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Co-localization of islet amyloid polypeptide and insulin in the B cell secretory granules of the human pancreatic islets

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Summary. Islet amyloid polypeptide is a novel 37 amino-acid-residues polypeptide which has been isolated from amyloid deposits in an insulinoma, and in human and cat islets of Langerhans. The molecule has 46% homology with the calcitonin gene-related peptide. Light microscopy examination of the pancreas shows that islet amyloid polypeptide immunoreactivity is restricted to the islet B cells. The present study utilized a rabbit antiserum against a synthetic peptide corresponding to positions 20-29 of islet amyloid polypeptide, a sequence without any amino-acid identity with calcitonin gene-related peptide. By applying the immunogold technique at the ultrastructural level, it was shown that both insulin and islet amyloid polypeptide immunoreactivity occurs in the central granular core of the human B cell secretory granules, while the A cells remain unlabelled. The demonstration that islet amyloid polypeptide is a granular protein of the B cells may indicate that it is released together with insulin. Further studies are necessary to evaluate the functional role of islet amyloid polypeptide.

Key words: Islet amyloid polypeptide, Pancreatic islets, B cells, Ultrastructure, Immunocytochemistry.

Isolation and chemical characterization of amyloid deposits from a human insulinoma has revealed the existence of a previously unknown peptide designated Islet (or Insulinoma) Amyloid Polypeptide (IAPP) [1, 2]. Subsequently, it was shown that the amyloid fibrils in the islets of Langerhans, which is a most characteristic morphological finding in Type 2 (non-insulin-dependent) diabetes mellitus, has identical chemical composition [3, 4]. IAPP is also the constituent of the fibrils in feline islet amyloid, which is mainly seen in old diabetic cats [2, 3]. IAPP consists of 37 amino-acid residues and has 46% identity with the neuro-peptide calcitonin gene-related peptide (CGRP) [2]. The function of IAPP is not yet known.

Light microscopic examination of the human pancreas demonstrates that IAPP and insulin antisera label identical islet cells [2, 4-6]. The aim of the present study was to clarify the subcellular localization of IAPP in the normal human pancreatic islet B cells.

Subjects, materials and methods

Tissues

Apparently normal human pancreata were obtained from three patients (two males, 45 and 70 years old and one female, 72 years old) undergoing pancreatic resection due to carcinoma. The material used was taken from a non-affected part of the pancreas. The patients did not suffer from any metabolic disease and no amyloid was found in any of the three pancreata. For *light microscopy* the samples were fixed in 4% formaldehyde, dehydrated in ethanol and embedded in paraffin. For *electron microscopy* the samples were (a) fixed in 2% glutaraldehyde for 6 h at +20°C, postfixed in 1% osmium tetroxide for 1 h at +20°C and embedded after a conventional protocol in Agar 100 (an epoxy resin of Epon type, Agar Aids Ltd, Stansted, Essex, UK) (7), (b) fixed in 4% paraformaldehyde/0.5% glutaraldehyde for 6 h and embedded as a), or (c) fixed in 4% paraformaldehyde/0.5% glutaraldehyde for 2 h at +4°C, dehydrated in 50-95% ethanol fol-

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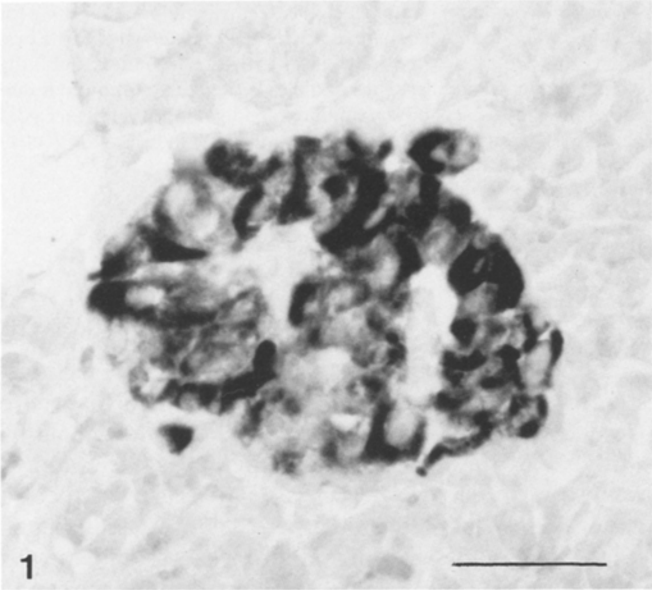
Fig. 1. Light microscopical (LM) view of the pancreas where an islet of Langerhans is labelled with anti-IAPP antisera. Bar = 50 μm; ×410

