Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 3881–3885, June 1987
Medical Sciences

Amyloid fibrils in human insulinoma and islets of Langerhans of the diabetic cat are derived from a neuropeptide-like protein also present in normal islet cells

(islet B cells/calcitonin gene-related peptide/diabetes mellitus)

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Communicated by Jan G. Waldenström, February 9, 1987 (received for review November 24, 1986)

ABSTRACT **Amyloid deposits localized to the islets of Langerhans are typical of non-insulin-dependent human diabetes mellitus and of diabetes mellitus in adult cats. Amyloid deposits also commonly occur in insulin-producing pancreatic tumors. We have purified a major protein—insulinoma or islet amyloid polypeptide (IAPP)—from human and cat islet amyloid and from amyloid of a human insulinoma. IAPP from human insulinoma contained 37 amino acid residues and had a theoretical molecular mass of 3850 Da. The amino acid sequence is unique but has >40% identity with the human calcitonin gene-related peptide. A partial amino acid sequence of cat islet IAPP corresponding to positions 1–27 of human insulinoma IAPP was identical to the human IAPP except for substitutions in three positions. An antiserum raised to a synthetic human insulinoma IAPP-(7–17) undecapeptide showed specific immunohistochemical reactivity with human and cat islet amyloid and with islet B cells. The significance of this pancreatic neuropeptide-like protein is unknown, but it is suggested that it may exert an important endocrine regulatory effect.**

Non-insulin-dependent diabetes mellitus (NIDDM) is characterized by an impaired insulin response to elevated glucose levels. In contrast to insulin-dependent diabetes mellitus (IDDM), this is not primarily due to a loss of B cells even though the total B-cell mass is moderately diminished in NIDDM (1, 2). Despite the apparent multifactorial nature of the pathogenesis of NIDDM and regardless of whether B-cell dysfunction is primary or secondary, the most substantial and uniform morphologic aspect of this disease with respect to the islets of Langerhans is the deposition of amyloid. Such deposits, exclusively limited to the islets of Langerhans, occur in >90% of patients with NIDDM and in >65% of adult diabetic cats (3–5). Islet amyloid also occurs less frequently and to a lesser extent in old persons and adult or aged cats without this disease (3, 4, 6). In contrast, islet-amyloid deposits are not found in IDDM.

The significance of the islet amyloid has been a matter of discussion since its description in 1900 (7). Although islet amyloid probably is a sign of impaired islet cell function associated with the development of NIDDM, chemical analysis of islet-amyloid proteins has not been reported previously. The principal obstacle to chemical characterization of islet amyloid has been related to the difficulty in solubilization (depolymerization) of the islet-amyloid fibrils for protein purification (8). This property, which contributes to the elusive nature of islet amyloid, is in direct contrast with that of the two major systemic forms of amyloid (i.e., secondary amyloid, designated AA, and primary amyloid, designated AL). Both of these types form fibril suspensions in distilled water and can be depolymerized effectively in 6 M guanidine hydrochloride. These properties of systemic forms of amyloid have allowed purification and direct chemical analysis of the fibril proteins.

