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Rat amylin: Cloning and tissue-specific expression in pancreatic islets

(amyloid/diabetes)

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ABSTRACT **Amyloid deposits in the islets of Langerhans of the pancreas are a common finding in non-insulin-dependent diabetes mellitus. The main protein constituent of these deposits is a 37-amino acid peptide known as amylin that resembles calcitonin gene-related peptide, a neuropeptide. We have isolated cDNA clones corresponding to the rat amylin precursor from an islet cDNA library and we show that this peptide is encoded in a 0.9-kilobase mRNA that is translated to yield a 93-amino acid precursor. The amylin peptide is bordered by dibasic residues, suggesting that it is proteolyzed like calcitonin gene-related peptide. The peptide sequences flanking the amylin sequence do not resemble the calcitonin gene-related peptide flanking sequences. RNA hybridization studies show that amylin mRNA is abundant in the islets of Langerhans but is not present in the brain or seven other tissues examined. Dietary changes, such as fasting or fasting and refeeding, have little effect on amylin mRNA expression. This tissue specificity suggests that amylin is involved in specific signaling pathways related to islet function.**

The pancreas of patients with non-insulin-dependent diabetes mellitus (NIDDM) contains deposits of amyloid, an extracellular protein matrix with unique staining characteristics (1–4). Small deposits of amyloid are also found in the pancreas of an elderly patient without diabetes; however, in patients with NIDDM they occur earlier and are more widespread. Cooper and coworkers (5, 6) and Westermark *et al.* (7, 8) have purified and sequenced the peptide that is the major component of islet amyloid in NIDDM. This peptide, known as amylin, diabetes-associated peptide (DAP), or islet amyloid polypeptide (IAPP), is a 37-amino acid peptide that is 50% identical to calcitonin gene-related peptide (CGRP). Johnson *et al.* (9) have found amylin immunoreactive material in normal islets within insulin-containing secretory granules of the β cells. In the pancreas from a diabetic patient, immunoreactive material is abundant and primarily located extracellularly in amyloid deposits (10). Sanke *et al.* (11) have isolated a cDNA for the precursor of islet amyloid from human insulinoma. They found this mRNA encoded an 89-amino acid peptide that included the human amylin sequence. Low levels of this RNA were detected in one insulinoma but RNA from the islets was not examined and only a limited number of extrapancreatic tissues were studied. Therefore, the tissue distribution of the amylin mRNA remains unknown.

Effects of amylin and CGRP have been observed that are potentially relevant to diabetes and islet function. In pancreatic exocrine cells CGRP suppresses amylase release (12). Intravenous administration of CGRP suppresses basal insulin levels and glucose-stimulated insulin secretion (13). Leighton and Cooper (14) found that CGRP inhibits insulin-stimulated glycogen synthesis in skeletal muscle, although it did not affect glycolysis. A comparable inhibition was observed using amylin purified from the pancreas of diabetic patients or synthetic amylin (14, 15). No effect was seen in adipose tissue. These studies suggest that amylin may affect insulin secretion and the sensitivity of peripheral tissues to insulin.

To define the primary structure of amylin in the islets, we have isolated and characterized cDNAs encoding rat amylin from an islet cDNA library.¶ Sequence analysis shows that a 0.9-kilobase mRNA encodes a 93-amino acid precursor that is proteolyzed to yield the final amylin product. RNA expression studies show that this molecule is expressed at high levels in the islets of Langerhans of normal animals but is not detected in the brain or any of seven other tissues in the rat. Amylin thus appears to be a normal constituent of the β cell that is processed in a fashion similar to other peptide hormones and is secreted with insulin from the islet.