Fig. 2. LM view of the pancreas where an islet of Langerhans is labelled with anti-insulin antisera. Fig. 1 and Fig. 2 show the same islet in consecutive sections. Bar = 50 μm; ×410

Fig. 3. Two B cells with their typical vesicular granules containing angular or rodlike, crystalline cores (arrows, see also Fig. 4) and one A cell in the lower left corner. The A cell granules are round with a highly electrondense core surrounded by an eccentric halo (arrowhead, see also Fig. 5). IAPP (5 nm gold particles) is demonstrated exclusively in the B cell granules together with insulin (15 nm gold particles). Neither of these peptides were found anywhere else in the islets but in the B cell granules. Bar = 0.3 μm; ×43300

Fig. 4. Detail of a B cell with its granules filled with labels visualizing IAPP (5 nm gold particles, arrow) and insulin (15 nm gold particles, arrowhead). Bar = 0.2 μm; ×76800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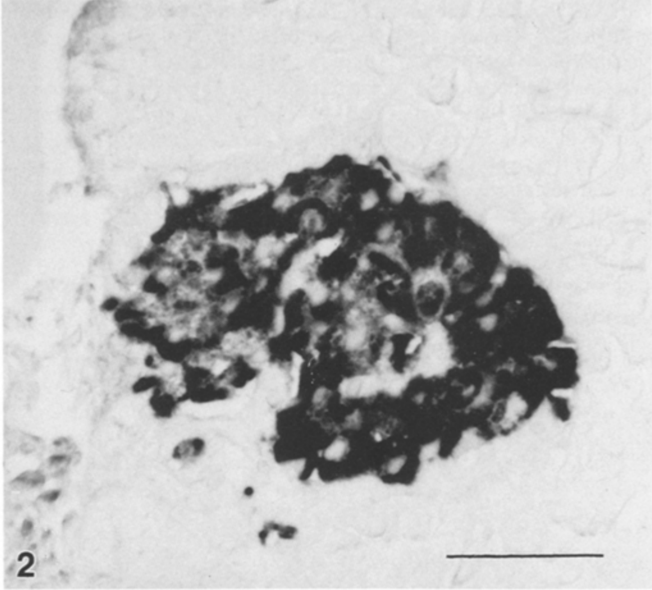