In all instances, amyloid is a pathologic deposit of polymerized small proteins that form β-pleated-sheet fibrils (9). Many different types of amyloid exist, and these deposits can occur systemically or be localized to a single tissue (10). Each form of disease is characterized by discrete fibril proteins, and until now seven different proteins have been shown to form amyloid fibrils *in vivo* (11–17). These biochemical forms of amyloid that have been characterized to date are (*i*) protein AL (derived from immunoglobulin light chains) found in the amyloid of primary amyloidosis and in multiple myeloma patients, (*ii*) protein AA in secondary (reactive) amyloidosis, (*iii*) prealbumin (transthyretin) in forms of hereditary polyneuropathy and senile systemic amyloidosis, (*iv*) β_2-microglobulin in the amyloid associated with chronic hemodialysis, (*v*) gamma trace in the syndrome of hereditary cerebral hemorrhage with amyloidosis, (*vi*) procalcitonin in medullary carcinoma of the thyroid, and (*vii*) β protein in Alzheimer's disease. Amyloid localized to polypeptide hormone-producing tissues has been proposed to consist of polymerized hormones (18, 19). This is best supported by amino acid sequence analysis showing that amyloid fibrils in human medullary carcinoma of the thyroid are derived from procalcitonin (13). Amyloid deposits are common in insulin-producing tumors (19), and there is a close relationship between the amyloid fibrils and the B cells in the islets of Langerhans as well as in the tumors. Therefore, it has been believed that the amyloid in these locations is derived from insulin or its precursors (18). However, we have shown (20) that the major insulinoma amyloid fibril protein [named insulinoma or islet amyloid polypeptide IAPP§] is a previously unknown peptide with some N-terminal amino-acid-sequence identity to the neuropeptide identified as calcitonin gene-related peptide (CGRP) (20). We now have determined the amino acid sequence of human insulinoma IAPP and also have purified a major similar peptide from the islet amyloid of a diabetic cat. Partial amino acid sequence analysis of the peptide isolated from the diabetic cat shows that it corresponds to the human insulinoma IAPP.

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Abbreviations: NIDDM, non-insulin-dependent diabetes mellitus; IDDM, insulin-dependent diabetes mellitus; IAPP, islet amyloid peptide; CGRP, calcitonin gene-related peptide.

§IAPP refers to the amyloid fibril protein of the insulinoma, of the islets of Langerhans in human type 2 diabetes, and of the islets of Langerhans in the diabetic cat.

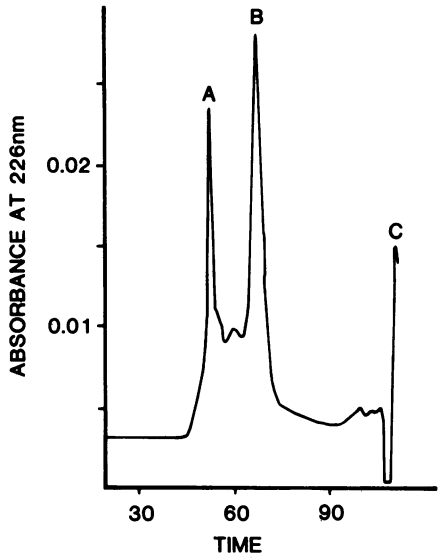

FIG. 1. Gel permeation HPLC of purified cat islet amyloid. There is one major retarded protein, peak B. Peak A is the breakthrough volume, and peak C is the salt front. Separation of human insulinoma and islet amyloid material gave virtually identical patterns. Time is in minutes.

MATERIALS AND METHODS

Purification of amyloid fibrils from a human insulin-producing pancreatic tumor and cat and human pancreases has been described (20, 21). Briefly, tumor or pancreatic tissue was homogenized repeatedly in normal saline containing 0.05 M sodium citrate, followed by repeated homogenizations in distilled water. Amyloid-enriched pellets obtained by this procedure were defatted and then treated with 6 M guanidine hydrochloride in 0.1 M Tris·HCl buffer (pH 8.0) at room temperature overnight. This treatment did not dissolve the amyloid but further purified it. After that the residual pellet was washed in distilled water, and solubilization was achieved by treatment with concentrated formic acid for 12–15 hr at room temperature. Complete or nearly complete solubilization of the amyloid was confirmed by the absence of Congophilic material in the small amounts of undissolved pellet material obtained after centrifugation at 20,000 rpm (Beckman A-20 rotor) for 1 hr. The supernatant was dried under nitrogen and redissolved in 1% $NaDodSO_4$/0.05 M sodium phosphate buffer, pH 7.5. Further purification of human insulinoma IAPP, human islet IAPP, and cat islet IAPP was obtained by gel permeation HPLC. In all cases, a 0.2-ml sample containing 25–100 μg of protein was injected into HPLC apparatus consisting of a LKB 2150 pump and a LKB 2152 LC controller equipped with a TSK G2000 SW (7.5 × 600 mm) column. Elution was performed with 0.1% $NaDodSO_4$/0.05 M sodium phosphate buffer, pH 7.5, at a flow rate of 0.2 ml/min. The elution profile was monitored at 226 nm with an LKB 2158 SD wavelength detector. $NaDodSO_4$/PAGE was performed as described (22).

