for blocking and subsequently with guinea pig anti-Noc2 IgG (18 μg/ml) antibody for 12 h. The site of antigen-antibody reaction was detected by incubation in goat anti-guinea pig IgG labeled with 15 nm colloidal gold particles (BBInternational, Golden Gate, UK). The sections without any counterstaining were

observed under the electron microscope.

RT-PCR analysis

Four small pieces (about 2 mm x 2 mm in size) of a mouse pancreas were sampled, and total RNA was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The RNA was reverse-transcribed by ReverTra Ace (Toyobo, Osaka, Japan), and the resultant cDNA was subjected to PCR using Taq polymerase (Roche, Penzberg, Germany). PCR primers for amylase, insulin 2, and Noc2 were designed such that the amplified regions spanned an intron in the gene, 5'-GGCTCATCCTTATGGATTCA-3' and 5'-GACATCACAGTATGTGCCAG-3', 5'-CCCTGCTGGCCCTGCTCTT-3' and 5'-AGGTCTGAAGGTCACCTGCT-3', and 5'-GCAGTGGAAATGATCAGTGG-3' and 5'-CATCACGTTCCTCTGCATTG-3', respectively. The PCR condition was as follows: denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72 °C for 45 sec over 40 cycles.

Results

Western Blot

Immunoblotting using the guinea pig antibody against Noc2 detected intensely immunoreactive bands at 38 kDa in isolated pancreatic islets, the pancreas, MIN6 cells (mouse insulin-secreting cell line), and the adrenal gland (Fig. 2). The immunoreactive proteins contained in some tissues appeared as two closely related bands, represented by the blotting of pancreatic islets and pancreas. Less intensely immunoreactive bands for Noc2 appeared at the same position in lanes of the pituitary gland, thyroid gland, and stomach. However, no significant bands were visible in lanes of the salivary gland, duodenum, ovary and testis.

Immunohistochemistry

When immunostaining results were compared between two antibodies raised in a rabbit and a guinea pig, excepting a few cases, both antibodies exhibited essentially the same reactivities. The positive reactivities obtained by immunohistochemistry corresponded well with the results of Western blotting. For single immunostaining, the results obtained with the rabbit antibody are shown in the figures, while for double immunostaining, either of two antibodies was used to accord to the types of combined anti-hormone antibodies. In all immunoreactive cells, Noc2 immunoreactivity was localized in the cytoplasm, and there was none in the nucleus. The immunoreactivities disappeared in all tissues of Noc2-knockout mice, as shown in pancreatic islets (Fig. 3a, b).

In the pancreas, only the islets of Langerhans were stained positively, and the exocrine portions including the duct system were free from any immunoreactivity (Fig. 3a). All islet cells were more or less immunolabeled, but several endocrine cells

located at the periphery of islets showed **a** more intense reaction. Double staining by the use of anti-Noc2 and either the anti-glucagon or anti-insulin antibody revealed that the intensely stained cells at the periphery of islets corresponded to glucagon-secreting cells (Fig. 4a-c). Insulin cells occupying the central region of islets were stained with different intensities, more remarkably so with the use of the rabbit antiserum (Fig. 4d-f).

The adrenal gland exhibited an intense immunoreactivity for Noc2 in the medulla, but the cortical endocrine cells were negative in reaction (Fig. 5a). All medullary chromaffin cells, namely A cells and NA cells, were immunolabeled with the anti-Noc2 antibody. In the pituitary gland, Noc2 immunoreactivity was restricted to the adenohypophysis (anterior and intermediate lobes), where almost all endocrine cells were positive with different intensities (Fig. 5b). The thyroid gland contained scattered immunoreactive cells (Fig. 5c), identified as calcitonin/CGRP-containing parafollicular cells by double staining (Fig. 6a-c). The thyroid follicular epithelial cells and parathyroid endocrine cells lacked any Noc2 immunoreactivity. No immunoreactivity for Noc2 was detected in the pineal glands.

The alimentary canal exhibited immunoreactive cells scattered in the epithelium from the stomach to the large intestine. In the acid-secreting mucosa of the stomach, small and round Noc2-immunoreactive cells were dispersed predominantly in the bottom of the gastric glands. Double staining showed these immunoreactive cells to be ghrelin-containing endocrine cells (Fig. 7a-c). Since the Noc2-immunoreactive cell population always predominated, a population of Noc2-positive cells without ghrelin immunoreactivity were intermingled with ghrelin-immunoreactive cells here. Histamine-containing ECL cells, a representative endocrine cell type in the acid-secreting area, were not immunoreactive for Noc2 in double staining combined with the antibody against its synthesizing enzyme, HDC (data not shown). The

duodenum, jejunum, ileum, and colon also contained numerous Noc2-positive cells scattered in the intestinal villi (Fig. 8a) and crypts, and were identified as gut endocrine cells by their positive reactivity for chromogranin A, an endocrine cell marker (data not shown). No significant immunoreactivity for Noc2 was recognized in other epithelial cells throughout the alimentary canal. Positive staining for Noc2 was found in neuronal cell bodies in the submucous and myenteric nerve plexuses only when the sections were stained with the antibody raised in a guinea pig.

