Purification and characterization of a peptide from amyloid-rich pancreases of type 2 diabetic patients

(amino acid sequence/calcitonin gene-related peptide/insulin A chain/Alzheimer disease/pancreatic islet)

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ABSTRACT Deposition of amyloid in pancreatic islets is a common feature in human type 2 diabetic subjects but because of its insolubility and low tissue concentrations, the structure of its monomer has not been determined. We describe a peptide, of calculated molecular mass 3905 Da, that was a major protein component of amyloid-rich pancreatic extracts of three type 2 diabetic patients. After collagenase treatment, an extract containing 20–50% amyloid was solubilized by sonication into 70% formic acid and the peptide was purified by gel filtration followed by reverse-phase high-performance liquid chromatography. We term this peptide diabetes-associated peptide, as it was not detected in extracts of pancreas from any of six normal subjects. Diabetes-associated peptide contains 37 amino acids and is 46% identical to the sequences of rat and human calcitonin gene-related peptide, indicating that these peptides are related in evolution. Sequence identities with conserved residues of the insulin A chain were also seen in a 16-residue segment. On extraction, the islet amyloid is particulate and insoluble like the core particles of Alzheimer disease. Their monomers have similar molecular masses, each having a hydrophobic region that can probably form β -pleated sheets. The accumulation of amyloid, including diabetes-associated peptide, in islets may impair islet function in type 2 diabetes mellitus.

The occurrence of amyloid in the islets of Langerhans is a major pathological feature of type 2 diabetes. Hyalinization of islets was the lesion that first indicated islet pathology produced diabetes mellitus (1, 2). Histological evidence on the amyloid nature of the hyaline was obtained in 1943 (3). This was later confirmed by alkaline Congo red staining (4) and by the finding of fibrillar structure on electron microscopy (5).

Although islet amyloid is found in the majority of patients with type 2 diabetes (6, 7), it is also found in approximately 14–18% of elderly “normal” subjects (8, 9). This has raised the possibility that it is a nonspecific feature of aging. However, a recent study suggests that amyloid is specific for type 2 diabetes and is rarely found in control subjects (6). Amyloid in normal subjects could represent undiagnosed diabetes in the normal population since diabetes cannot be excluded retrospectively, and autopsy studies of hospital patients could include a bias to a diabetes-enriched population. The presence of islet amyloid in nondiabetics in autopsy-based studies may be spurious and based on sampling from biased populations (10, 11). Islet amyloid also occurs spontaneously in the diabetic monkey *Macaca nigra* (12) and in the diabetic domestic cat (13). In humans, amyloid is found more frequently in older diabetic patients (14) and in those with more severe diabetes as shown by requirement for insulin therapy (9, 15).

Although a decrease in insulin secretion appears to be inherited in type 2 diabetes (16, 17), there is only a modest reduction of β cells with estimates of decrease ranging between 15% (6) and 40% (7); this is probably insufficient to account for the impaired insulin secretion found (18). The deposition of amyloid might impair the function of islets and could be a major factor in the pathogenesis of type 2 diabetes.

Previous attempts at characterization of islet amyloid have been hindered by its insolubility and low concentration in the pancreas. Studies based on chemical extraction (19) and on immunohistochemistry (20) have suggested that it may contain either whole insulin or the B chain of insulin.

We have purified and characterized a peptide that we term diabetes-associated peptide (DAP), as it was present in pancreases from each of three diabetic patients with islet amyloid but not in any of the pancreases from six normal subjects. This peptide has significant sequence identity to calcitonin gene-related peptide (CGRP) and weaker identity to the A chain of insulin. Quantitative studies of protein yields indicated DAP is the major protein constituent of islet amyloid.

MATERIALS AND METHODS

Human Subjects. Pancreases from three type 2 diabetic and six nondiabetic patients were obtained at autopsy and frozen at -20°C until extraction. Islet amyloid was detected in tissue fixed in 150 mM NaCl/10% formalin by light microscopy after hematoxylin and eosin staining and was confirmed after staining with alkaline Congo red by the demonstration of green birefringence by microscopy under polarized light. Only the pancreases from diabetic patients contained amyloid.

