Islet amyloid polypeptide/amylin messenger RNA and protein expression in human insulinomas in relation to amyloid formation

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Abstract

Objective: Islet amyloid polypeptide (IAPP), also named amylin, is the predominant protein component of amyloid deposits in human islet β cell tumours of the pancreas (insulinomas). IAPP is co-produced with insulin by islet β cells. We investigated IAPP expression in relation to insulin expression and to amyloid formation in eleven insulinomas.

Design and methods: RNA and protein extracts were prepared from the same pieces of tumour tissue, and from specimens of two normal human pancreata. IAPP and insulin mRNA and peptide content were quantified using Northern blot analysis and radioimmunoassay (RIA) respectively. Molecular forms of IAPP immunoreactivity were analysed by reversed-phase high-performance liquid chromatography (HPLC). The presence of islet hormones and of amyloid was assessed by (immuno)histochemical staining of paraffin sections. Plasma levels of IAPP and insulin prior to tumour resection were determined by RIA.

Results: IAPP and insulin mRNA and peptide content varied widely between the tumour specimens, and there was considerable intratumour heterogeneity of peptide content. HPLC analysis indicated correct proteolytic processing of the IAPP precursor protein. Amyloid deposits were detected only in the three tumours with the highest IAPP content. In contrast to insulin, plasma levels of IAPP were not elevated in the insulinoma patients.

Conclusions: The spectrum of hormone production by insulinomas cannot be inferred from only a few tissue sections due to intratumour heterogeneity. Expression of the IAPP and insulin genes is not coupled in insulinomas, which produce properly processed mature IAPP. In addition to IAPP overproduction, additional factors such as intracellular accumulation of IAPP are involved in amyloidogenesis in insulinomas.

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Introduction

Insulinoma is the most common pancreatic endocrine tumour, with an estimated annual incidence of four cases per million (1). In over 50% of insulinomas investigated, amyloid deposits have been demonstrated (2–4). The major protein component of these deposits was identified as islet amyloid polypeptide (IAPP) (5, 6). A peptide with the same amino acid (aa) sequence was purified from pancreatic islet amyloid in patients with type 2 (non-insulin-dependent) diabetes mellitus (7) and was named amylin (8).

Islet amyloid polypeptide is a 37 aa polypeptide, which has 43% and 46% aa sequence identity with calcitonin gene-related peptide-I and -II respectively (9). IAPP is

produced in islet β cells and co-secreted with insulin in response to glucose and other β cell secretagogues (10, 11). Human IAPP is synthesized as part of an 89 aa precursor protein (preproIAPP) which includes a 22 aa amino-terminal signal peptide for transport through the endoplasmic reticulum (12, 13). Proteolytic cleavage of the 67 aa propeptide (pIAPP) at pairs of basic aa residues yields mature IAPP(1–37), as well as amino- and carboxy-terminal flanking peptides (N-pIAPP of 9 aa and C-pIAPP of 16 aa respectively) (Fig. 1).

Several potential physiological functions of IAPP have been reported which include inhibition of insulin secretion from β cells and inhibition of actions of insulin on glucose metabolism (14).

Met Gly Ile Leu Lys Leu Gln Val Phe Leu Ile Val Leu Ser Val Ala Leu Asn His Leu
 Lys Ala Thr Pro Ile Glu Ser His Gln Val Glu Lys Arg *Lys Cys Asn Thr Ala Thr Cys*
Ala Thr Gln Arg Leu Ala Asn Phe Leu Val His Ser Ser Asn Asn Phe Gly Ala Ile Leu
Ser Ser Thr Asn Val Gly Ser Asn Thr Tyr Gly Lys Arg Asn Ala Val Glu Val Leu
Lys Arg Glu Pro Leu Asn Tyr Leu Pro Leu

Figure 1 Amino acid (aa) sequence of the 89 aa precursor protein of human islet amyloid polypeptide (preproIAPP). The sequence of IAPP(1–37) is given in italic bold type and the amino- and carboxy-terminal flanking peptides of 9 and 16 aa (N-pIAPP and C-pIAPP) are underlined.

The purpose of the present study was to examine relationships between IAPP and insulin expression and their relations with the presence of amyloid deposits in endocrine pancreatic tumours clinically diagnosed as insulinomas. Expression was studied at the immuno-histochemical, mRNA and peptide level and molecular form(s) of IAPP were characterized using reversed-phase high-performance liquid chromatography (HPLC).

