SPANDIDOS PUBLICATIONS

Insulinoma-associated protein 2-deficient mice develop severe forms of diabetes induced by multiple low doses of streptozotocin

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Abstract. Insulinoma-associated protein 2 (IA-2) is the major autoantigen that contributes to the pathogenesis of type 1 diabetes (T1D). IA-2-deficient (IA-2$^{-/-}$) mice showed impaired insulin secretion after intraperitoneal injection of glucose as well as elevated glucose level in a glucose tolerance test. Despite the fact that 70% of newly diagnosed T1D patients have an antibody against IA-2, the role of IA-2 in the pathogenesis of T1D is largely unknown. In this study, the sensitivity to diabetes induced by streptozotocin (STZ) of IA-2$^{-/-}$ mice was compared with that of wild-type (WT) mice. STZ injection to IA-2$^{-/-}$ mice caused significant elevation of blood glucose and depressed insulin concentration in the pancreas. Furthermore, abnormal ultrastructure in the ß cells of the IA-2$^{-/-}$ mice was revealed by electron microscopy, showing a decreased number of insulin containing vesicles and dilation of the ER-Golgi complex. These results demonstrated that IA-2$^{-/-}$ mice had higher sensitivity to STZ, suggesting a role of IA-2 not only in the secretion but also in the production of insulin.

Introduction

Type 1 diabetes (T1D) is an autoimmune-mediated metabolic disease. The pancreatic ß cells of the patients are destroyed by a cell-mediated autoimmune process, in which autoantibodies react against a group of autoantigens including insulin, GAD, insulinoma-associated protein (IA) -2 and -2ß (1). Among these autoantigens, IA-2 has been identified to be associated with high risk of T1D progression (2). At the clinical onset of T1D, over 70% of the patients have autoantibodies against IA-2 (3).

IA-2 [also known as islet cell antigen 512 (ICA512)] is an integral membrane glycoprotein that is predominantly expressed in the insulin secreting ß cells and the neuroendocrine cells, such as neurons of the brain, especially in the hippocampus, pituitary, and endocrine organs such as the thyroid and adrenal glands (4-6). IA-2 belongs to the protein tyrosine phosphatase (PTP) family, and is characterized by an intracellular domain containing a PTP domain, one transmembrane domain and one extracellular domain directed to the luminal of the secretory granules (4,7-9). However, IA-2 does not have phosphatase activity against the common PTP substrates (7,9). The physiological role of IA-2 and its relationship to T1D are still unclear.

IA-2$^{-/-}$ mice showed abnormality in insulin secretion after intraperitoneal (i.p.) injection of glucose, although they had no other abnormalities in physical and histological studies compared with wild-type (WT) mice. The nonfasting blood glucose level was slightly higher in IA-2-deficient (IA-2$^{-/-}$) mice than in WT mice but still remained in the normal range. In the glucose tolerance test, the blood glucose level remained higher in the first 30 min after glucose injection in IA-2$^{-/-}$ mice compared to WT mice. Isolated pancreatic islets from the IA-2$^{-/-}$ mice showed significantly inhibited insulin secretion in response to glucose stimulation (10).

In order to reveal the role of IA-2 in the pathogenesis of T1D, we compared IA-2$^{-/-}$ and WT mice in the issue of their sensitivity to streptozotocin (STZ)-induced T1D. The lack of IA-2 leads to a severe diabetic symptom in IA-2$^{-/-}$ mice compared to WT mice after i.p. injection of STZ. The reduced pancreatic insulin level in IA-2$^{-/-}$ mice suggests that the production of insulin was affected due to the IA-2 deficiency.

Materials and methods

Animals. IA-2$^{-/-}$ mice were developed at the National Institute of Dental Research, National Institutes of Health, USA (10). IA-2$^{-/-}$ mice were backcrossed to C57BL/6 mice (CLEA