Amino Acid Sequence Analysis. Material from the major retarded peaks obtained with gel-permeation HPLC was precipitated with 9 volumes of ethanol at −20°C. The precipitate was dried, dissolved in 50% trifluoroacetic acid, and applied to an Applied Biosystems (Foster City, CA) 470 A gas-phase sequenator. The phenylthiohydantoin-derivatized amino acid residues were determined on line by an Applied Biosystems 120 A phenylthiohydantoin analyzer. For determination of cysteine residues, 5 μg of human IAPP was reduced and alkylated with iodo[^{3}H]acetic acid (New England Nuclear) (23). The alkylated protein was applied to the sequenator as described, and the released amino acid derivatives were analyzed in a β counter.

Amino acid compositions were determined after acid hydrolysis for 24 hr.

Immunohistochemistry. An undecapeptide corresponding to positions 7–17 of the human insulinoma IAPP was synthesized (custom synthesis by Cambridge Research Biochemicals, Harston, England) and conjugated to keyhole limpet hemocyanine. The dissolved conjugated peptide mixed with Freund's complete adjuvant was injected intra- and subcutaneously into a guinea pig, followed by weekly injections of the immunogen in Freund's incomplete adjuvant. Serum was harvested 1 week after the fourth injection. Human and cat pancreases shown by Congo red staining to have large amounts of islet amyloid, and surgical specimens of normal human pancreas were fixed in 4% formaldehyde solution and Bouin's fluid, embedded in paraffin, and sectioned. Deparaffinized sections were studied by the peroxidase-antiperoxidase method (24) with primary antiserum dilutions of 1:50 to 1:800. Normal guinea pig serum and primary antiserum absorbed with the synthetic peptide (300 μg per ml of diluted antiserum) were used for controls. A sequential immunocytochemical- and silver-staining technique was applied to the same sections to identify the type of endocrine cells of the islets displaying immunoreactivity (25).

RESULTS

Repeated homogenizations of insulinoma and pancreatic tissue in normal saline followed by guanidine hydrochloride extraction resulted in amyloid that was nearly pure microscopically in both pellet materials. Although the amyloid was insoluble in guanidine hydrochloride, it was possible to dissolve it in concentrated formic acid. After solubilization in

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                                                    10                                  20
Human IAPP Lys-Cys-Asn-Thr-Ala-Thr-Cys-Ala-Thr-Gln-Arg-Leu-Ala-Asn-Phe-Leu-Val-His-Ser-Ser-

Cat   IAPP Lys- ? -Asn-Thr-Ala-Thr- ? -Ala-Thr-Gln-Arg-Leu-Ala-Asn-Phe-Leu-Ile-Arg-Ser-Ser-

                                                    30
          Asn-Asn-Phe-Gly-Ala-Ile-Leu-Ser-Ser-Thr-Asn-Val-Gly-Ser-Asn- ? -Tyr

          Asn-Asn-Leu-Gly-Ala-Ile-Leu
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FIG. 2. The amino acid sequence of human insulinoma IAPP compared to the N-terminal sequence of cat islet IAPP. Human IAPP (2.5 nmol) was applied, and 273 pmol of lysine was recovered after the first degradation, giving an initial yield of 11%. The average repetitive yield was 90%, and 2.2 pmol was recovered of residue 37 (tyrosine). The yield of the N-terminal lysine in cat IAPP was low (20 pmol) compared to the yield of asparagine in position 3 (52.5 pmol). The repetitive yield in the sequencing of cat IAPP was 89%, and the yield of leucine in position 27 was 2.4 pmol. Position 7 of human IAPP is cysteine and not serine as previously reported (20).