METHODS

Screening of cDNA Library. A rat pancreatic islet cDNA library (16) was screened with the 54-residue oligonucleotide (5′-RTGIACTARRAARTTIGCYARYCGYTGIGTIGCRCA-IGTIGCIGTRTTRCAYTT-3′, where R is adenosine or guanosine and Y is thymidine or cytidine) that is complementary to the nucleotide sequence for amino acids 1–18 of the human amylin sequence. The oligonucleotide was 5′-end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase and hybridized to the filters in 6× SSC (1× SSC is 0.15 M NaCl/15 mM sodium citrate, pH 7.0) containing denatured salmon testes DNA at 200 μg/ml, 5× Denhardt's solution (1× Denhardt's solution is 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin), and 0.1% $NaDodSO_4$ at 42°C. The filters were washed in 2× SSC/0.1% $NaDodSO_4$ at 42°C. Positive clones were plaque-purified and the cDNA inserts were either amplified with oligonucleotides corresponding to the vector sequences flanking the cloning site (17) and subcloned into M13 vectors or subcloned directly from the phage DNA. DNA was sequenced by the dideoxynucleotide chain-termination procedure with the universal primer or synthetic oligonucleotides corresponding to the amylin sequence. The DNA was sequenced on both strands in multiple clones.

RNA Hybridization Studies. Rat islets were prepared from male Wistar rats by collagenase digestion and Ficoll density

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Abbreviations: NIDDM, non-insulin-dependent diabetes mellitus; CGRP, calcitonin gene-related peptide; DAP, diabetes-associated peptide.

¶The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04544).

gradient centrifugation (18). For the analysis of brain RNA, brains were dissected into four regions; (*i*) the cerebellum, (*ii*) the cerebral cortex, (*iii*) the midbrain, hippocampus, and hypothalamus, and (*iv*) the medulla oblongata and pons (19). RNA was prepared by the guanidinium isothiocyanate/cesium chloride procedure (20). Poly(A)$^+$ RNA was prepared by oligo(dT)-cellulose chromatography. Aliquots of poly(A)$^+$ RNA were denatured with glyoxal, separated by electrophoresis in a 1.5% agarose gel, and transferred to Biotrans nylon membranes (ICN). Filters were hybridized in a solution containing 50% (vol/vol) formamide, 5× SSC, salmon testes DNA at 100 μg/ml, 5× Denhardt's solution, and 0.1% $NaDodSO_4$ using single-stranded cDNA probes prepared from a M13 template at 3×10^6 cpm/ml, as described (21), that had a specific activity of 10^9 cpm/μg. The amylin probe was primed with synthetic oligonucleotides at positions +90, +360, and +720 (see Fig. 1) to span the entire length of the amylin cDNA. Filters were washed in 0.1× SSC/1.0% $NaDodSO_4$ at 60°C. Control hybridizations were done with a human β-actin probe (22) or a rat insulin probe (23).

RESULTS

Positive clones were identified at a frequency of ≈1 in 1000 clones when the pancreatic islet cDNA library was screened with the 54-residue amylin oligonucleotide. Five of the largest clones were subcloned into M13 vectors and sequence analysis showed that all of these clones were identical except for the lengths of the 5′ and 3′ ends. The 897-nucleotide sequence of the amylin cDNA is shown in Fig. 1. The correct identity of these clones was confirmed by the presence of a sequence encoding a peptide quite similar to the 37-amino acid human amylin sequence contained within an open reading frame of 93 amino acids. The amylin sequence is flanked by dibasic sequences that are presumed sites of proteolysis and carboxyl-terminal amidation (24). Untranslated regions of 87 and 588 nucleotides were present at the 5′ and 3′ ends, respectively. The 5′ ends of the cDNA clones were at positions −87, −70, −68, −31, and −18. The 3′ ends of three clones were defined and were at position +721 in two and position +801 in the other.

The precursor molecule consists of three regions—an amino-terminal domain, the central peptide domain, and a carboxyl-terminal domain. This is very similar to the structure of α and β CGRPs (25). The 37-amino acid amino-terminal end of the amylin precursor is shorter than the corresponding regions of the CGRPs, which are 80–86 amino acids long. In this region amylin does not share any homology with the CGRP sequences, although α and β CGRP are 53% identical with each other in this region. The central amylin sequence is flanked by proteolytic cleavage sites just as in the CGRP precursors. The 16-residue carboxyl-terminal end is somewhat longer than the 4-amino acid carboxyl-terminal ends present in the CGRP sequences.