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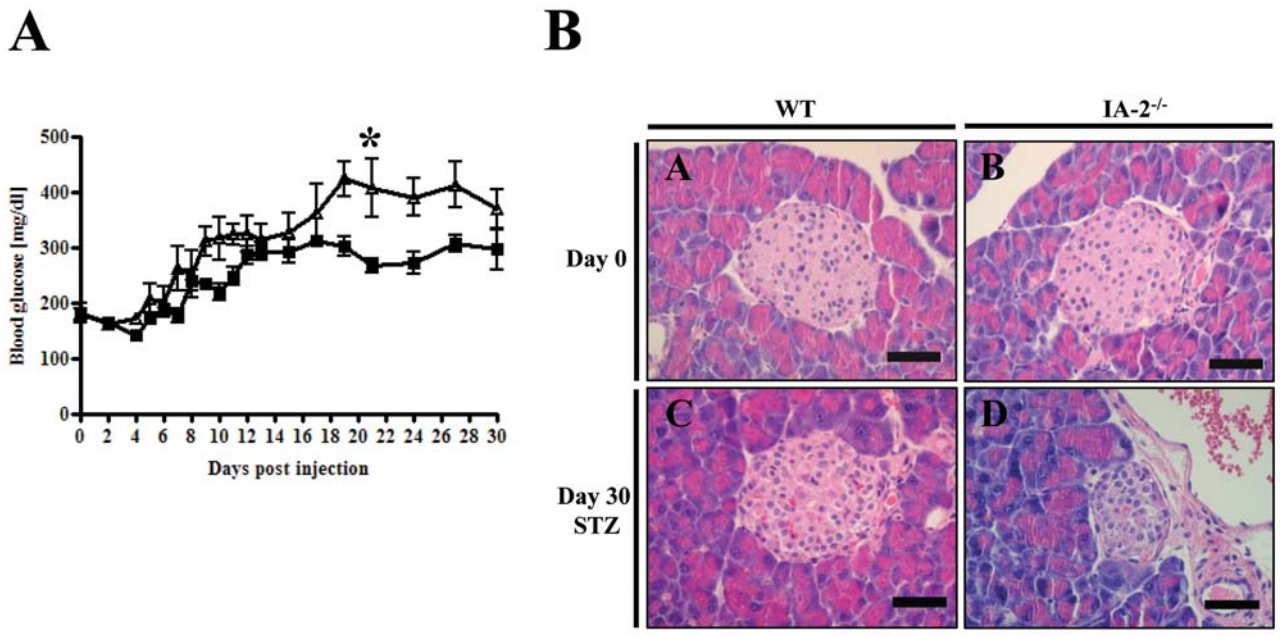

Figure 1. (A) Blood glucose levels in WT and IA-$2^{-/-}$ mice treated with multiple low doses of STZ. The results represent the average value of 5 mice in STZ treated group. (△IA-$2^{-/-}$ mice; ■WT mice). Data are means ±SE. *p<0.05. (B) Histological analysis of the pancreatic islets. Five mice in each group were sacrificed and the tissues were dissected for formalin fixation. H&E staining was performed for WT mice (A, C), and IA-$2^{-/-}$ mice (B, D). Bar, 50 μm.

Japan, Inc., Tokyo, Japan) for 8 generations. All mice were bred and maintained in a temperature- and light-controlled room and were allowed free access to a standard rodent chow diet and water. C57BL/6 mice were used as the WT mice.

Induction of diabetes. Six-week-old male mice received i.p. injections of 50 mg/kg streptozotocin (Sigma Aldrich Japan KK, Tokyo, Japan) dissolved in 0.1 M sodium citrate buffer, pH 4.5, over 5 consecutive days. The blood glucose levels were measured on days 1, 3, 5, 6 to 15, 17, 19, 21, 24, 27, and 30 after STZ injection by measuring a drop of blood from the tail vein using the blood glucose meter and test chips (Medisafe Reader, Terumo, Tokyo, Japan). Animals were sacrificed at day 15 or 30 after the start of STZ injections. All the experiments followed the principles of the University of Tokyo for laboratory animals.

Histological and immunohistochemical analysis. Pancreatic tissues were fixed in formalin for 24 h. They were dehydrated, penetrated and embedded in paraffin. The blocks were cut into 4 μm sections and used for hematoxylin and eosin (H&E) staining. For immunohistochemistry, the sections were deparaffinized and incubated with PBS-T. Endogenous peroxidase was blocked by incubation with 0.3% H_2O_2 in methanol for 1 h. After washing with PBS-T, the sections were blocked with Block Ace (Dainippon Pharmaceutical, Osaka, Japan) for 1 h at room temperature. The sections were incubated overnight at 4˚C with primary antibodies against insulin (Santa Cruz Biotechnology, Inc.), and glucagon (R&D Systems). Secondary antibodies were from the Histofine Simple Stain MAX-PO (M) and (R) (Nichirei Corporation, Tokyo, Japan). Antibody reactions were visualized using 3'-diaminobenzidine tetrahydrochloride (DAB). Sections were counterstained with hematoxylin.