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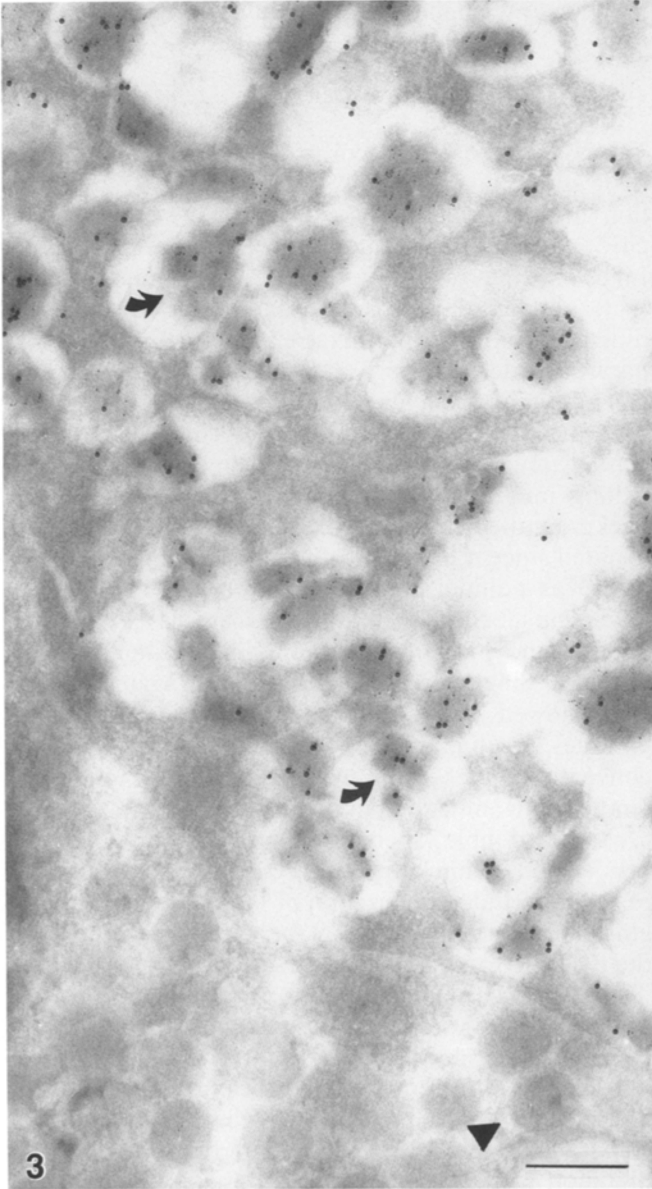
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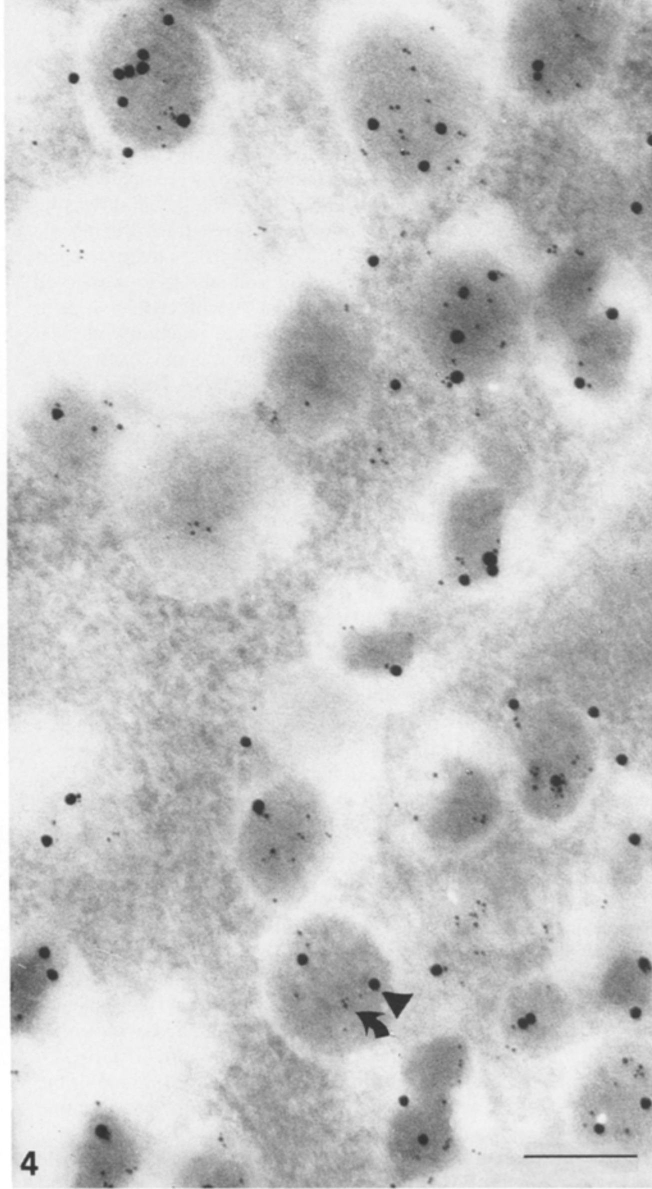
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lowed by embedding in Lowicryl K4M (Agar Aids Ltd.). During the dehydration step the temperature was lowered from +4° C to −10° C. Infiltration and photopolymerization (UV-light, 360 nm) took place at −10° C [8-12]. In all three electron microscopy embedding protocols a 0.1 mol/l cacodylate buffer, pH 7.2, containing 0.1 mol/l sucrose was used. Levels in the blocks containing islets of Langerhans were found with the help of semithin sections stained with toulidine blue and ultrathin sections were cut with a diamond knife on an LKB Ultrotome No IV (LKB-Produkter AB, Bromma, Sweden) and placed on formvar-coated nickel grids.