An alignment of the rat and human amylin precursor sequences is shown in Fig. 2. The amino-terminal domain is hydrophobic and consistent with a signal sequence in a secreted protein. The rat and human sequences are 54% identical in this region. In the central peptide domain, the rat and human sequences are 84% identical. The rat amylin sequence is 51% identical to the α and β CGRP sequences. The carboxyl-terminal domain is 62% identical between the rat and human amylin precursors. In the human amylin cDNA Sanke *et al.* (12) noted that the 3′ untranslated region contained a repetitive *Alu* element; however, no repetitive sequence is found in the rat cDNA.

Previous studies have identified (9, 10) immunoreactive amylin in pancreatic tissue; however, expression in other tissues was not examined. Based on the similarity with CGRP and the predominant site of synthesis of CGRP in the nervous system (25), we examined the expression of mRNA for the amylin precursor in islets and extrapancreatic tissues, including the central nervous system. Poly(A)$^+$ RNA was prepared from isolated pancreatic islets and tissues including the brain, lung, heart, adrenal, testes, kidney, intestines, and liver and examined by Northern blot analysis. The brain was dissected into four regions so as to sample different regions known to express the CGRPs. As shown in Fig. 3, an intense amylin signal was detected in islet RNA at 0.9 kilobase. Faint bands were also seen at 2.1, 2.3, and 3 kilobases. No amylin mRNA was detected in any of the other tissues examined. After hybridization with the amylin cDNA, the filter was

```
        ACGCTTGGGCTGTAGTTCCTGAAGCTTCAGGCTGCCAGCACACTATCTGTTATTGCTGCCACTGCCCACTGAAAGGGATCTTGAGAC  -1

    ATGAGGTGCATCTCCAGGCTGCCAGCTGTTCTCCTCATCCTCTCGGTGGCACTCGGCCACTTGAGAGCTACACCTGTCGGAAGTGGTACC  90
 1  MetArgCysIleSerArgLeuProAlaValLeuLeuIleLeuSerValAlaLeuGlyHisLeuArgAlaThrProValGlySerGlyThr

    AACCCTCAGGTGGACAAACGGAAGTGCAACACAGCCACATGTGCCACACAACGTCTGGCAAACTTCTTGGTTCGCTCCAGCAACAACCTT  180
31  AsnProGlnValAspLysArgLysCysAsnThrAlaThrCysAlaThrGlnArgLeuAlaAsnPheLeuValArgSerSerAsnAsnLeu

    GGTCCAGTCCTCCCACCAACCAATGTGGGATCCAATACATATGGGAAGAGGAATGTGGCAGAGGATCCAAATAGGGAATCCCTGGATTTC  270
61  GlyProValLeuProProThrAsnValGlySerAsnThrTyrGlyLysArgAsnValAlaGluAspProAsnArgGluSerLeuAspPhe

    TTACTCCTGTAAAGTCAATGTACTCCCGTATCTCTTATTACTTCCTGTGTAAATGCTCTGATGATTTCCTGAATAATGTAACAGTGCCTT  360
91  LeuLeuLeu***

    CAACGTGCCTGTGCTTGCTGTGTTTGTAAATTCTTATTCTAAGACGTGCTTTAAACTGAGTGTTGATAAAGGTCAGGGTGAATACCTCTC  450

    TAATCACAACATGTTCTTGGCTGTACATCGATATCGTAGGAACACTTAAAATTTCTGTTTTTACCTTGTAACTCTATGACTCAAGTTTAA  540

    CAATAAAGGAGGGCGTGGGATGGTGGACTTGAAAAGTCATTAACAGCTCATAGTAAATTTCTGATTCTAGACATTGGAAAGCAAGCATTC  630

    TAAATACCTGCTAAGATGACATTTCAAAATAAATTAGCAAAATCTTGTACTTATTTAATTAAACTTTTTAATTCTATTTCTTGTTGTCCT  720

    TGTTGGTAGTAAGAGGCTGTTTTAAAGGATAAAGGTTGTTTGTATCCCACAAAAAAAAAA  810
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FIG. 1. Nucleotide and predicted amino acid sequence of rat amylin precursor cDNA. The first nucleotide of the initiation methionine in the predicted protein precursor is position +1. The 5′ untranslated region of the cDNA is numbered −1 to −87. The 93-amino acid precursor is translated beneath the nucleotide sequence. The 37-amino acid region homologous to human amylin is underlined. Nucleotide numbers are indicated to the right of the sequence. The amino acid numbers are indicated to the left of the sequence.

NH_2-TERMINAL DOMAIN (1-37)

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RAT DAP     MRCISRLPAVLLILSVALGHLRATPVGSGTNPQVDKR
HUMAN DAP   M-GILKLQVFLIVLSVALNHLKATPIES---HQVEKR
```