Besides the endocrine tissues, granular duct cells in the submandibular gland were intensely immunoreactive for Noc2 (Fig. 8b). Another immunoreactivity for Noc2 was found in the striated ducts of the submandibular gland as well as the parotid and sublingual glands, where only the apical cytoplasm of some duct cells was immunolabeled (Fig. 8c). Double staining by the use of Noc2 and EGF antibodies in the submandibular gland labeled the same regions, namely the granular ducts (Fig. 9a-c). The Noc2 immunoreactivity exhibited a coarse-granular appearance, suggesting a subcellular localization of Noc2 associated with granular components, in contrast to the diffuse EGF immunoreactivity. The Noc2 immunoreactivity was not detected in acinar cells of any salivary gland.

Immunoelectron microscopy

We investigated the subcellular localization of Noc2 in pancreatic islets and the submandibular gland by the pre-embedding silver-intensified immunogold method. In pancreatic islets, gold particles showing the existence of the Noc2 immunoreactivity were localized along the granular membrane in α (Fig. 10a,b) and β cells (Fig. 10c). No significant labeling was visible in the plasma membrane or cell organelles, including the Golgi apparatus. The localization of Noc2 along the limiting membrane of

secretory granules in islet cells was confirmed by the post-embedding immunogold method (Fig. 10d). In the granular ducts of the submandibular gland, gold particles also gathered along the limiting membrane of large electron-dense granules (Fig. 10e, f). Here, we observed the slight aggregation of gold particles in the apical cytoplasm of the granular ducts and striated portion but could not relate this immunoreactivity to any special structures such as vesicles or the plasma membrane.

Expression of Noc2 in the exocrine pancreas

RT-PCR was performed to determine the expression of Noc2 in the exocrine pancreas (Fig. 11). Among the four cDNA samples generated from four small pieces of the pancreas, one cDNA (designated cDNAEX1) was found positive for amylase but negative for insulin2 and glucagon (data not shown), indicating that cDNAEX1 is purely derived from the exocrine pancreas rather than the endocrine pancreas. Using cDNAEX1 as a template, the expression of Noc2 mRNA was detected, confirming that Noc2 is expressed in exocrine cells.

Discussion

Rab proteins play a leading role in the final steps of regulated secretion (Burgoyne and Morgan 2003). To manifest action, the active GTP-bound Rab protein utilizes specific effector proteins possessing a Rab-interacting domain linked to the granular/vesicular membrane or cytoskeleton. Accumulating evidence suggests the direct involvement of Rab effector proteins, such as rabphilin3, granuphilin and Noc2, in the exocytosis of cytoplasmic granules in amine/peptide-secreting endocrine cells (Yi et al. 2002; Fukuda et al. 2004; Matsumoto et al. 2004). Noc2 is unique among the Rab partners in its being small in molecular size and devoid of domains with predictable functions, apart from the Rab-binding motif (Cheviet et al. 2004b). The identification of Rab proteins as counterparts of Noc2 is still controversial. The first investigation of Noc2 failed to demonstrate any interaction between Noc2 and Rab3A under conditions in which rabphilin3 binds Rab3A, despite its homology with rabphilin3 (Kotake et al. 1997). Subsequent studies reported that Noc2 could bind to all of the Rab3 isoforms (Rab3A-D) in COS-1 cells in a GTP-dependent manner (Mastumoto et al. 2004), or to Rab3A in PC12 cells (Haynes et al. 2001). On the other hand, Noc2 was shown to interact preferentially with Rab27, the closest homologue of the Rab3 subfamily, rather than with Rab3A *in vitro* (Cheviet et al. 2004a; Fukuda et al. 2004). The inconsistent findings on the relationship between Noc2 and Rab proteins may reflect the facts that a cell type possesses different sets of Rab and effector proteins, and that a single Rab protein can interact with multiple effector proteins, showing different expression patterns according to cell types (Fukuda 2003; Cheviet et al. 2004b).