Table 1. Amino acid compositions of IAPP purified from amyloid of a human insulinoma, of human islets of Langerhans, and of feline islets of Langerhans

	Amino acid composition of IAPP		
	Insulinoma	Human islet	Feline islet
Asx	5.4 (6)	4.8	4.0
Thr	4.1 (4)	3.6	2.7
Ser	5.2 (5)	4.2	3.0
Glx	1.8 (1)	1.8	2.7
Pro	0.0 (0)	Trace	1.6
Gly	3.3 (3)	4.2	3.2
Ala	3.8 (4)	4.2	3.2
Cys	1.8 (2)	ND	ND
Val	2.1 (2)	2.4	2.2
Met	0.0 (0)	0.0	0.0
Ile	1.0 (1)	1.2	2.0
Leu	2.9 (3)	3.1	4.1
Tyr	0.8 (1)	0.9	1.3
Phe	1.7 (2)	1.8	1.6
His	0.8 (1)	1.2	0.6
Lys	1.1 (1)	0.9	2.0
Arg	1.2 (1)	1.3	1.8

Values are residues per molecule. Values from the amino acid sequence of human insulinoma IAPP are within parentheses. Cysteine was determined after performic acid oxidation. ND, not determined.

concentrated formic acid, the lyophilized material also was soluble in $NaDodSO_4$.

High-performance gel-permeation chromatography of islet amyloid from both the human insulinoma and human and cat pancreas resulted in elution profiles with major singlet retarded protein peaks (Fig. 1). The automated gas-phase sequencing resulted in the release of one amino acid residue in each step. The amino acid sequence of human insulinoma IAPP and partial amino acid sequence of cat islet IAPP resulting from this analysis are shown in Fig. 2. Automated amino acid sequence analysis of human insulinoma IAPP was run for 40 cycles, but no amino acid residue appeared after position 37. No amino acid residues were found in positions 2 and 7, but the N-terminal amino acid analysis of the reduced and alkylated human IAPP released labeled amino acid after the second and seventh cycle, indicating cysteine in these positions. The yield of phenylthiohydantoin-derivatized amino acid in the last positions of human IAPP was low, and the amino acid residue in position 36 could not be determined. Based on the amino acid composition (Table 1), human IAPP contains two glutamic acid/glutamine residues, while only one was identified by sequence analysis. Therefore, it is possible that position 36 contains a glutamic acid or glutamine residue. Based on amino acid sequence analysis, the calculated molecular mass of human insulinoma IAPP is ≈3850 Da. This is in agreement with the $NaDodSO_4$/PAGE of the purified and reduced human IAPP, which showed one band with an apparent molecular mass of <10,000 Da. The determined amino acid sequence is in good agreement with the amino acid composition of purified IAPP (Table 1) and also of the crude amyloid fibril preparation (20), which indicates that IAPP is the major amyloid protein.

Human insulinoma IAPP shows a 46% identity with one of the two human CGRPs (β-CGRP, ref. 26; Fig. 3). Amino acid sequences with complete identity to human β-CGRP exist at positions 2–7, 11–13, and 30–34. Proportionately, greater identity with CGRP is apparent with the N-terminal portion of the human insulinoma IAPP molecule.

The result of the N-terminal amino acid sequence analysis of the cat islet amyloid protein is also shown in Fig. 2. Automated sequence analysis yielded conclusive amino acid residues in positions 1–27 of the cat islet IAPP except for positions 2 and 7. This sequence is identical to the human insulinoma IAPP with the exception of three amino acid substitutions at positions 17, 18, and 23. This incomplete cat islet IAPP sequence represents a 44% identity with the same region of human β-CGRP.