Materials and methods

Tissues

Two normal human pancreas specimens were obtained at autopsy, and served as a reference for protein levels in non-tumour pancreas tissue, as well as a positive control for the immunohistochemical detection of islet hormones and endocrine markers. Pancreatic neoplasms were from eleven patients operated on between 1986 and 1994. One patient (patient a) suffered from multiple endocrine neoplasia type 1 (MEN 1), and one

patient (patient k) had a malignant islet cell tumour with liver metastases.

Samples of each tumour were snap frozen in liquid nitrogen immediately after surgical resection and stored at -80°C until use. Parallel samples were fixed in buffered formalin for 24 to 48 h and embedded in paraffin.

Histopathology and immunohistochemistry

Besides haematoxylin and eosin staining, Congo red (CR) staining of tumour sections was performed for the detection of amyloid deposits (15). These CR-stained sections were examined by polarized light microscopy, which reveals an apple-green birefringence of amyloid deposits, and by UV light microscopy, visualizing auto-fluorescent CR-stained material.

Immunohistochemical staining on paraffin sections was performed as described (16). The presence of chromogranin, glucagon, gastrin, Ki 67 antigen, insulin and IAPP was determined with the antibodies listed in Table 1, and semiquantitatively scored from 0

Table 1 Antibodies used in immunohistochemistry.

Antibody	Specificity	Clone or code no.	Dilution	Source
Monoclonal				
Anti Ki 67	345 and 395 kDa nuclear antigen of proliferating cells (G1, S, G2, M phases)	MIB-1	2 $\mu\text{g/ml}$	Immunotech, Marseille, France
Polyclonal				
Rabbit anti-chromogranin A	C-terminal region of chromogranin A	A430	1:500	DAKO, Glostrup, Denmark
Rabbit anti-glucagon	Porcine/human glucagon	A565	1:1000	DAKO, Glostrup, Denmark
Rabbit anti-gastrin	Non-sulphated I and sulphated II forms of gastrin-17 and -34	A568	1:2000	DAKO, Glostrup, Denmark
Guinea pig anti-insulin	Porcine/human insulin	A 564	1:1000	DAKO, Glostrup, Denmark
Rabbit anti-IAPP	As described	K1338	1:800	van Hulst <i>et al.</i> (22)

(negative) to 1–5%, 6–10%, 11–25%, 26–50%, 51–75% or 76–100%, indicating the percentage of positive tumour cells in a representative region of the stained tissue section analysed.

Tissue and plasma extraction

Tissue was powdered in liquid nitrogen and two portions were separated for extraction of total cellular RNA (17) and polypeptides respectively. The latter were extracted overnight in acidified ethanol, lyophilized and prepared for radioimmunoassay (RIA) (18). The DNA content in tissue pellets was measured (19) as a reference for peptide quantification in the tissue extracts. For the determination of IAPP levels in the circulation, EDTA-plasma of insulinoma patients was extracted with acid-acetone (11).

Northern blot analysis

Samples of 20 µg total cellular RNA were analysed by Northern blotting as described (13). A 588 base pairs human IAPP probe and a 751 base pairs human insulin probe were described previously (20). An 18S ribosomal RNA (rRNA) probe was provided by GWM Swart (Department of Biochemistry, University of Nijmegen, The Netherlands). The probes were labelled with [α -³²P]dCTP (Amersham, Little Chalfont, Bucks, UK) using a Prime-a-Gene labelling system (Promega Corporation, Madison, WI, USA). Hybridization conditions were described previously (21). For quantitation of mRNA, IAPP and insulin probes were dot-blotted in increasing amounts of 1, 5, 10, 50, 100, 500, 1000 and 5000 pg and hybridized together with the Northern blots. Quantitation of hybridization signals was performed by phosphor-imaging and Image-Quant version 3.3 software (Molecular Dynamics, Inc. GmbH, Krefeld, Germany). To correct for different amounts of RNA loaded on the gel, hybridization signals were normalized to the 18S rRNA hybridization signal.