CENTRAL PEPTIDE DOMAIN (38-74)

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RAT DAP     KCNTATCATQRLANFLVRSSNNLGPVLPPTNVGSNTY
HUMAN DAP   KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY
RAT αCGRP   SCNTATCVTHRLAGLLSRSGGVVKKDFVPTNVGSKAF
RAT βCGRP   SCNTATCVTHRLAGLLSRSGGVVKKNFVPTNVGSKAF
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COOH-TERMINAL DOMAIN (75-93)

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RAT DAP     GKRNVAEDPNREESLDFLLL
HUMAN DAP   GKRNAVEVLKREPLNYLPL
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FIG. 2. Alignment of rat and human amylin precursors and rat CGRP sequences. The three domains of amylin are listed with the aligned amino acid sequences of rat and human amylin shown. In the central peptide domain, the amino acid sequences of rat α and β CGRP are also included. Amino acids that are shared between rat amylin and either human amylin or the CGRPs are shaded. Amino acids are given in the single-letter code.

hybridized with a cDNA probe for actin. Actin mRNA was detected in all the RNA samples tested, although the size and abundance of the actin RNA varied in the different tissues.

RNA was also examined in islets from animals that were fed ad lib, fasted for 48 hr, or fasted and refed for 4 hr prior to sacrifice. The filter was hybridized with an amylin probe (Fig. 4 *Left*) and an insulin probe (Fig. 4 *Right*). Although a quantitative comparison between the insulin and amylin RNA levels cannot be made because of differences in the specific activities and nature of the probes, it is apparent that insulin is far more abundant than amylin in the islets. Changes in amylin mRNA relative to insulin mRNA were estimated by scanning densitometry of the autoradiograms. In response to fasting, amylin RNA fell ≈30% and did not increase after a short refeeding period.

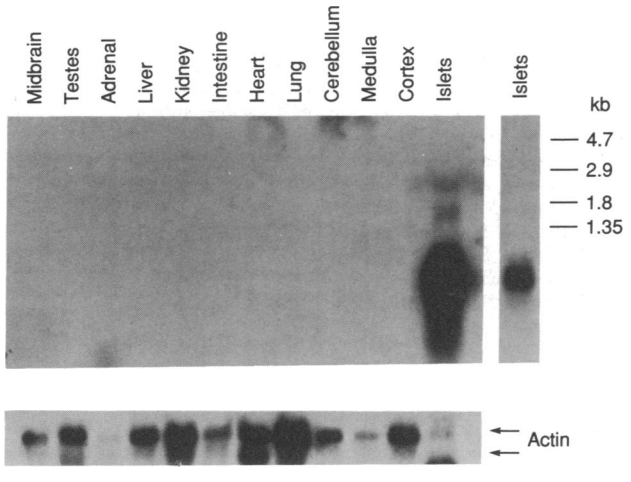

FIG. 3. Tissue-specific expression of amylin precursor mRNA. (*Upper*) Poly(A)$^+$ RNAs (3–5 μg) from the indicated tissues were denatured, separated by gel electrophoresis, transferred to nylon membranes, and hybridized to a single-stranded ^{32}P-labeled probe complementary to amylin RNA. The filter was exposed to Kodak XAR-5 film for 24 hr with an intensifying screen or for 2 hr for the lane of islet RNA on the far right (labeled Islets). The sizes of RNA markers are indicated on the right. (*Lower*) After analysis with the amylin probe, the filter was hybridized with a single-stranded cDNA probe for human β actin and washed. The filter was exposed to XAR-5 film for 16 hr with an intensifying screen to detect the actin signal. kb, kilobases.