The present immunoblot analysis revealed the predominant expression of Noc2 in endocrine tissues at the protein level. Previously, RNA blot analysis by Kotake et al. (1997) showed that Noc2 mRNA was expressed at very high levels in pancreatic islets,

at moderate to high levels in the adrenal gland, pituitary gland, and ovary, and at low levels in the testis. The tissue distribution of the Noc2 protein by the present Western blotting largely accords with the data obtained from the Northern blot analysis. The 38-kDa Noc2-immunoreactive protein contained in endocrine tissues is consistent with a major immunoreactive band detected in mouse MIN6 cells (insulin cells) and rat PC12 cells (Kotake et al. 1997). Using the specific antibodies against mouse Noc2, the present immunohistochemical study is the first to identify the cell types expressing Noc2, namely the pancreatic endocrine cells, pituitary endocrine cells, adrenal chromaffin cells, thyroid C cells, and gastrointestinal endocrine cells. The failure to detect the immunoreactive bands in the immunoblotting of the salivary glands may be due to the sparsity of Noc-2 immunoreactive cells as compared with total tissue masses. All endocrine cell types in the adenohypophysis, pancreatic islets, and adrenal gland exhibited Noc2 immunoreactivity. Thus, the cellular distribution of Noc2 is broader than in the other two endocrine-type Rab effector proteins: granuphilin, which is detected in pancreatic β-cells and the pituitary gland (Wang et al. 1999; Yi et al. 2002), and Rim2, which is expressed in insulin cell lines, the pituitary gland, and PC12 cells, but not in the adrenal gland (Ozaki et al. 2000). The association of Noc2 with secretory granules has been demonstrated in insulin-secreting β-cell lines (Cheviet et al. 2004a) and PC12 cells (Fukuda et al. 2004) by confocal laser microscope, electron microscope, and subcellular fractionation. The present electron microscopic observation revealed the subcellular localization of Noc2 associated with the granular membrane in pancreatic endocrine cells *in vivo*. These immunoblot and immunohistochemical analyses of Noc2 protein lead us to the conclusions that Noc2 is the Rab effector protein shared by most of the amine/peptide-secreting endocrine cells and that it is involved in the trafficking of secretory granules.

The uneven intensities or the lack of Noc2 immunoreactivity in some amine/peptide-secreting endocrine cells may contribute to a better understanding of the functions of Noc2. The intensities of Noc2 immunoreactivity in the endocrine cells differ from cell to cell, as was clearly shown by the immunostaining of pancreatic β cells. This finding suggests that the content of Noc2 in cells changes during the secretory process, and that the difference in expression level is associated with the secretory phase of endocrine cells. On the other hand, Noc2 expression in the gastric endocrine cells was characterized by cell type-specific localization. In acid-secreting mucosa of the stomach, histamine-secreting ECL cells and ghrelin-secreting endocrine cells (A-like or X cells, Date et al. 2000) are the predominant types among endocrine cells; only the latter expressed Noc2, implying the importance of Noc2 in the secretion of some specific hormones.

Besides endocrine tissues, only the striated duct of the salivary glands, especially the granular ducts of the submandibular gland, possess Noc2 immunoreactivity. The expression of Noc2 in the ducts of salivary glands has been confirmed at the mRNA level by *in situ* hybridization analysis (Teramae H, unpublished data). Our electron microscopic observation demonstrated the granular membrane-associated localization of Noc2 immunoreactivity in granular duct cells, as in pancreatic endocrine cells. The granular duct of the rodent submandibular gland is unique, secreting several growth factors such as NGF, EGF, and IGF-I. Secretion of these growth factors is primarily an exocrine function, but some amount of secreted substances may be transported inside the body to regulate the growth of epithelial cells and nerves in an endocrine or paracrine manner (Garrett 1998). Nevertheless, the duct cell of salivary glands is an exceptional case for the production of Noc2, as both our immunohistochemical analyses and the conventional Northern blotting by Kotake (1997) failed to detect any significant