Amyloid Extraction. Extraction of amyloid by a standard method for soluble amyloid, using distilled water (21), was unsuccessful so the method below was developed. Whole pancreases were homogenized in ice-cold 150 mM NaCl, 1:4 (wt/vol) and a pellet was obtained by centrifugation (Sorvall RC-5B centrifuge, GS-3 head, $10,000 \times g$, 30 min, 4°C). The fat and supernatant layers were discarded, and the process, beginning with homogenization, was repeated twice. Crude lyophilized collagenase (EC 3.4.24.3; Boehringer Mannheim, Product 103586) was dissolved 1:100 (wt/vol) in buffer comprising 50 mM Tris-HCl/150 mM NaCl/3 mM CaCl_2 /2% (vol/vol) Nonidet P-40, pH 7.4, and the insoluble material was removed by centrifugation (Beckman L8-70M centrifuge, SW 40 Ti rotor, $150,000 \times g$, 2 hr). Aliquots of the crude pancreatic homogenate were heated to 70°C for 10 min and incubated with the collagenase supernatant (1:10, wt/vol) for 20 hr at 37°C with continuous vigorous shaking. Aliquots were then pelleted in siliconized microcentrifuge tubes for 10

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Abbreviations: DAP, diabetes-associated peptide; CGRP, calcitonin gene-related peptide.

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min at $11,200 \times g$, the supernatant was discarded, and the wet pellet was washed twice with 150 mM NaCl and once with distilled water. Staining of aliquots from amyloid-containing diabetic pancreases with alkaline Congo red revealed that 20–50% of the residual material was in the form of particles of amyloid (average diameter, 10–30 μm). This was not seen in any of the pancreatic extracts from amyloid-negative nondiabetic subjects. The solubility of the amyloid was assessed by shaking continuously in a variety of solvents for 2 days, with repelleting at $11,200 \times g$ for 10 min, followed by microscopy of an aliquot after alkaline Congo red staining and protein analysis of the supernatants.

The amyloid in particulate form was solubilized by sonication (M.S.E. Sonic disintegrator, model 150W; wavelength, 8 μm , 20 kHz) into 70% (vol/vol) formic acid at 1:4 (wt/vol). Ultrasound was delivered in four 30-s bursts with cooling in a dry ice/ethanol bath for 15 s after each burst. The formic acid was immediately removed by rotary evaporation to near dryness in a Savant Speed Vac (Strattech Scientific, London), and the amyloid was resolubilized in 6 M guanidine hydrochloride/0.2 M $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$, pH 7.5, with constant shaking for 1 hr.

Peptide Purification and Amino Acid Analysis and Sequencing. Initial protein separation of the solubilized amyloid was done by high-performance liquid chromatography (HPLC) on Zorbax GF-450 and GF-250 gel filtration columns (250×9.4 mm; Du Pont) in series in a Waters system with mobile phase 6 M guanidine hydrochloride/0.2 M $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$, pH 7.5. The runs were monitored at 280 nm. Desalting of guanidine-containing samples from the gel filtration system by dialysis (Spectropor 6 dialysis tubing, 1 kDa cut-off; Pierce and Warriner, Chester, U.K.) into various solvents gave high and variable losses of protein as judged by quantitative Waters "Pico-Tag" analysis, so samples from the gel filtration system were injected directly onto a Partisil-10 ODS-3 reverse-phase HPLC column (300×4 mm; Whatman). The mobile phase was 1% trifluoroacetic acid, with linear gradient elution using acetonitrile (5–80% over 45 min) monitored at 280 nm. Quantitative protein determinations of all peaks and amino acid compositions were made using a Waters Pico-Tag amino acid analysis system (22). Protein sequencing of the major peaks was performed on an Applied Biosystems 470A protein sequencer (23) using the 02CPTH cycle in the Version 2.0 software (Applied Biosystems, Foster City, CA). Phenylthiohydantoin-derivatized amino acids were identified by HPLC.