Antisera

A synthetic peptide, corresponding to positions 20-29 of human IAPP ($IAPP_{20-29}$) was produced. The molecule was linked to key-hole limpet haemocyanin (KLH), mixed with Freund's complete adjuvant and injected in rabbits every third week for 7 months, whereafter antiserum (AA 90) was obtained. Guinea pig antiserum to insulin was purchased from Dakopatts (Santa Barbara, Calif, USA).

Immunocytochemistry

Light microscopy. Sections of the paraffin-embedded material were deparaffinised, treated with 0.3% hydrogen peroxide and incubated over-night with antiserum to $IAPP_{20-29}$ (AA 90), diluted 1:400 in 0.05 mol/l Tris-HCl buffer, pH 7.2, with 0.15 mol/l NaCl (TBS), or antiserum against insulin, diluted 1:1000 in TBS, in a moist chamber at +20° C. Serial dilution tests determined the optimal concentration of the antisera. As intermediate antibodies sheep anti-rabbit IgG and sheep anti-guinea pig IgG was used. The sections were incubated in the PAP-complex, diluted 1:80 in TBS, before the labelling was visualized by using 30% 3,3-diaminobenzidine-tetrahydrochloride (DAB) (Sigma, St. Louis, MO, USA) in TBS with 0.02% hydrogen peroxide as chromogen [13]. Normal guinea pig and rabbit sera were used in the control experiments. Absorption of AA 90 with $IAPP_{20-29}$ prior to the immunolabelling procedure abolished the reaction with islet B cells, while absorption with KLH and insulin was uneffective. Absorption of insulin antiserum with recombinant DNA human insulin (Humulin; Kabi-Vitrum, Stockholm, Sweden) also abolished the reaction with islet B cells, while absorption with $IAPP_{20-29}$ was ineffective.

Electron microscopy. Immunocytochemical labelling for electron microscopy was done after a protocol of De Mey [14] modified by Lukinius et al. [7]. Sections from all three fixation/embedding protocols were used and etching in saturated sodium-metaperiodate (Merck, Darmstadt, FRG) for 0, 3, 5 and 10 min was performed. After careful rinsing in water, the sections were blocked with normal goat serum (National Veterinary Institute, Uppsala, Sweden) diluted 1:20 in TBS with 0.1% BSA (Bovine serum albumine type V, Sigma, St. Louis, MO, USA) for 30 min at +20° C. The sections were drained and incubated with antiserum against $IAPP_{20-29}$ diluted 1:800 in TBS-0.1% BSA at +4° C over-night, drained again and incubated for 2 h at +20° C with antiserum against insulin (1:1000 in TBS-0.1% BSA). After careful rinsing in TBS containing 0.1%, 0.2% and finally 1.0% BSA, the sections were incubated for 2 h at +20° C with goat anti-rabbit IgG conjugated to 5 nm colloidal gold (GAR-G5, Janssen Pharmaceutica N.V., Beerse, Belgium) diluted 1:20 in TBS, pH 8.2, with 1% BSA. After draining, the grids were incubated with goat anti-guinea pig IgG conjugated to 15 nm colloidal gold (GAGp-G15, Janssen Pharm.), diluted 1:20 in TBS, pH 8.2, with 1% BSA for 2 h at +20° C. Finally, the grids were rinsed and counterstained with uranyl acetate and Reynolds lead citrate. Grids were also single-labelled with either $IAPP_{20-29}$ antiserum/GAR-G15 or insulin antiserum/GAGp-G15. As control, incubation with the primary antiserum was omitted or the primary antiserum was replaced by the homologous non-immune serum. Otherwise sections were treated as described above.

To obtain optimal results, all buffers were passed through sterile filters (pore diameter 0.20 μm) and any aggregates in the protein- and gold/protein solutions were pelleted by centrifugation at 2000 × *g* for 15 min. Ealier double-labelling experiments have shown that the best results are acquired when incubation with the secondary antibody conjugated to the smallest gold particles is done first, followed by incubation with the secondary antibody conjugated to the bigger gold particle [7]. Examination of the results was done in a Jeol 100 C electron microscope.