expression of Noc2 in other exocrine cells and tissues. In contrast, Northern blot analysis in a recent study revealed an extensive expression of Noc2 mRNA in visceral organs including the liver, lung, kidney, heart, and testis as well as an intestinal epithelial cell line (Caco-2 cell) (Manabe et al. 2004). In support of this finding, we also detected Noc2 expression in the exocrine pancreas by RT-PCR analysis. Considered together, these facts indicate that exocrine tissues other than the granular ducts of salivary gland also contain Noc2, though its expression levels are not high. The phenotype of Noc2-knockout mice is characterized by the remarkable accumulation of secretory granules in exocrine cells rather than in endocrine cells (Matsumoto et al. 2004). A marked accumulation of secretory granules was found in the exocrine pancreas, salivary gland, gastric chief cells, intestinal Paneth cells, and duodenal Brunner's gland. Other experiments done by Matsumoto et al. (2004) using chimeric mice between wild-type GFP-transgenic mice and $Noc2^{-/-}$ mice showed that the morphological changes of pancreatic acinar cells in these knockout mice were primarily due to the lack of Noc2. In accord with the morphological changes, there was no amylase secretion from pancreatic acinar cells of $Noc2^{-/-}$ mice in response to secretagogues. Since Rab3D, originally identified in fat cells, is rich in pancreatic and parotid gland acinar cells and gastric chief cells (Ohnishi et al. 1996; Tang et al. 1996; Valentijin et al. 1996), these exocrine cells may well regulate secretion by a combination of Rab and its effector protein, in which Noc2 is a potent candidate for the Rab-binding protein. However, the genetic deletion of Rab3D in mice resulted in normal pancreatic amylase secretion and only a mild change in the morphology of the secretory granules of pancreatic acinar cells (Riedel et al. 2002), suggesting that Rab3D is not an essential component of the exocytotic machinery in exocrine cells. In contrast, Noc2, even in low concentration, might play a crucial role by acting at the final

step in the release of secretory products.

The predominant distribution of Noc2 in endocrine tissues and the affinity to Rab proteins (Rab3 and Rab27) suggests critical roles in the regulation of hormone secretion. Indeed, transfection of the Noc2 gene rescued impaired insulin secretion in cultured pancreatic islets of $Noc2^{-/-}$ mice, while the transfer of mutant Noc2 gene without the ability to bind Rab3 isoforms did not (Matsumoto et al. 2004). However, under *in vivo* normal conditions, the defect in Noc2 did not impair insulin secretion, in contrast to the severely impaired exocytosis in the exocrine glands (Matsumoto et al. 2004). Morphologically, there were no abnormalities in cell mass, cellular arrangement, or ultrastructure of the pancreatic β-cells in $Noc2^{-/-}$ mice, as assessed by immunohistochemistry and electron microscopy. This non-critical phenotye in β-cells may be explained by the multi-regulation of insulin secretion by different sets of Rab/effector. Endocrine cells including pancreatic β cells possess some potential Rab effectors other than Noc2, such as granuphilin (Wang et al. 1999; Yi et al. 2002) and Rim2 (Ozaki et al. 2000), which were identified first in β cells and are suggested to be present in other amine/peptide-secreting endocrine cells. The normal insulin release in response to glucose and the normal morphology of β-cells in Noc2-knockout mice suggests a compensation for the Noc2 deficiency by granuphilin and Rim2 although the expression levels of granuphilin and Rim2, as assessed by RT-PCR, were similar in the knockout mice (Matsumoto et al. 2004). In contrast, since exocrine cells may possess Noc2 only as a Rab effector protein, the deletion of Noc2 might well result in a serious defect in regulated exocrine secretion.

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Figure legends

Fig. 1 The underlined amino acid sequence (C-terminal 248-302) of mouse Noc2 protein was used as an antigen for production of the antibody. This sequence of Noc2 shows a low homology to the corresponding region of mouse rabphilin3 (identical amino acids are indicated by *asterisks*).

Fig. 2 Western blot analysis using a guinea pig antibody against Noc2. Pancreatic islets, the pancreas, MIN6 cell, and adrenal gland show a major immunoreactive band at 38 kDa. Less intensely immunoreactive bands are visible at the same position in the pituitary gland, thyroid gland, and stomach.

Fig. 3a, b Immunostaining for Noc2 in the pancreas of wild-type and Noc2-deficient mice. Nearly all endocrine cells of pancreatic islets in a normal mouse are stained positively with more intense reactivity at the periphery of the islets (**a**). No immunoreactivity is recognizable in the pancreatic islets from a $Noc2^{-/-}$ mouse (**b**). *Bars* 50 μm

Fig. 4a-f Double immunostaining of Noc2 and glucagon or insulin in pancreatic islets (confocal laser scanning microscope). Intensely Noc2-immunopositive cells at the periphery of the islets correspond to glucagon cells (**a**-**c**). Insulin cells are stained positively for Noc2 with different intensities (**d**-**f**). **c** and **f** are merged images. *Bars* 50 μm

Fig. 5a-c Immunostaining of the adrenal gland, pituitary gland, and thyroid gland.