The peroxidase-antiperoxidase technique was used with the antiserum directed to the synthetic peptide segment (positions 7–17) of human insulinoma IAPP. An intense immunoreactivity was demonstrated in B cells of normal human pancreatic islets and in both human and cat islet amyloid deposits (Fig. 4). Pancreatic exocrine parenchyma remained completely unstained. Immunoreactivity of the islet cells and islet amyloid was abolished when the primary antiserum was absorbed with the synthetic peptide and when

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                                        10                  20
Human αCGRP (28)            A C D T A T C V T H R L A G L L S R S G

Human βCGRP (26)            A C N T A T C V T H R L A G L L S R S G

Human insulinoma IAPP       K C N T A T C A T Q R L A N F L V H S S

Cat islet IAPP              K X N T A T X A T Q R L A N F L I R S S


                                        30
Human αCGRP                 G V V K N N F V P T N V G S K A F

Human βCGRP                 G M V K S N F V P T N V G S K A F

Human insulinoma IAPP       N N F G A I L S S T N V G S N X Y

Cat islet IAPP              N N L G A I L
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FIG. 3. Amino acid sequence of human insulinoma IAPP compared to the two human CGRPs and to the sequenced part of cat islet IAPP. Areas in boldface type indicate identity between IAPP and CGRP. The sequenced parts of cat and human IAPP are identical except for positions 17, 18, and 23. X, unidentified amino acid.

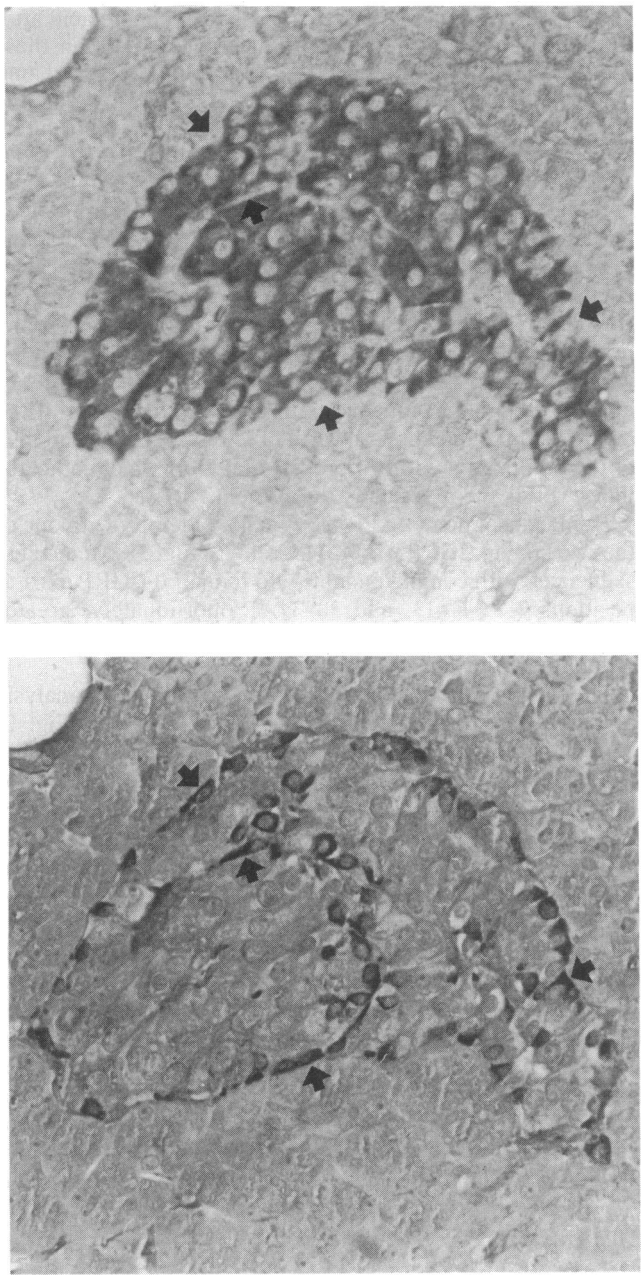

FIG. 4. Sequential immunohistochemical staining with anti-synthetic-IAPP-(7–17) antiserum (*Upper*) and silver staining (*Lower*). The silver-staining technique stains the A cells, which remain unstained with the immunohistochemical method. The islet B cells react strongly with the antiserum. Four of the silver-positive and IAPP-negative cells are indicated by arrows. (×500.)

the primary antiserum was replaced by normal guinea pig serum.