Results

Light microscopy

Examination of routinely embedded paraffin sections, stained with haematoxylin/eosin, confirmed that there were no abnormalities in the pancreata used in this study. With the peroxidase anti-peroxidase method, it was shown that the $IAPP_{20-29}$ antiserum labelled a major cell population in the islets of Langerhans (Fig. 1). The same labelling pattern was also shown with the insulin antisera (Fig. 2). In the control experiments there was no labelling with any of the two antisera.

Electron microscopy

The conventional protocol with fixation in glutaraldehyde and osmium tetroxide followed by 100% dehydration and embedding in epoxy resin gave a very unspecific and sparse labelling when IAPP antisera was used. Likewise, the result was poor when paraformaldehyde/glutaraldehyde fixation and epoxy resin embedding was used. The mild fixation for 2 h at +4° C in 4% paraformaldehyde/0.5% glutaraldehyde, followed by embedding in the hydrophilic low temperature embedding media Lowicryl K4M, preserved the IAPP antigenicity much better and was used in the study. The labelling intensity was high and there was almost no background. Etching in sodium-metaperiodate was avoided since it had a negative effect on the specificity as well as a diminishing effect on the labelling.

Single labelling with antisera against IAPP showed specific and prominent immunoreaction in the B cell granules. Single labelling with antisera against insulin also showed specific immunoreaction in the B cell granules. Insulin antigenicity was preserved both in the conventional embedded material as well as in the low temperature embedded material.

In the double labelling experiments the gold markers (5 and 15 nm respectively) used for identification of the subcellular localization of IAPP and insulin were almost exclusively found in the secretory granules of the B cells. Mostly, there was an obvious co-localization of IAPP and insulin immunoreactivity in the same granules. The gold-particles were predominantly localized to the electron-dense central core of the granules leaving the peripheral electron lucent space unlabelled (Figs. 3 and 4). Any noteworthy unspecific background labelling that could interfere with the interpretation of the results was not observed. The A cells

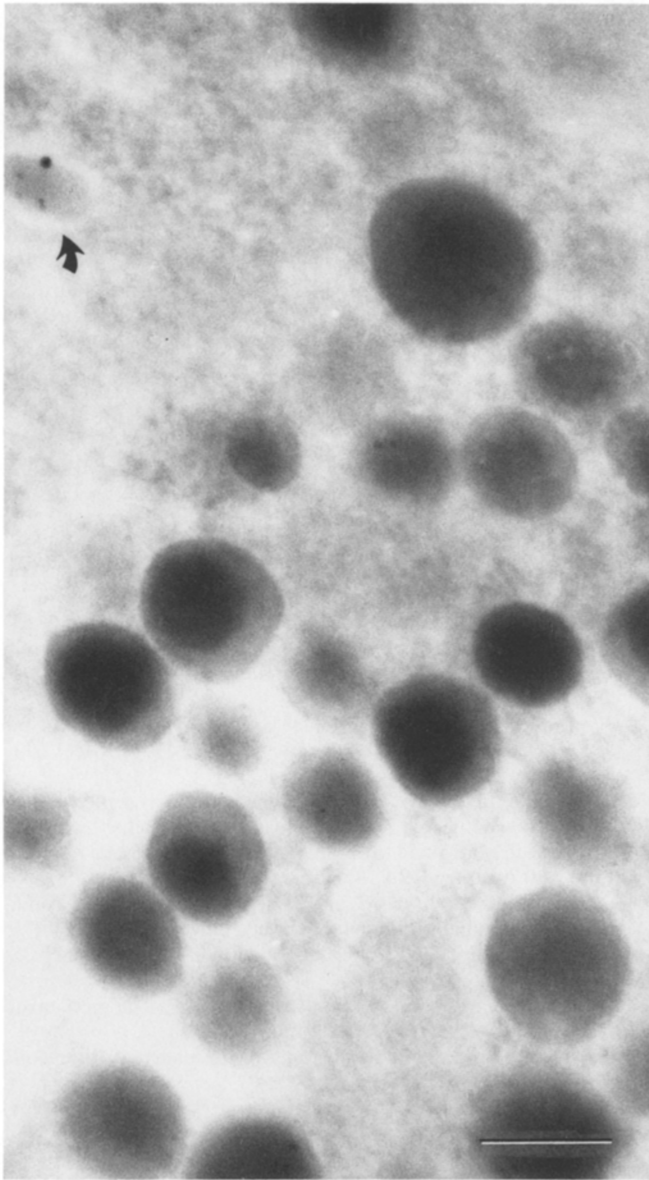

Fig. 5. Detail of an A cell completely lacking any label for IAPP or insulin. Note the B cell granule in the upper left corner containing labels for both IAPP and insulin (arrow). Bar = 0.2 um; × 87000