The adrenal gland (**a**) contains immunoreactive cells only at the medulla (*M*) (*C* cortex). In the pituitary gland **(b)**, immunoreactivity is seen in the anterior (*A*) and intermediate lobes (*I*) but not in the posterior neurohypophysis (*P*). The thyroid gland (**c**) shows scattered immunoreactive cells. *Bars* 50 μm in **c**; 20 μm in **b**, **c**

Figs. 6a-c and 7a-c Double immunostaining of Noc2 and bioactive peptides in the thyroid gland and stomach. CGRP and Noc2 are colocalized in parafollicular cells of the thyroid gland (Fig. 6**a**-**c**). In the gastric gland (Fig. 7**a**-**c**), all ghrelin-containing cells are immunoreactive for Noc2 and intermingled with some ghrelin-negative Noc2-expressing cells (*arrows*). *Bars* 20 μm in Fig. 6; 50 μm in Fig. 7

Fig. 8a-c Immunohistochemistry of Noc2 in the intestine and salivary glands. Scattered endocrine cells in the intestinal villi are immunoreactive with the basal cytoplasm (**a**). Secretory cells in the granular ducts of the submandibular gland (**b**) show a positive immunoreactivity and are granular in appearance. The sublingual gland (**c**) shows slight immunoreactivity at the apical portions of striated ducts. *Bars* 20 μm in **a**; 50 μm in **b**, **c**

Fig. 9a-c Double immunostaining of Noc2 and EGF in the submandibular gland. Granular ducts of the submandibular gland contain immunoreactivities for both EGF and Noc2. **c** is a merged image. *Bar* 200 μm

Fig. 10a-f Immunogold methods showing the subcellular localization of Noc2 in pancreatic islets and granular ducts of the submandibular gland (a-c and e-f: pre-embedding method, d: post-embedding method). In pancreatic glucagon cells, the

gold particles appear on the limiting membrane of dense-cored granules, though not on all of the granules (**a**, **b**). Most of the insulin-containing granules show Noc2 immunolabeling on the limiting membrane of secretory granules (*arrows* in ç̃The perigranular localization of Noc2 in insulin cells is confirmed by the post-embedding method (*arrows* in **d**). Secretory cells in the granular duct of the submandibular gland contain gold-labeled large secretory granules (**e, f**). Areas enclosed by squares (**a**, **e**) are magnified in **b** and **f**, respectively. *Bars* 1 μm in **a**, **e**, **f**; 0.5 μm in **b**, **c, d**

Fig. 11 RT-PCR analysis of Noc2 in the exocrine pancreas. Noc2 and amylase but not insulin2 were detected by RT-PCR analysis in one of four cDNA samples generated from the mouse pancreas. Water (H_2O) and MIN6 cDNA were used as PCR templates for negative and positive controls, respectively.