Reversed-phase high-performance liquid chromatography

Tissue extracts were subjected to reversed-phase HPLC on a Nucleosil 100–7C18 column (Machery-Nagel, Düren, Germany) as described previously (18). Mean recoveries of synthetic human IAPP and C-pIAPP were $87.5 \pm 9.1\%$ ($n=4$) and $91.2 \pm 2.9\%$ ($n=2$) respectively.

Radioimmunoassays

Immunoreactive IAPP and insulin were measured by RIA as described (11). For the IAPP RIA, IAPP antiserum K1338 to human IAPP was used (22). In

normal subjects, plasma levels of IAPP ranged from non-detectable (<1.1 pmol/l) to 14 pmol/l (11).

Immunoreactive C-pIAPP was determined by RIA using antiserum K1352 (20, 22). This antiserum was obtained by subcutaneous injection of a New Zealand white rabbit with 35 µg synthetic C-pIAPP (preproIAPP (74–89); Peninsula Laboratories, Belmont, CA, USA) conjugated to bovine thyroglobulin (Sigma, St Louis, MO, USA) using 1% glutaraldehyde. Booster injections were administered every three weeks. The antiserum collected after the 11th injection was used at a final dilution of 1:250 000. Antibody-bound and free radioligands were separated using donkey anti-rabbit coated cellulose (IDS, Boldon, UK) as antibody-immunoprecipitating reagent. The detection limit of the RIA (B_0-2 sd) was 0.30 ± 0.14 fmol/tube ($n=6$).

Statistical analysis

Results were calculated as means $\pm$ sd. Relationships between IAPP and insulin mRNAs and their corresponding peptides were analysed using Pearson's correlation coefficient, and P values <0.05 were considered statistically significant.

Results

Patients, histopathology and immunohistochemistry

Clinical characteristics and preoperative plasma levels of immunoreactive IAPP and insulin are depicted in Table 2. In most patients plasma levels of insulin were elevated, but those of IAPP did not differ from healthy subjects.

All tumours analysed had features of islet cell tumours. Ten tumours were benign (islet cell adenoma), whereas the tumour of patient k appeared to be malignant (islet cell carcinoma) because of the occurrence of liver metastases. The diameter of the tumours ranged from 1 to 9 cm (Table 3).

Immunohistochemical analysis of the majority of tumour specimens was performed in detail (Table 3, Fig. 2). Insulin was detected in a low percentage of tumour cells in eight of the ten specimens investigated. Eight of the eleven tumours stained for IAPP and the percentage of positive cells ranged from 5 to 100%. IAPP producing tumour cells were found as foci in the tumour of patient i and only in the peripheral area of the tumour in patient j. In the tumour sections of all the other patients IAPP producing cells were evenly distributed. In some tumours, IAPP was also demonstrated in connective tissue stroma. These IAPP immunoreactive structures were identified as amyloid deposits (Figs 2 and 3). All of the ten specimens investigated stained for chromogranin. Two of the ten tumours stained for glucagon, albeit in a low

Figure 2 Paraffin sections of insulinoma tissue from patient c stained with antibodies to (a) insulin, (b) IAPP, (c) chromogranin A, and (d) Ki 67 nuclear antigen. In panels (a) and (b), arrows indicate moderately immunoreactive cells lying between numerous weakly immunoreactive or negative tumour cells. Amyloid deposits (*) are weakly IAPP-immunoreactive. In panel (c), many tumour cells are stained for chromogranin A, in accordance with the endocrine nature of the tumour. In panel (d), the nuclei of three tumour cells are Ki 67-immunoreactive (arrows), whereas amyloid deposits (*) are negative with this antibody. (Magnification: 150-fold).

percentage of cells, and six of the ten tumours investigated stained for gastrin. In general the number of cell divisions, as determined with Ki 67 antigen antibody, was small, except for the islet cell carcinoma from patient k.

Tumours from patients a, b and c showed hyalinization of connective tissue stroma with Congo red-positive fibrillar material deposits. Congo red autofluorescence revealed fine needle-like structures located within or between tumour cells and apparently confluent into the amyloid deposits (Fig. 3).

Analysis of insulinoma extracts

Northern blot analysis of insulinoma RNAs revealed a large variation of IAPP and, to a lesser extent, of insulin gene expression (Fig. 4). The ratio between IAPP and insulin mRNA content ranged from 0.01 to 5.29 (Table 4).

