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Aromatic-L-amino-acid decarboxylase, a pyridoxal phosphate-dependent enzyme, is a β-cell autoantigen

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ABSTRACT **Different autoantigens are thought to be involved in the pathogenesis of insulin-dependent diabetes mellitus, and they may account for the variation in the clinical presentation of the disease. Sera from patients with autoimmune polyendocrine syndrome type I contain autoantibodies against the β-cell proteins glutamate decarboxylase and an unrelated 51-kDa antigen. By screening of an expression library derived from rat insulinoma cells, we have identified the 51-kDa protein as aromatic-L-amino-acid decarboxylase (EC 4.1.1.28). In addition to the previously published full-length cDNA, forms coding for a truncated and an alternatively spliced version were identified. Aromatic L-amino acid decarboxylase catalyzes the decarboxylation of L-5-hydroxytryptophan to serotonin and that of L-3,4-dihydroxyphenylalanine to dopamine. Interestingly, pyridoxal phosphate is the cofactor of both aromatic L-amino acid decarboxylase and glutamate decarboxylase. The biological significance of the neurotransmitters produced by the two enzymes in the β cells remains largely unknown.**

The cell-specific destruction of the insulin-producing pancreatic β cells in insulin-dependent diabetes mellitus (IDDM) is mediated by autoimmune mechanisms. Glutamate decarboxylase (GAD), an enzyme catalyzing the synthesis of γ-aminobutyric acid (GABA) from glutamate, has been identified as a major autoantigen (1). Autoantibodies against this enzyme are present in up to 80% of the patients with IDDM (2, 3), and prediabetic individuals often exhibit reactivity against GAD before the appearance of any clinical manifestations of the disease (4). Patients with IDDM are prone to develop autoimmunity against other organs—e.g., Addison disease and Graves disease (5). In the rare disease autoimmune polyendocrine syndrome type I (APS I), an autoimmune attack against the parathyroid glands, the adrenal glands, and the gonads begins in childhood (6), whereas IDDM may occur at a higher age (5, 6). Nonendocrine symptoms—e.g., chronic mucocutaneous candidiasis, chronic active hepatitis, alopecia, vitiligo, and malabsorption—are also frequent manifestations of the disease (6). In the adrenal cortex (7) and gonads (8), cytochrome P450 side-chain cleavage enzyme—the catalyst of the rate-limiting step in the steroid synthesis—is the major autoantigen. GAD is recognized in the insulin-producing β cells by a majority of sera from APS I patients (9, 10). In addition, strong reactivity against a 51-kDa protein of previously unknown identity has been observed in all APS I sera examined (9). The 51-kDa protein has now been identified by immunoscreening of a cDNA library constructed from a rat insulinoma cell line.†

MATERIALS AND METHODS

Subjects and Sera. Sera were obtained from seven patients with diagnosed APS I. Clinical characteristics of six of these patients have been described previously (9). The seventh patient was a 17-year-old woman with candidiasis, hypoparathyroidism, Addison disease, gonadal insufficiency, and hypothyroidism. Normal goat serum was obtained from Dakopatts (Glostrup, Denmark), and a specific rabbit serum against aromatic-L-amino-acid decarboxylase (AADC, EC 4.1.1.28) (anti-AADC) was purchased from Biogenesis (Poole, Dorset, U.K.).

Construction of RINm 5F cDNA Library. The LiCl/urea method (11) was used to prepare total cellular RNA from the rat insulinoma cell line RINm 5F. Cells were cultured in Ham's F12 medium (Nord Vacc, Skärholmen, Sweden) supplemented with nonessential amino acids and 10% fetal calf serum (FCS; Biochrom, Berlin). Poly(A)$^+$ RNA was purified with Oligotex-dT (Qiagen, Chatsworth, CA). cDNA was synthesized and the library in the λ ZAP II vector was constructed according to instructions of the supplier (Stratagene), except for size fractionation of the cDNA, which was performed on a continuous 5–20% potassium acetate gradient. Five milliliters of gradient was overlaid with 100 μl of 10 mM Tris·HCl and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4, containing the synthesized cDNA. After centrifugation for 3 hr at 50,000 rpm (230,000 × *g*) in an SW 50.1 rotor (Beckman) at 22°C, 0.5-ml fractions were collected and analyzed by electrophoresis in a 1.0% agarose gel. Fractions containing cDNAs larger than 1000 bp were pooled and ligated into λ ZAP II. The completed library, containing 4.5×10^6 recombinant clones, was amplified once.