were unreactive to both IAPP and insulin antisera (Fig. 5).

Light microscopical experiments with the PAP technique, and electron microscopical experiments with the immuno-gold technique, confirm that there is no cross-reactivity between the secondary antibodies. In the control experiments where the primary antisera were omitted no labelling occured.

Discussion

The functional significance of IAPP is unknown. However, several facts indicate that IAPP possesses a hormonal function, namely: (a) the 46% amino-acid homology with CGRP, a recently identified neuropeptide that is present in certain restricted areas of the central and peripheral nervous system [15, 16], (b) its occurrence in the islet B cells and co-localization with insulin in the secretory granules, (c) the amyloid in the C cell tumour of the thyroid gland consists of a calcitonin-related protein, probably procalcitonin [17], (d) IAPP is expressed as a 89 amino-acid residue molecule consistent with a preprohormone (C. Betsholtz et al. to be published) and (e) IAPP has been shown to be a potent inhibitor of both basal and insulin-stimulated rates of glycogen synthesis in stripped rat soleus muscle in vitro [18]. It is possible that IAPP is co-released with insulin from the B cells on stimulation.

The findings in the present investigation clearly indicate that the IAPP immunoreactivity in human B cell granules is restricted to the electron-dense core. In a previous electron microscopic study performed on cat islet B cells, IAPP immunoreactivity was localized to the outer, translucent zone of the secretory granules [5]. This discrepancy cannot be explained at present but species differences may exist, and fixation and processing can result in redistribution of soluble proteins which would explain the differences between the present and previous results. The antisera used in both the studies had also been raised to different parts of the IAPP molecule. In the present study we used antiserum to $IAPP_{20-29}$, while in the previous study antiserum to $IAPP_{7-17}$ was employed. While the sequence of $IAPP_{20-29}$ is unique to IAPP, $IAPP_{7-17}$ is > 50% identical to CGRP [2]. However, at the light microscopical level the two different IAPP antisera gave identical staining of islets in human pancreas.

Recently, it has been shown that the human B cells display chromogranin immunoreactivity [19]. The chromogranins comprise a family of acidic high molecular weight polypeptides, originally isolated from the granules of the adrenal medulla [20–23]. The levels of chromogranin in serum are elevated in patients with a great variety of neuroendocrine tumours and the chromogranins are regarded as putative peptide hormones [19, 24]. Apparently the B cell granules are the storage site of several peptides in addition to C-peptide and insulin. Although speculative, it is reasonable to assume that at B cell granular exocytosis, a spectrum of peptide molecules are released into the circulation. Such a theory has major clinical implications since, from the morphological point of view, Type 1 (insulin-dependent) diabetes mellitus is a disease characterized by pronounced B cell deficiency [25–29]. This means that deficiency of substances other than insulin may also be of some significance for the metabolic disturbances and late complications occurring in many diabetic individuals.

IAPP was originally identified by chemical analysis of amyloid from a human insulinoma, and subsequently from insular amyloid [1–4]. Antiserum to IAPP reacts both with B cells and the islet amyloid deposits [5, 6]. In Type 2 diabetes, IAPP immunoreactivity in the B cells is diminished while IAPP is deposited as

amyloid fibrils in the islets [6]. Islet amyloid deposits occur in addition to humans in a limited number of mammalian species e.g. monkeys [30] and cats [31], in association with a form of diabetes which resembles human Type 2 diabetes. This indicates that the ability of IAPP to polymerize to amyloid is associated with the development of Type 2 diabetes. More information about species variations in the amino acid sequence and metabolic pathways of IAPP may increase the knowledge about the pathogenesis of Type 2 diabetes.

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