Insulin detection. Mice were sacrificed and blood was sampled intracardinally. Serum insulin was measured using insulin ELISA kit (Morinaga Institute of Biological Sciences Inc., Japan). The pancreatic insulin was also measured using the same kit. The pancreas was homogenized in HCl-ethanol Buffer (7.5 ml HCl, 370 ml 95% ethanol) and further diluted to 1:1000 weight/volume.

Electron microscopy analysis. The pancreases were harvested from IA-$2^{-/-}$ and WT mice, and they underwent glutaraldehyde fixation. The ultrastructure of the pancreatic cells was analyzed using the transmission electron microscopic method (Chobikeitai Laboratory, Yokohama, Japan).

Statistics. Results were shown as means ±SE. The data were analyzed by the repeated measures (blood glucose) or non-repeated measures (insulin) ANOVA followed with Bonferroni test. Significance was defined as *p<0.05 and **p<0.01.

Results

IA-$2^{-/-}$ mice showed higher blood glucose level than WT mice after STZ treatment. From the first day of the injection of STZ, the blood glucose levels were measured for 30 days (Fig. 1A). At day 0, the blood glucose concentration was 180±17.2 mg/dl in WT mice. In IA-$2^{-/-}$ mice, it was 180±29.6 mg/dl. At day 5 after the first injection of STZ, the blood glucose levels started to increase in both mice. At day 19, the blood glucose levels increased to 415±55.2 mg/dl in IA-$2^{-/-}$ mice, while in WT mice, it was 301±30.0 mg/dl (Fig. 1A). At day 21, a significant difference was detected. After that, the blood glucose level of IA-$2^{-/-}$ mice remained higher than that of WT mice although significance was not recorded. During the 30-day observation, the blood glucose levels were higher in