Concentrations of immunoreactive IAPP and insulin in extracts of specimens of two normal human pancreata were 22.2 and 26.4 fmoleq IAPP/ μ g DNA and 0.7 and 4.7 pmoleq insulin/ μ g DNA respectively. In the insulinoma tissue extracts both immuno-

reactive IAPP and insulin varied substantially, their ratio ranging from 0.004% to 78.8% (Table 4). A statistically significant correlation was detected between mRNA and peptide levels of IAPP ($r=0.70$, $P<0.02$), whereas no correlation was found between mRNA and peptide levels of insulin. Ratios between IAPP and insulin mRNA only revealed a statistically significant correlation with the corresponding peptide ratios when insulinoma patient c (see below) was omitted from the calculation ($r=0.72$, $P<0.02$). In five tumours immunoreactive C-pIAPP was detected next to IAPP. In these tumours, levels of C-pIAPP revealed a statistically significant correlation with those of IAPP ($r=0.91$, $P<0.04$). An exceptional case was the insulinoma of patient c, in which no immunoreactive C-pIAPP was detected, despite a high IAPP content. In this tumour, the low IAPP mRNA level was also not in agreement with the high IAPP peptide level. Insulinoma extracts from patients a to f, as well as a normal human pancreas extract, were analysed by reversed-phase HPLC to examine the molecular form(s) of immunoreactive IAPP and C-pIAPP in these tissues. In all extracts, the majority of immunoreactive IAPP (ranging from 50.1 to 84.5%)

Figure 3 Paraffin section of insulinoma tissue from patient a stained with Congo red. (a) Low magnification (50-fold) shows tumour cells around stroma focally birefringent under polarized light. (b) Higher magnification (150-fold) of the marked central area in (a) shows small patches of green birefringence of the amyloid under polarized light. The same area as in (b) is shown in (c) under ordinary light, and in (d) under UV-light. (e) Higher magnification (450-fold) of (d) shows fine fluorescent needles protruding from or situated in tumour cells and apparently confluent into the Congo red positive stroma.

and of immunoreactive C-pIAPP (ranging from 87.4 to 100%) had the retention time of synthetic amidated IAPP(1–37) and C-pIAPP respectively (Fig. 5). Two smaller peaks of immunoreactive IAPP (around fractions 30 and 36 respectively) were observed both in the normal human pancreas and in 5 of the 6 insulinomas. The smaller peak of immunoreactive IAPP around fraction 30 co-eluted with immunoreactive C-pIAPP. In the insulinoma extract of patient a, small peaks of immunoreactive IAPP and C-pIAPP were detected around

fraction 70. Another peak of immunoreactive IAPP (around fraction 47) was only detected in insulinoma tissue extracts of patients a, c, e and f.

Discussion

Immunohistochemistry

Immunohistochemical analysis confirmed that all tumours investigated originated from the endocrine

Table 2 Sex, age and fasting plasma levels of IAPP and insulin (determined prior to surgery) of insulinoma patients.

Insulinoma patient	Sex (M/F)	Age (years)	IAPP (pmoleq/l)	Insulin (pmoleq/l)
a*	M	17	10.6	ND
b	F	42	9.3	154
c	F	66	5.5	88
d	F	80	10.5	176
e	F	26	9.6	213
f	M	46	10	176
g	M	21	8.4	51
h	M	43	<2	418
i	F	67	6.7	1356
j	M	33	1.7	139
k	M	51	8.6	572
Upper limit of plasma levels in normal subjects			14	110

* Patient with MEN 1; ND = not done.

pancreas. Several islet hormones were demonstrated, with a considerable inter- and intra-tumour variation in the number and distribution of cells producing these hormones. Focal localization of IAPP and insulin producing cells in the tumours of patients a and g, or insufficient sensitivity of IAPP and insulin immunohistochemistry, may explain the absence of IAPP and insulin staining cells in selected tissue sections, in the face of detectable amounts of both peptides in extracts of tissue specimens of the same tumours. Alternatively, or in addition, secreted peptides present in blood vessels may contribute to the levels of immunoreactive peptides measured in extracts, whereas immunohistochemistry detects the cellular peptides.