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  230                                                                295
Rabphilin3  TRRASEARMSTAARDSEGWDHAHGGGTGDTSRSPAGLRRANSVQAARPAPAPVPSPAPPQPVQPGP
             *  * * *    *        *  *      * **             * *
   Noc2     PRGDSGASM-ESPRLGPARPPSHLSGSQSSLGSEAGTGATEPQGGTPAQPEPRVPGKRHTWATPRY
  238                                                                302
```

Fig. 1 Teramae et al.

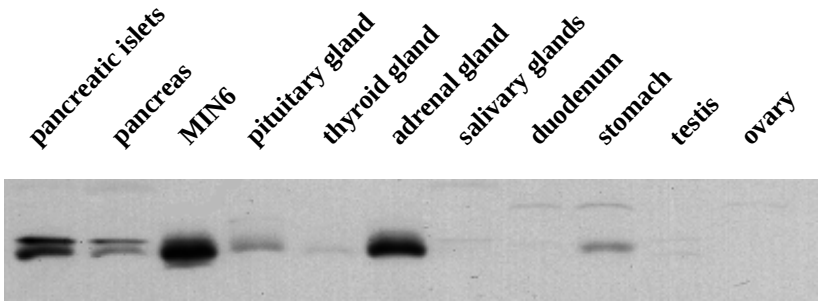


Fig. 2 Teramae et al.

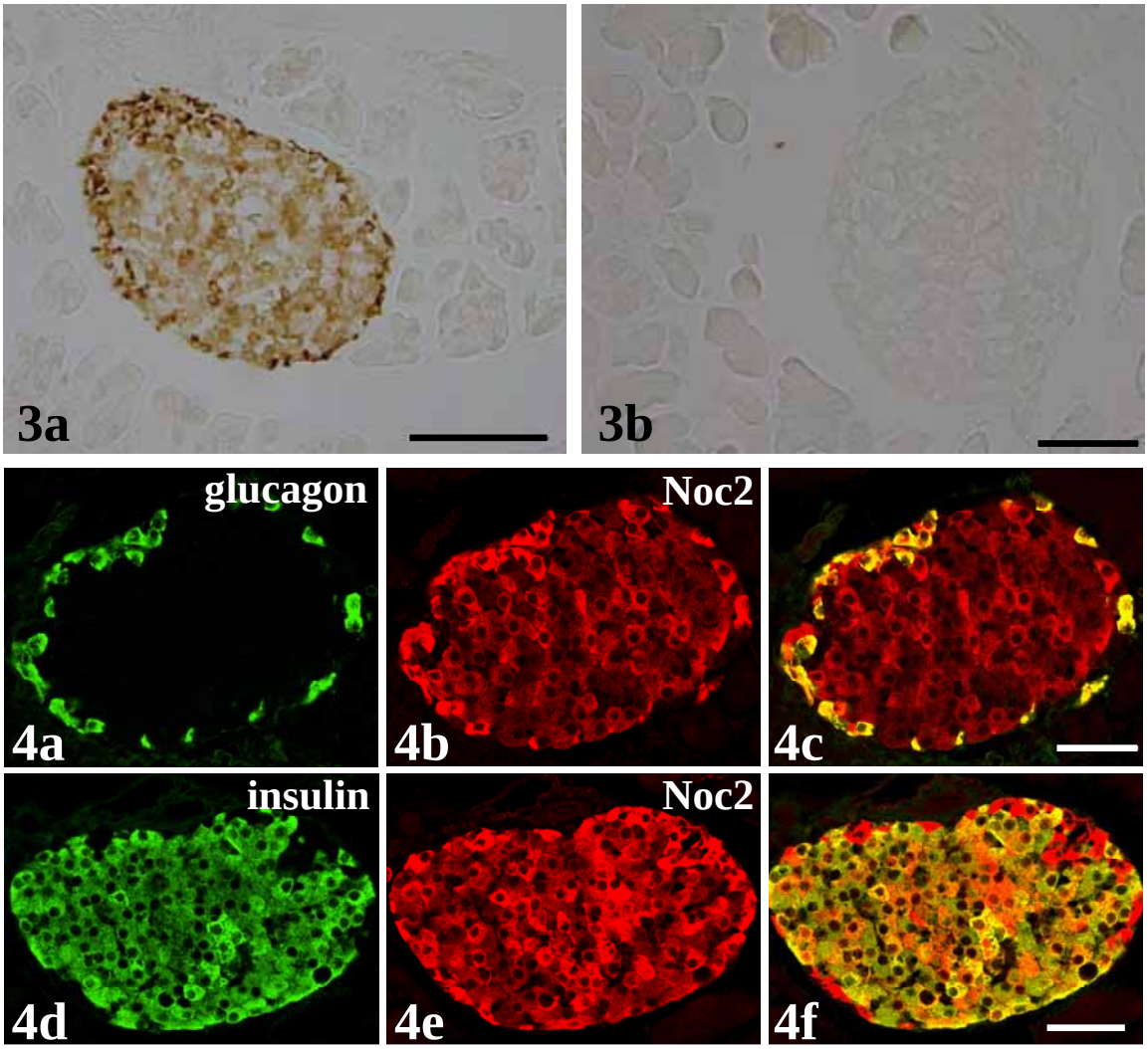