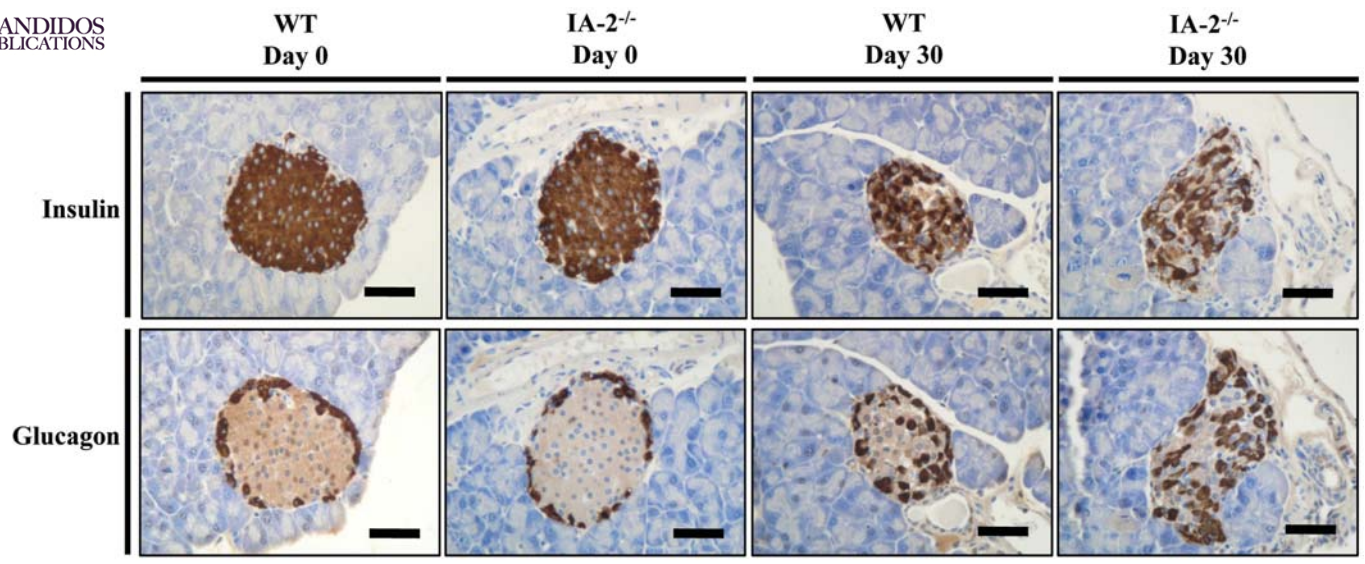

Figure 2. Immunostaining of insulin and glucagon in IA-2$^{-/-}$ and WT mice pancreas after STZ injection. A representative of five mice in each group is shown. No difference of the distribution of insulin and glucagon was seen between the WT and IA-2$^{-/-}$ mice. On day 30, a decrease in insulin positive cells and an increase of glucagon positive cells were seen in both the WT and IA-2$^{-/-}$ mice. The glucagon positive cells were seen in the center of the islets instead of their normal distribution in the edge. Bar, 50 μm.

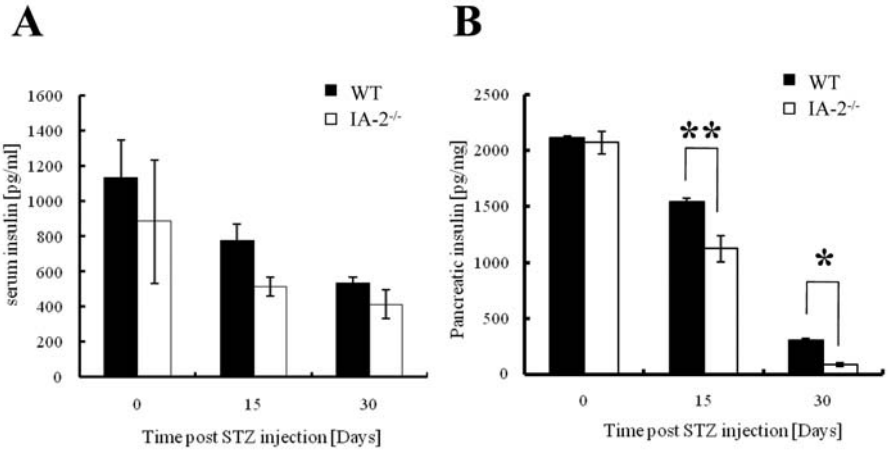

Figure 3. Changes of insulin levels in the serum and pancreas at days 0, 15, and 30 after STZ injection. Serum insulin level (A) and pancreatic insulin level (B) were detected by ELISA. The average insulin levels were lower in the STZ-treated IA-2$^{-/-}$ mice both in the serum and pancreas. On day 15 and 30, the insulin levels in IA-2$^{-/-}$ mice were significantly lower than that of WT mice. Data are means ±SE. *p<0.05, **p<0.01.

IA-2$^{-/-}$ mice than that of WT mice, indicating a higher tendency to STZ-induced diabetes.

IA-2$^{-/-}$ and WT mice displayed similar level of ß cell loss after STZ administration. To evaluate the ß cell loss between IA-2$^{-/-}$ and WT mice after the STZ administration, H&E staining of the pancreas was performed. At day 0 of STZ injection, there were no differences between the IA-2$^{-/-}$ and WT mice. Loss of cells was clearly seen in the islets 30 days after STZ treatment both in the WT and IA-2$^{-/-}$ mice (Fig. 1B). The number of ß and α cells was evaluated by immunohistochemistry using anti-insulin and -glucagon antibodies. There was clear damage of ß cells at day 30 after the treatment of multiple low doses of STZ (Fig. 2, upper panel). However, α cells appeared in the central area of islets instead of only on the edge as revealed by the staining of glucagon (Fig. 2, lower panel). To evaluate the damage of STZ to ß cell in IA-2$^{-/-}$ and WT mice, a binary of the picture of the islet was made using Image J software. The ratio of insulin positive area and glucagon positive area was calculated. However, there was no significant difference between the IA-2$^{-/-}$ and WT mice (data not shown).