Analysis of insulinoma extracts

Messenger RNA and peptide levels of IAPP and insulin, as well as ratios between IAPP and insulin, both at the

mRNA and peptide level, showed enormous variation between the tumour specimens. This supports the notion that the expression of the two genes is not necessarily coupled (23–25). Levels of immunoreactive IAPP and insulin were elevated in about 50% of insulinoma specimens, as compared with the normal human pancreas. A correlation was found between mRNA and peptide levels of IAPP, but not of insulin. Uncontrolled insulin release, also through a constitutive pathway, and/or decreased storage capacity for insulin in tumour cells may account for this difference (26).

O'Brien *et al.* (24) postulated distinct populations of islet β cells that contain different relative amounts of IAPP and insulin, and Pipeleers (27) reported functionally heterogeneous β cell populations in the human pancreas. Large differences in ratios between IAPP and insulin in the insulinoma specimens investigated in this study indicate that these tumours may have originated from different types of β cells.

Table 3 Histopathology and immunohistochemistry of the insulinomas investigated.

Insulinoma patient	Diameter of tumour(s) (cm)	Insulin ^a	IAPP ^a	Chromogranin ^a	Glucagon ^a	Gastrin ^a	Ki 67 ^a antigen	Hyalinization (+/–)	Congo red (+/–)
a	3	0	0	+ ^b	0	+ ^b	ND	+	+
b	2	+ ^b	51–75	ND	ND	ND	ND	+	+
c	1	6–10	11–25	76–100	0	6–10	1–5	+	+
d	1.1	6–10	76–100	76–100	0	0	1–5	–	–
e	2	1–5	6–10	76–100	1–5	11–25	6–10	–	–
f	1.3	6–10	76–100	51–75	0	1–5	1–5	–	–
g	4	0	0	76–100	0	0	1–5	–	–
h	1.5	6–10	6–10	76–100	0	0	1–5	–	–
i	9	6–10	6–10	76–100	6–10	6–10	1–5	–	–
j	3	1–5	76–100	76–100	0	0	1–5	–	–
k	ND	6–10	0	76–100	0	6–10	11–25	–	–

^a Percentage of stained cells; ^b Percentage of immunoreactive cells not determined; ND = not done.

Figure 4 Northern blot analysis of total cellular RNA (20 µg) from insulinoma specimens of patients a to k. Northern blots were consecutively hybridized with a human IAPP probe detecting the human IAPP mRNAs of 0.8, 1.6 and 2.1 kb (38) as indicated by the arrows (top panel); with a human insulin probe detecting the human insulin mRNA of approximately 0.7 kb (39) (middle panel); and with an 18S rRNA probe (bottom panel). Dot-blot of unlabelled human IAPP and human insulin probes were used to quantitate mRNA hybridization signals, which were subsequently normalized to the 18S rRNA hybridization signal (see Materials and methods and Table 4).

Reversed-phase HPLC revealed that the predominant immunoreactive IAPP and C-pIAPP components in insulinoma tissue extracts had the retention times of the corresponding synthetic peptides, indicating proper processing of preproIAPP in β cell tumours. Minor components, some of which may contain both IAPP and C-pIAPP immunoreactivity, were detected in some tumour extracts and may correspond to aberrant or partial proteolytic processing products of preproIAPP. The presence of mature IAPP(1–37) as the major

immunoreactive IAPP component in insulinomas was also reported by Bretherton-Watt *et al.* (28), using gel permeation chromatography.

Plasma IAPP and insulin levels

In insulinoma patients the plasma levels of IAPP, in contrast to those of insulin, were within the normal range. Stridsberg *et al.* (29) detected an elevated plasma level of IAPP in only 1 of 11 insulinoma

Table 4 Ratio between concentrations of IAPP and insulin mRNAs, concentrations of insulin, IAPP, and its C-terminal flanking peptide (C-pIAPP), and the ratio between IAPP and insulin in insulinoma tissue extracts.