DISCUSSION

Amyloid is recognized by its electron microscopic appearance and typical staining properties with Congo red and other dyes. The unique ultrastructural component of all forms of amyloid, regardless of organ, species, or pathogenesis, is the amyloid fibril. This is formed by the polymerization of small repetitive peptide units arranged in β-pleated-sheet configuration. The β-pleated-sheet fibrils appear to be responsible for the unique tinctorial and optical properties of all forms of amyloid. To date, seven different proteins have been shown to give rise to amyloid fibrils systematically or locally. To these we now add IAPP.

The chemical nature of amyloid localized to the pancreatic islets of individuals with NIDDM or insulinomas has not been elucidated previously because of difficulties associated with purification of the subunit protein. However, it was obvious that information regarding the molecular origin of this form of amyloid may provide important insights regarding the pathogenesis of diabetes mellitus associated with aging. Islet amyloid highly resembling the human form is common in old cats with a form of diabetes that resembles NIDDM (27). Preliminary chemical and immunological studies of cat islet amyloid have shown properties comparable with human islet amyloid (21).

Of interest and possibly of great significance is the finding that the previously unidentified peptide IAPP from the human and cat has >40% identity with human CGRP (26, 28). CGRP is a recently identified neuropeptide that is present in certain restricted components of the central and peripheral nervous system (29, 30). The name of this neuropeptide indicates that it is a product of the same gene as calcitonin, and the ultimate difference in gene expression is dependent on differential posttranscriptional RNA splicing events (28, 29). The overall distribution of CGRP-immunoreactive neurons and pathways suggested functions related to nociception, ingestive behavior, and modulation of the autonomic and endocrine systems. It was demonstrated that CGRP exerted significant effects on blood pressure and blood catecholamine levels when administered to rats (31). Administration of CGRP intracerebroventricularly induced a prompt rise in plasma noradrenaline levels and dose-related increases of mean arterial pressure and heart rate (31).

In our study with the peroxidase-antiperoxidase technique, intense immunoreactivity was demonstrated in normal human pancreatic islet B cells and in both human and cat islet amyloid deposits with primary antiserum to synthetic human insulinoma IAPP-(7–17). These results suggest that IAPP is released locally from islet cells. Interestingly, antiserum to synthetic rat CGRP-(23–37) was previously shown to react with nerve fibers in the exocrine and endocrine rat pancreas and also with a few intrainsular cells (30). Positions 23–37 of human CGRP contains a sequence of five amino acids (positions 30–34) identical to human and cat IAPP (Fig. 3) but contains no further homology. IAPP exists also in the rat (unpublished observation) and a crossreactivity of antibodies to CGRP-(23–37) with IAPP cannot be excluded.

We have not been able to obtain yet the amino acid sequence of IAPP from human islet amyloid, but its amino acid composition is similar to that of human insulinoma IAPP (ref. 20; Table 1). Therefore, there is little doubt that the human and cat islet amyloid is of identical chemical nature.

The potential biological and pathobiological significance of this previously unidentified neuropeptide-like substance with respect to pancreatic endocrine function and pathogenesis of NIDDM is interesting in light of a recent demonstration of amyloid deposits in pancreatic nerves and ganglia of diabetic cats (32). These amyloid deposits were shown to have the same histochemical and immunohistochemical properties as islet amyloid and were in close association with ganglionic insulin-expressing cells. Although the role of IAPP in the islets is unknown, its conservation and partial identity with CGRP strongly indicate an important regulatory function.

This work was supported by the Swedish Medical Research Council (project nos. 5941 and 5817), the research fund of King Gustaf V, the Nordic Insulinfund, and Grant R01 DK36734 of the National Institute of Diabetes and Digestive and Kidney Diseases.

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