Pancreatic insulin level was significantly decreased in the IA-2$^{-/-}$ mice after STZ treatment. Since the blood glucose level of IA-2$^{-/-}$ mice was higher than that of WT mice after the STZ injection, the insulin level was detected both in the serum and pancreas. As time advanced, both serum and pancreatic insulin levels were decreased in both types of mice (Fig. 3). Although there was no significant difference in the serum insulin levels at days 0, 15 and 30, the average serum insulin level was lower in IA-2$^{-/-}$ mice than WT mice (Fig. 3A). Moreover, insulin levels in the pancreas were also lower in

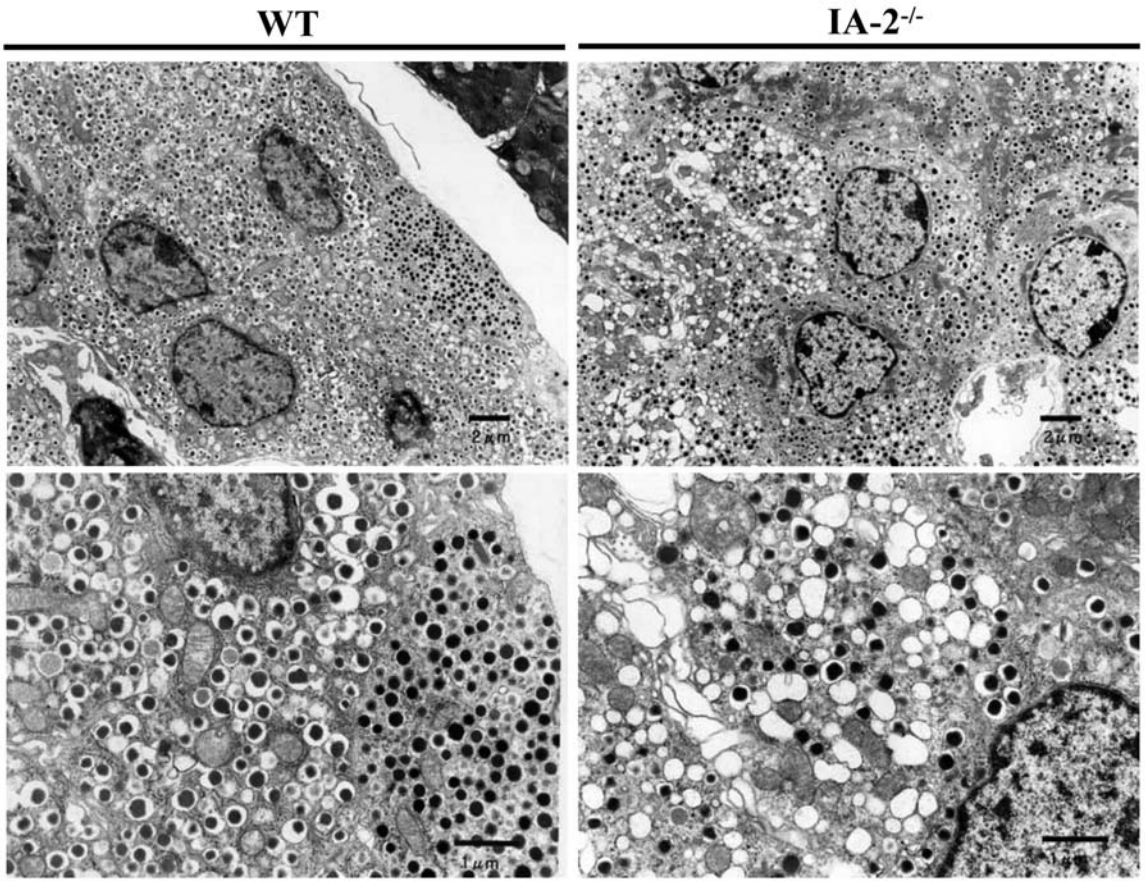

Figure 4. Ultrastructure of the pancreatic islet cells in STZ-untreated WT and IA-2$^{-/-}$ mice by transmission electron microscopy. Bar, 2 μm (upper); bar, 1 μm (lower).

IA-2$^{-/-}$ mice than WT mice. At days 15 and 30, significantly lower insulin levels were detected in the pancreas of IA-2$^{-/-}$ mice than WT mice (Fig. 3B).