Insulinoma patient	IAPP/insulin mRNA ratio	IAPP (fmoleq/µg DNA)	Insulin (pmoleq/µg DNA)	IAPP/insulin peptide ratio ($\times 10^{-2}$)	C-pIAPP (fmoleq/µg DNA)
a	5.29	523.3	9.4	5.6	128.8
b	1.60	438.8	49.5	0.9	51.1
c	0.43	551.4	0.7	78.8	<2.7
d	0.20	80.7	7.8	1.0	3.5
e	1.21	54.2	2.0	2.7	14.7
f	0.11	52.2	26.4	0.2	4.4
g	0.03	2.4	53.8	0.004	<1.4
h	0.17	13.3	2.1	0.6	<1.3
i	0.39	<2.1	0.6	<0.4	<1.4
j	0.01	<3.0	12.9	<0.02	<2.0
k	0.10	<4.6	0.1	<4.6	<2.3
Normal pancreas ($n = 2$)		22.2, 26.4	0.7, 4.7	3.17, 0.56	1.0, <1.0

Figure 5 Reversed-phase HPLC profiles of extracts from a normal human pancreas (NP) and six insulinomas (a to f). Individual fractions were analysed for immunoreactive IAPP (Δ) and C-plAPP ($\circ$). The acetonitrile gradient (AG) is represented by the dotted line and arrows indicate the retention times of synthetic human C-plAPP (I) and IAPP(1–37) (III), as well as of 125 I-human calcitonin gene-related peptide I (II) and 125 I-salmon calcitonin (IV).

patients investigated. The concentrations of these peptides in plasma may not reflect those in insulinoma extracts, because of cellular heterogeneity and different sizes of the tumours. Nevertheless, normal plasma levels of IAPP, in the presence of increased tumour IAPP content, suggests retention of IAPP by the tumour tissue.

IAPP expression in relation to amyloidogenesis

The highest concentrations of immunoreactive IAPP were revealed in insulinoma specimens of patients a, b

and c. The high IAPP mRNA content in the insulinomas of patients a and b indicate overproduction of IAPP. In insulinoma tissue of patient c, however, high IAPP concentrations were associated with low IAPP mRNA content and with undetectable immunoreactive C-plAPP. Remarkably, amyloid deposits were only detected in tumours of patients a, b and c, suggesting that amyloid deposition is associated with high local concentrations of IAPP. This agrees with a report by Tasaka *et al.* (30), demonstrating that also in patients with type-2 diabetes mellitus, amyloid deposition in

pancreatic islets was associated with a high pancreatic IAPP content. The discrepancy between IAPP mRNA and peptide contents in the insulinoma extract of patient c may be due to excessive intracellular accumulation of the peptide, inducing amyloid formation despite a low production. Thus, besides IAPP overproduction additional factors, such as increased cellular retention of IAPP, may be essential for amyloidogenesis in pancreatic tissue.

Novials *et al.* (25) demonstrated that exposure of isolated human islets to high glucose concentrations resulted in enhanced IAPP synthesis but impaired IAPP secretion. In transgenic mice overexpressing human IAPP mRNA as compared with endogenous mouse IAPP mRNA (20), elevated plasma levels of IAPP (up to 15-fold) were revealed when compared with non-transgenic animals. Islet amyloid was not demonstrated in these animals by histochemical staining or electron microscopy (20, 31). When pancreatic islets of these human IAPP transgenic mice were cultured in high glucose medium, intra- and extracellular amyloid fibrils were formed (32), probably as a result of enhanced intracellular accumulation of IAPP. Also in other human IAPP transgenic mouse models, islet amyloid formation has been associated with hyperglycaemia, suggesting the involvement of increased intracellular IAPP concentrations (33–35). Congo red staining or birefringence are inadequate for the detection of small deposits of amyloid fibrils. The autofluorescent properties of Congo red, however, served to detect small needle-like structures by using UV-light microscopy, suggesting an intracellular origin of amyloid deposits (4).

Conclusions

The present study demonstrates independent and heterogeneous expression of IAPP and insulin in human insulinomas. Therefore, immunohistochemical analysis of a few tissue sections does not necessarily reflect the concentrations of these peptides in different pieces of the same tumour.

HPLC analysis of tissue extracts revealed correct proteolytic processing of the IAPP precursor in insulinomas. In these islet β cell tumours the secretion of IAPP, however, seems impaired. Hyperglycaemia has been shown to promote intracellular accumulation of IAPP in human islet β cells (25), suggesting a defect in IAPP secretion also in patients with type-2 diabetes.

Amyloid was detected only in the three tumours with the highest IAPP content. Both in islet β cell tumours and in type-2 diabetes patients, impaired IAPP secretion is probably involved in amyloidogenesis, associated with β cell destruction (36, 37).

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