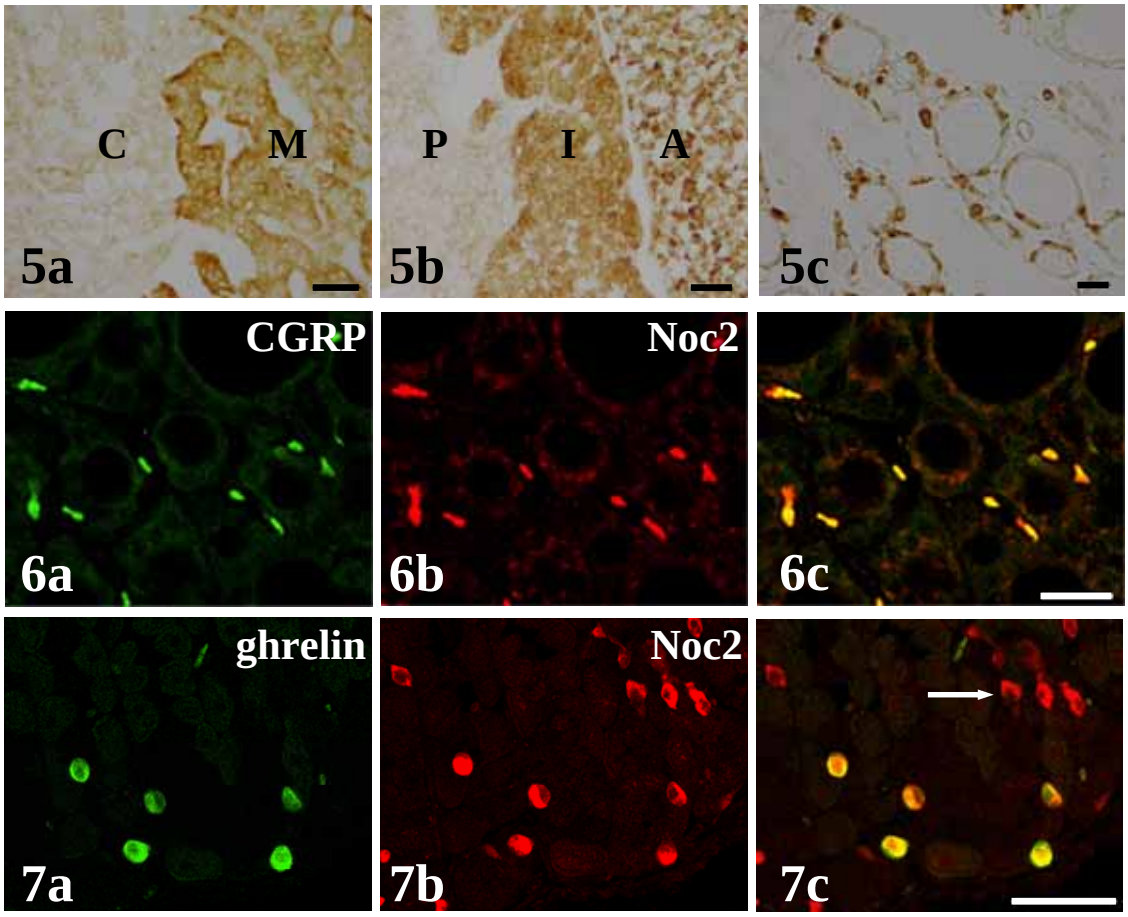
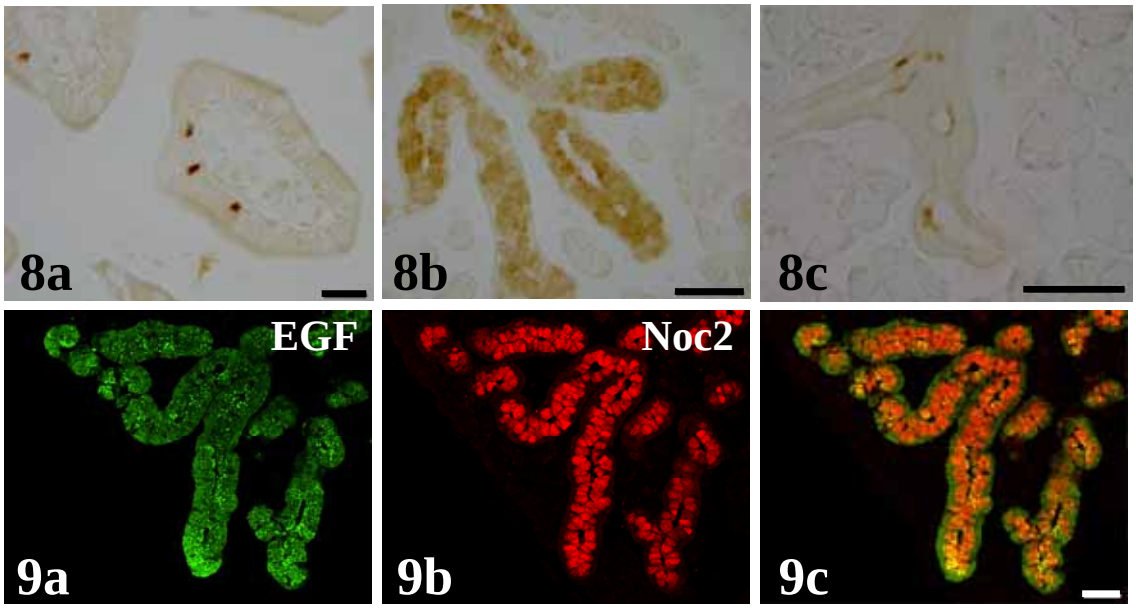


Fig. 3,4 Teramae et al.



Figs. 5-7 Teramae et al.



Figs. 8, 9 Teramae et al.

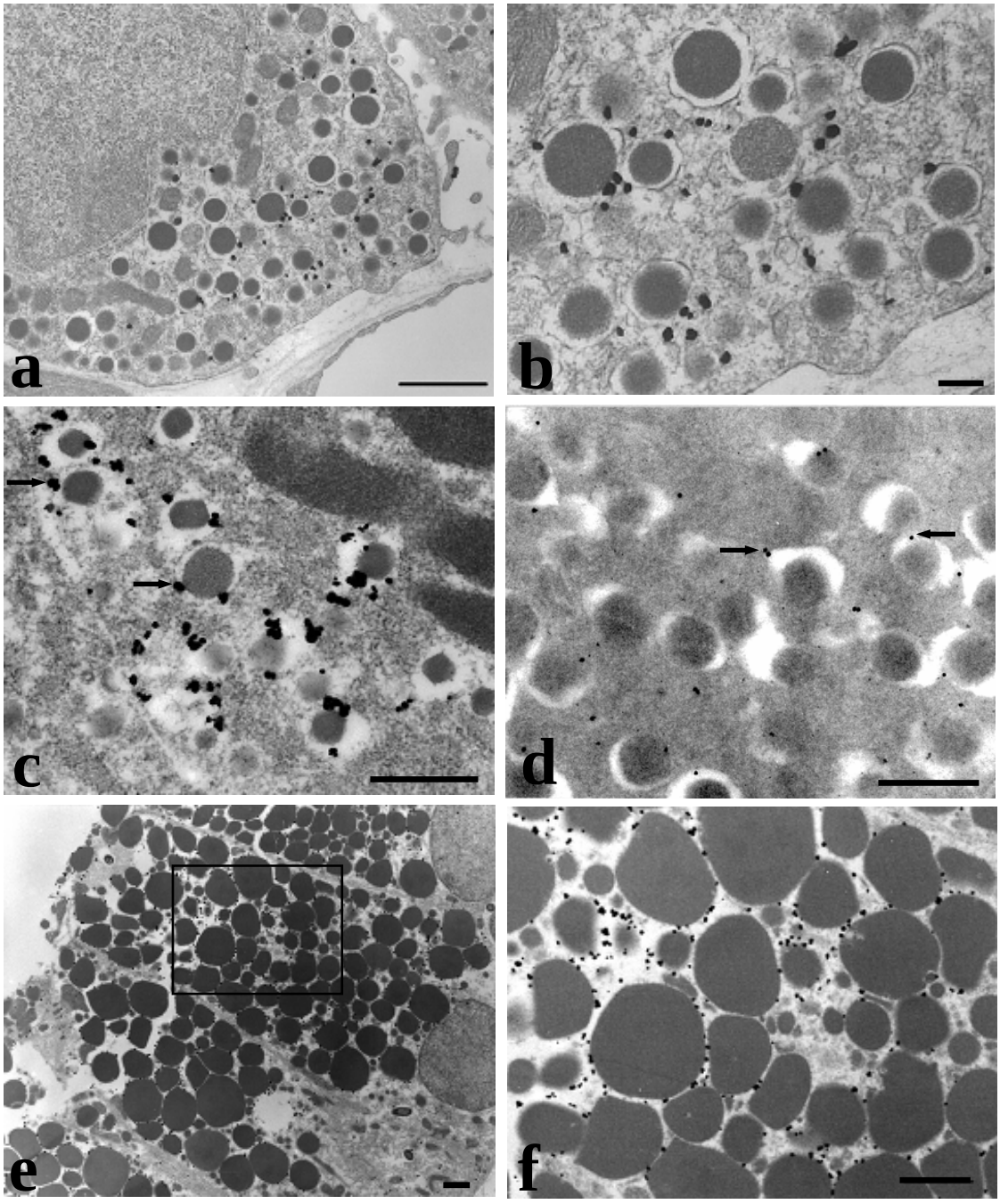


Fig. 10 Teramae et al.

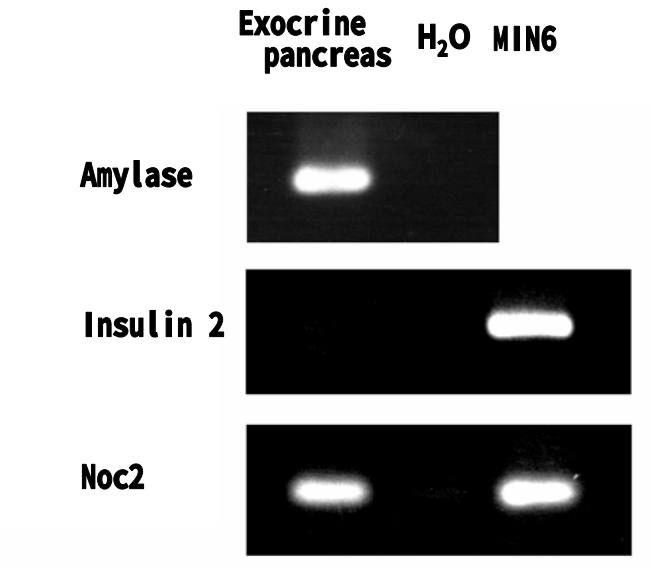


Fig. 11 Teramae et al.