Peptide Cleavage and Derivatization of the Disulfide Bond. Tryptic cleavage (L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin EC 3.4.21.4. Worthington) of the purified peptide was done for 3 hr at 37°C in 100 mM NH_4HCO_3 buffer, enzyme/substrate molar ratio, 1:100, with termination of reaction by addition of diisopropylfluorophosphate to 25 mM. Tryptic peptides were separated by reverse-phase HPLC on a Partisil-10 ODS-3 column, with a mobile phase of 0.1% trifluoroacetic acid, with linear gradient elution using acetonitrile (5–72% over 45 min) and monitoring at 206 nm. C-terminal identification with carboxypeptidase Y (EC 3.4.16.1) was performed in 0.2 M pyridine/acetic acid buffer (pH 5.5) with termination of reaction at 100°C for 2 min. Reduction and alkylation of cysteine residues was performed in 6 M guanidine hydrochloride/0.2 M Tris, pH 8.0/3 mM NaEDTA by the addition (to 20 mM) of dithiothreitol to ≈ 1 nmol of purified peptide, shaking for 3 hr, and subsequent addition of iodo[2- ^{14}C]acetic acid (54 Ci/mol; 1 Ci = 37 GBq) for 5 min, at 0°C in the dark, followed by freshly neutralized iodoacetic acid to 40 mM. Reduced and alkylated material was then repurified by HPLC reverse-phase chromatography (1% trifluoroacetic acid, 5–80% acetonitrile, 45 min, 280 nm) (see Fig. 1D).

RESULTS

Solubilization of Amyloid. After repeated homogenization and saline extraction, particulate amyloid remained a minor component in a matrix of collagen fibers. After extensive collagenase digestion, the amyloid became a major component of the residue, but further amyloid purification by differential or density-gradient ultracentrifugation was unsuccessful. The amyloid remained in particles similar in size and shape to the amyloid cores described in Alzheimer disease (24). The amyloid particles were insoluble in distilled water, 0.01 M HCl, 50% ethylene glycol, 10% dimethyl sulfoxide, 3 M potassium thiocyanate, 2% Nonidet P-40, 8 M urea/2% NaDodSO₄/0.2 M Tris-HCl, pH 8.0, and 1% deoxycholate, 10 mM diethylamine hydrochloride, pH 11.2. Incomplete solution was obtained with 6 M guanidine hydrochloride/0.2 M $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$, pH 7.5. Solubility was achieved by first disrupting the particles with ultrasound into 70% (vol/vol) formic acid followed by evaporation of formic acid and redissolution of the remaining material in 6 M guanidine hydrochloride/0.2 M $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$, pH 7.5.

Protein Separation. HPLC gel filtration of solubilized material (Fig. 1A) gave similar absorbance profiles for amyloid-positive and amyloid-negative tissue, but the front shoulder of the main peak (above bar in Fig. 1A) varied between diabetic and nondiabetic samples. Direct injection of sequential fractions onto the reverse-phase HPLC system was done with extracts from three diabetic and six normal pancreases and revealed a peak (peak 3, Fig. 1B) that was present in the amyloid-containing extracts and in none of the amyloid-negative tissue extracts from nondiabetic subjects. This major peak eluted at an acetonitrile concentration of 67.5%. The peak was isolated, reduced and alkylated, and then further purified on the same reverse-phase HPLC system. The reduced and alkylated peptide eluted at 64% acetonitrile concentration, which is consistent with the introduction of more polar carboxymethyl groups into the molecule (peak 3RA, Fig. 1D). Two minor peptides separated at this stage, both of which had different amino acid compositions from the major peak and were found to be amino terminally blocked on sequence analysis (peaks 4 and 5, Fig. 1D). Amino acid analysis of the major peak indicated a virtually pure peptide, ≈ 37 amino acid residues long, with the distinctive amino acid ratio of 6 mol of Asx to 1 mol of Glx. This pattern was identical in the major peptide purified from tissue from the three diabetic subjects but different from all other peptides of similar size (peaks 1 and 2, Fig. 1B and C), which were present in both the amyloid-positive and amyloid-negative material. All the other peptides were shown to be amino terminally blocked on amino acid sequence analysis. Quantitative amino acid analysis of all peaks on the reverse-phase column (Fig. 1B) indicated that the diabetes-specific peak (peak 3) was the major protein peak and accounted for 24% of the total proteinaceous material on the column.

Amino Acid Composition and Sequence. By amino acid analysis, the following residues per mol of reduced and alkylated DAP were found (values from amino acid sequence analysis are given in brackets): Asx, 6.1 [6]; Glx, 1.0 [1]; Ser, 4.1 [5]; Gly, 2.1 [2]; His, 1.0 [1]; Arg, 1.0 [1]; Thr, 5.1 [5]; Ala, 4.1 [4]; Pro, 0.2 [0]; Tyr, 1.0 [1]; Val, 2.0 [2]; Met, 0.0 [0]; Ile, 1.0 [1]; Leu, 3.0 [3]; Phe, 2.1 [2]; Lys, 1.0 [1]. Two Cys residues were determined as *S*-carboxymethylcysteine on sequence analysis only. The estimate of residues per mol is based on the assumption that there is 1 Arg residue per mol of DAP. As no tryptophan was detected on sequence analysis, and as the A_{280} of DAP is consistent with the amount of tyrosine measured by quantitative amino acid analysis, it is unlikely that DAP contains tryptophan.