DISCUSSION

We have found in rats that amylin is expressed solely in the islets of Langerhans where it is derived from a precursor molecule of 93 amino acids. This precursor appears to be processed by proteolysis in a manner similar to a variety of other peptide hormones, including the CGRPs (20). Amylin mRNA was found at high levels in isolated islets from nondiabetic animals, showing that this molecule is an abundant product of normal β cells. However, at the present time neither the function of amylin nor the cause for its deposition as amyloid in NIDDM is understood.

The localized expression of amylin within the islets suggests that its normal physiologic role is highly tissue-specific. In this regard the function of amylin should be distinct from CGRP for its actions to be specific. The relationship between amylin and CGRP may be similar to that between insulin and insulin-like growth factor I (IGF-I), hormones involved in carbohydrate metabolism and growth regulation, respectively. These peptide hormones and their respective recep-

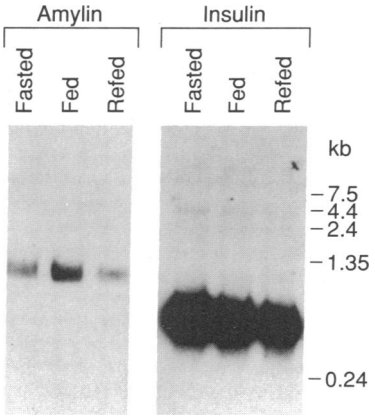

FIG. 4. Amylin mRNA expression in islets in response to dietary changes. Poly(A)$^+$ RNAs were prepared from islets of animals that had either been fed a diet ad lib, fasted for 48 hr, or fasted and then refed for 4 hr prior to sacrifice. The samples (3 μg) were separated by gel electrophoresis, transferred to nylon membranes, and hybridized to a single-stranded ^{32}P-labeled probe for amylin. After hybridization, the filter was washed and exposed to XAR-5 film for 2 hr. The filter was then stripped of the amylin probe and rehybridized with a ^{32}P-labeled insulin probe. After hybridization the filter was washed and exposed to XAR-5 film for 2 hr.

tors have similar structures. Because of these similarities, insulin at high concentrations can bind to the IGF-I receptor and IGF-I at high concentrations can bind to the insulin receptor and thus mimic each other's actions (26). We would predict that specific receptors exist for both amylin and CGRP in appropriate tissues that mediate the appropriate biologic actions of each of these peptides.

The role of amylin in NIDDM is also unclear, although there are other examples of amyloid deposition that may shed light on this process. In systemic amyloidosis proteolytic fragments of serum amyloid A or immunoglobulin light chains can be deposited (27). Amyloid can also result from amino acid substitutions in transthyretin (28) or the protease inhibitor γ-trace protein (29). Lastly, amyloid deposits in the brain in Alzheimer disease appear to be associated with altered proteolysis of a neuronal cell surface protein (30). Any of these mechanisms could be involved in the deposition of amylin within the diabetic pancreas. Early in NIDDM, insulin is secreted at a high rate. At this time amylin may also be secreted at a high rate and be prone to precipitate or undergo altered proteolysis. Glenner *et al.* (31) has shown that a fragment of amylin can form structures similar to amyloid *in vitro*. Once amylin is deposited as amyloid, it may contribute to the further decline of β-cell numbers and the decrease in insulin secretion from the β cells seen later in the course of NIDDM.

There are a wide variety of questions that need to be answered regarding the expression and function of amylin. The availability of the cDNA and the complete amino acid sequence of the precursor should permit further studies to address the regulation, processing, and function of this molecule. These studies should help clarify the role that amylin plays in normal islet function and in the pathogenesis of NIDDM.

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