The Golgi apparatus and insulin secretory granules are affected by deletion of IA-2. Despite the normal histological phenotype, IA-2$^{-/-}$ mice displayed impaired glucose tolerance ability and higher sensitivity to STZ-induced T1D. To further investigate the effect of IA-2 deficiency on insulin secretion, electron microscopy was performed on the pancreas taken from IA-2$^{-/-}$ and WT mice without STZ treatment. Impressively, in the WT mice, almost all the secretory granules were occupied. In contrast, in the IA-2$^{-/-}$ mice, a large number of empty secretory granules were observed. Furthermore, dilated ultrastructures that were expected to be components of the Golgi apparatus and endoplasmic reticulum (ER) were found in the IA-2$^{-/-}$ mice (Fig. 4). This phenomenon suggests the production of mature proteins, especially insulin in this case, was affected due to the deficiency of IA-2. Deficiency of IA-2 was suggested to affect the EG-Golgi traffic system in the pancreatic islet cells.

Discussion

IA-2$^{-/-}$ mice behaved abnormally with insulin secretion after i.p. injection of glucose. In addition, isolated pancreatic islets from the IA-2$^{-/-}$ mice showed significantly inhibited insulin secretion in response to glucose stimulation. However, without glucose stimulation, the IA-2$^{-/-}$ mice did not show abnormal blood glucose concentration (10). The function of IA-2 in the pathogenesis of T1D is still largely unrevealed. In this study, the sensitivity of IA-2$^{-/-}$ mice to STZ-induced diabetes was investigated.

After multiple-low-dose injection of STZ, IA-2$^{-/-}$ mice showed significantly higher blood glucose concentration than WT mice. Both IA-2$^{-/-}$ and WT mice demonstrated loss of ß cells after STZ injection (Fig. 2). However, the ratio of ß cells to α cells, as calculated by the areas taken using Image J, was the same between IA-2$^{-/-}$ and WT mice (data not shown). This result suggests that the levels of ß cell destruction after STZ injection are the same for IA-2$^{-/-}$ and WT mice. The STZ induced diabetes was caused by oxidative stress (11). The similar damage to ß cells by STZ in IA-2$^{-/-}$ and WT mice suggests the sensitivity of ß cells to STZ-induced oxidative stress were the same in the two types of mice. Then, the difference of blood glucose is not caused by the number of insulin producing cells, but relies on the difference of insulin secretion or production ability of individual ß cells between IA-2$^{-/-}$ and WT mice. In WT mice, even though the number was decreased, the remaining ß cells secreted enough insulin to keep the blood glucose normal. On the other hand, in IA-2$^{-/-}$ mice, the insulin secretion level per cells is low and not enough to control the glucose level. The electron microscopy of the ultrastructure of IA-2$^{-/-}$ and WT mice support this hypothesis.

periments, under non-STZ treatment condition, a ıber of empty secretory granules were found in the pancreatic cells of IA-2$^{-/-}$ mice (Fig. 4). Meanwhile, expanded membrane structures that resemble the ER-Golgi trafficking complex were found in the IA-2$^{-/-}$ mouse ß cells. Harashima *et al* demonstrated that IA-2 controls the number of insulin secreting granules in the insulinoma cell line MIN6 (12). Mziaut *et al* reported that IA-2 protein participated in the insulin gene transcription as well as controlling the transcription of the secretory granule genes, including IA-2 itself (13). On the glucose-stimulated exocytosis, the cytoplasmic/intracellular domain of IA-2 is cleaved and moved to the nucleus where it upregulates insulin transcription by binding to tyrosine phos-phorylated signal transducers and activators of transcription 5 (STAT5). In our experiments, ß cell of the IA-2$^{-/-}$ and WT mice reduced in number by the damage of STZ. The lower pancreatic insulin level of WT mice, as well as a larger number of empty secreting granules of ß cells in IA-2$^{-/-}$ mice suggests that the production of insulin in ß cells was impaired due to the lack of IA-2, *in vivo*. Moreover, the EM analysis demonstrated abnormality of the ER-Golgi trafficking components in the ß cell of IA-2$^{-/-}$ mice suggested that IA-2 not only functions in the production of insulin, but also in the packing or formation of insulin secretory granules. The balance of the vesicle conformation and the insulin transcription is damaged in the IA-2$^{-/-}$ mice. The role of IA-2 in the formation of insulin secretory granules revealed in this study will help in understanding the possible role of IA-2 in the formation of secretory granules as well as releasing of hormones and neuro-transmitters from neuroendocrine cells.

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