The sequence of DAP is given in Fig. 2a. Complete sequences were obtained from peptides from two patients.

FIG. 1. Purification and separation of the peptide. (A) HPLC gel filtration of extract from an amyloid-containing diabetic pancreas. DAP was present in the region indicated by the bar. (B) Reverse-phase HPLC in an acetonitrile gradient (dotted line) of diabetic amyloid extract. Unreduced DAP is present in peak 3. The elevation of base-line absorbance seen is produced by nonproteinaceous material, which may be lipid. (C) Reverse-phase HPLC of control, extract as in B. Peaks 1 and 2 in this profile correspond in elution time and amino acid composition to 1 and 2 in B. (D) Repurification of material in peak 3 (B) after reduction and alkylation, with conditions the same as in B. Peaks 4 and 5 have amino acid compositions distinct from that of 3RA, which is the reduced and alkylated DAP. Other peaks are either nonproteinaceous solutes or are part of the system. (E) Separation of peptides after tryptic digestion of reduced and alkylated DAP by reverse-phase HPLC. Peak 6 is the smaller more hydrophilic peptide DAP₁₋₁₁, and peak 7 is the larger C-terminal peptide DAP₁₂₋₃₇. All radiolabel was present in peak 6. Identity of peaks was confirmed by quantitative amino acid analysis and by sequencing. The ratio of peak heights is consistent with the relative lengths of the peptides.

The third pancreas reported was used in the development of the methods of purification of DAP. Consequently, sufficient peptide was obtained from this pancreas to allow confirmation of only the first seven amino acids of the sequence. Amino acid sequence analysis of repurified, reduced, and alkylated peptides (peak 3RA, Fig. 1D) from the amyloid-

positive tissue gave the same results in both pancreases from which full sequences were obtained. Peptides from both patients gave initial yields on the sequencer of 70–72% based on lysine at position one. The repetitive yield, based on alanine residues at positions 5 and 25, was 92.5%. Sequence analysis was also performed on unreduced peptide before and after tryptic digestion. This gave confirmatory sequence data except for residues 2 and 7, which were not seen in the unreduced peptide. No free sulfhydryl groups could be radiolabeled, thereby suggesting that Cys-2 is disulfide linked to Cys-7. The repetitive yield was poor in this region on sequencing of the unreduced peptide, being 87% based on alanine residues at positions 5 and 8. Confirmatory N-terminal sequence data (residues 1–7) were obtained from the extract of an amyloid-containing pancreas from the third diabetic subject.

After tryptic digestion and reverse-phase chromatography of reduced and alkylated DAP, two peptides were recovered (Fig. 1E). Amino acid and sequence analysis showed these to be peptides corresponding to DAP₁₋₁₁ and DAP₁₂₋₃₇. Their relative positions on the gradient were consistent with their primary structures and their relative peak heights with the known length of the peptides. It is therefore likely that DAP is 37 amino acids long, although this could not be confirmed by C-terminal identification with carboxypeptidase Y. The calculated molecular mass of DAP is 3905 Da.

Database search (Fast P) of the National Biomedical Research Foundation data base, using recommended parameters, indicated significant sequence identity between DAP and CGRP (Fig. 2b), and to a lesser degree between DAP and the A chain of human and guinea pig insulin (Fig. 2c). These sequences were subjected to statistical analysis by the ALIGN program (27, 28), using the mutation data matrix (250 PAMs) with a bias of +6 and a break penalty of +6. One hundred random runs were performed to establish the mean random scores. The alignment scores between DAP and the other peptides were 8.40 (human CGRP-1), 10.03 (rat CGRP-1), 4.39 (guinea pig insulin, A-chain), and 3.75 (human insulin) SD units. Secondary structure prediction (29) and hydropathy analysis (30) were also performed (results not shown). These studies suggest a tendency to β -sheet formation in the middle of the molecule where DAP is hydrophobic.

DISCUSSION

We have described the purification and structural characteristics of a peptide, DAP, which has been extracted from the amyloid-rich pancreases of three type 2 diabetic subjects and completely characterized in two, with identical partial characterization in the third. In spite of careful searching by microscopy, by quantitative amino analysis of all extracted peaks separated on reverse-phase HPLC, and by amino acid sequence analysis of peaks eluted near the position of DAP, neither particulate amyloid nor DAP was detectable in any of six amyloid-negative pancreases from nondiabetic subjects. In another study, we have shown that pancreatic islet amyloid deposits were found in 22 of 24 type 2 diabetic subjects and were not present in 10 age-matched control subjects (25). The islet amyloid of all 22 diabetics showed CGRP immunoreactivity that was blocked by preabsorption of three different CGRP antisera with CGRP carboxyl-terminal peptide 28–37 or with extracted DAP.

DAP shows highly significant sequence similarities to the two human CGRP peptides (31, 32) and to rat CGRP (33) (Fig. 2b). Weaker similarity to the insulin A chain is seen, but the match gains some credence since it occurs with residues that are conserved between species in the insulin molecule. An evolutionary relationship between insulin, DAP, and CGRP would be of interest but is not clearly established by the current findings. All these molecules share the common

FIG. 2. (a) Structure of DAP. (b) Primary structure of DAP compared with that of human CGRP-2, human CGRP-1, and rat CGRP-1. Amino acid identity between peptides is indicated by boxes. The segment of rat CGRP-1 underlined (residues 28–37) is the sequence of the synthetic peptide antigen, an antibody against which demonstrated immunoreactivity in islet amyloid (25). Dotted boxes indicate areas of displaced homology. (c) Comparison of primary structures of DAP and the A chains of guinea pig and human insulins. Numbering of residues is the same as for insulin. A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. Dashed boxes represent areas of conservative (26) amino acid substitution.

feature of an intrachain disulfide bridge toward the N terminus.

An amyloid protein has been extracted from an insulinoma, and sequencing of the amino-terminal 19 amino acids shows the same sequence as DAP, except for a serine instead of cysteine at position 7 (34). Further studies are needed to determine whether the insulinoma peptide is the same as DAP.

Immunohistochemical localization studies demonstrate CGRP-containing nerve fibers in the central nervous system (33), in nerve fibers throughout the cardiovascular system (35), and in some cells in the thyroid (36) and pancreatic islets (37). However, the similarity of DAP to CGRP raises the possibility that immunohistochemical identification of CGRP in the pancreas may in fact be due to detection of cross-reacting determinants on DAP (25).

The accumulation of amyloid in the islet may have a fundamental role in the pathogenesis of type 2 diabetes. The presentation of an inherited disease in middle or old age might relate to the slow accumulation of amyloid interfering with islet function. On the other hand, amyloid accumulation may be a secondary phenomenon resulting from disordered islet function and may have no direct pathogenic role. Formation of amyloid could occur if a mutant gene of DAP were present that facilitated β -pleat formation (25).

The islet amyloid resembles that isolated from amyloid plaques of Alzheimer disease in several respects. Both amyloids remain particulate after extraction and both are insoluble in most protein solvents. The A4 protein of Alzheimer disease [43 amino acids (38)] is similar in length to DAP, whereas the monomers comprising most other amyloids have significantly greater molecular weights. Both the A4 protein (39) and DAP have hydrophobic regions with coincident regions of predicted β -sheet formation in the

secondary structure, which may account for their insolubility. There are no significant primary structural homologies between the A4 protein and DAP.

The structural similarity between DAP and CGRP raises the possibility that DAP has endocrine, paracrine, or neurotransmitter activity. Human CGRP has been shown to be a potent vasodilator in several experimental models (40) and may have a functional role in cerebrovascular regulation (41). DAP may be involved with regulation of islet blood flow, although it might have different activities, including the suppression of insulin secretion as suggested for CGRP (37).

Note Added in Proof. Since the submission of this paper, peptides have been isolated from the amyloids of a human insulinoma and the islets of Langerhans of a diabetic cat (42). A peptide from the islets of Langerhans of a human type 2 diabetic has also been partially characterized (43). The reported sequences of the peptides from the insulinoma and human islet amyloid are identical to the sequence reported above in all residues assigned. The feline sequence varies in 3 of 27 residues compared with the human sequence; this probably represents evolutionary change.

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