Immunoscreening of the Library. We plated 5×10^5 plaque-forming units (pfu) of the amplified library on *Escherichia coli* XL-1 at a density of 350 pfu/cm^2. After 3.5 hr of culture at 42°C, plates were overlaid with nitrocellulose filters (Hybond C, Amersham) previously soaked with 10 mM isopropyl β-D-thiogalactopyranoside (IPTG; Sigma) and cultured for another 3.5 hr at 37°C. The filters were carefully removed from the plates and washed in 15 ml of 20 mM Tris·HCl, pH 7.5/0.1% gelatin/0.05% Tween-20 (TBS-GT) three times for 5 min. Nonspecific protein binding was blocked with 15 ml of 1% gelatin in 20 mM Tris·HCl, pH 7.5, for 1 hr, after which the filters were washed in TBS-GT as above. To reduce unspecific binding of the secondary antibody during color development, the filters were incubated with normal goat serum at a dilution of 1/1000 in TBS-GT for 30 min. After a third wash cycle, filters were incubated overnight with sera diluted 1/5000 in TBS-GT, from one of the patients. Filters were washed in 20 mM Tris·HCl, pH 7.5, supplemented with 0.1% gelatin and then incubated with alkaline phosphatase-conjugated goat anti-human IgG for 1.5 hr prior to color development with a *p*-nitrobluetetrazolium chloride (NBT)/5-bromo-4-chloro-3-

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Abbreviations: IDDM, insulin-dependent diabetes mellitus; GAD, glutamate decarboxylase; GABA, γ-aminobutyric acid; APS I, autoimmune polyendocrine syndrome type I; AADC, aromatic-L-amino-acid decarboxylase; FCS, fetal calf serum.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. U31884).

indoyl phosphate/toluidine (BCIP) system (Bio-Rad). Positive clones were rescreened until pure isolates were obtained.

DNA Sequence Analysis. The nucleotide sequence of positive cDNA clones was determined by the dideoxynucleotide method (12) after subcloning into M13 derivatives. Sequence analysis was performed with the Applied Biosystems automatic DNA sequenator 373A, using T7 DNA polymerase (Sequenase) with either -21M13 dye primer (Applied Biosystems) or unlabeled synthesized internal primers (Scandinavian Gene Synthesis, Köping, Sweden) together with dye terminators (Applied Biosystems).

Recombinant AADC. Clone 3.1 was ligated into the *Not* I and *Kpn* I sites of the pBK-CMV vector (Stratagene) for transient expression in COS cells. COS cells (2×10^6) were cultured to 80% confluence, harvested by trypsin treatment, washed twice in 10 ml of Dulbecco's modified Eagle's medium (DMEM; Biochrom) buffered with 10 mM Hepes, pH 7.4, and finally resuspended in 200 μl of the same medium. Fifty micrograms of vector was added to the cells. After incubation on ice for 10 min, electroporation was performed with a Bio-Rad Genepulser at 200 V and 500 mF. The cells were incubated on ice for another 10 min and then seeded in DMEM containing 10% FCS. After 48 hr of culture, the cells were used in immunoprecipitation experiments.

Isolation of Islets of Langerhans. Islets of Langerhans from Wistar–Furth rats were manually isolated after collagenase digestion (13) and cultured for 48 hr in RPMI 1640 medium (Biochrom) containing 11 mM glucose prior to metabolic labeling.

Immunoprecipitation. RINm 5F cells, transfected COS cells, and isolated islets were washed twice in methionine-free RPMI 1640 medium and then cultured for 6 hr in a medium supplemented with [^{35}S]methionine (>1100 Ci/mmol; Amersham; 1 Ci = 37 GBq). After washing in nonradioactive medium as above, cells and islets were lysed for 1 hr at 4°C in a buffer of 20 mM Tris·HCl, pH 8.2/1.0% Triton X-100 (Eastman Kodak)/2 mM phenylmethylsulfonyl fluoride (PMSF; Sigma)/20 mM NaCl/1% Trasylol (Bayer, Leverkusen, Germany). Insoluble membrane debris was removed by centrifugation at 100,000 rpm (350,000 × *g*) in a TLA-100.2 rotor (Beckman) for 15 min. Immunoprecipitations and SDS/PAGE were performed as described previously (14).

RESULTS

Identification of the 51-kDa Antigen as AADC. A cDNA library from RINm 5F rat insulinoma cells constructed in λ ZAP II was screened with high-titer sera (1/5000 dilutions) from patients with APS I. Four positive clones were identified and rescreened until pure isolates were obtained. Nucleotide sequence analyses revealed that all clones coded for the pyridoxal phosphate-dependent enzyme AADC. Clones 3.1 and 5.1 were identical to the reported full-length cDNA (15) coding for a protein of 480 amino acids with a calculated molecular mass of 54 kDa, whereas clones 10.5 and 10.7 corresponded to forms not previously described (Fig. 1). Clone 10.5 had a unique 3′ untranslated sequence downstream of an

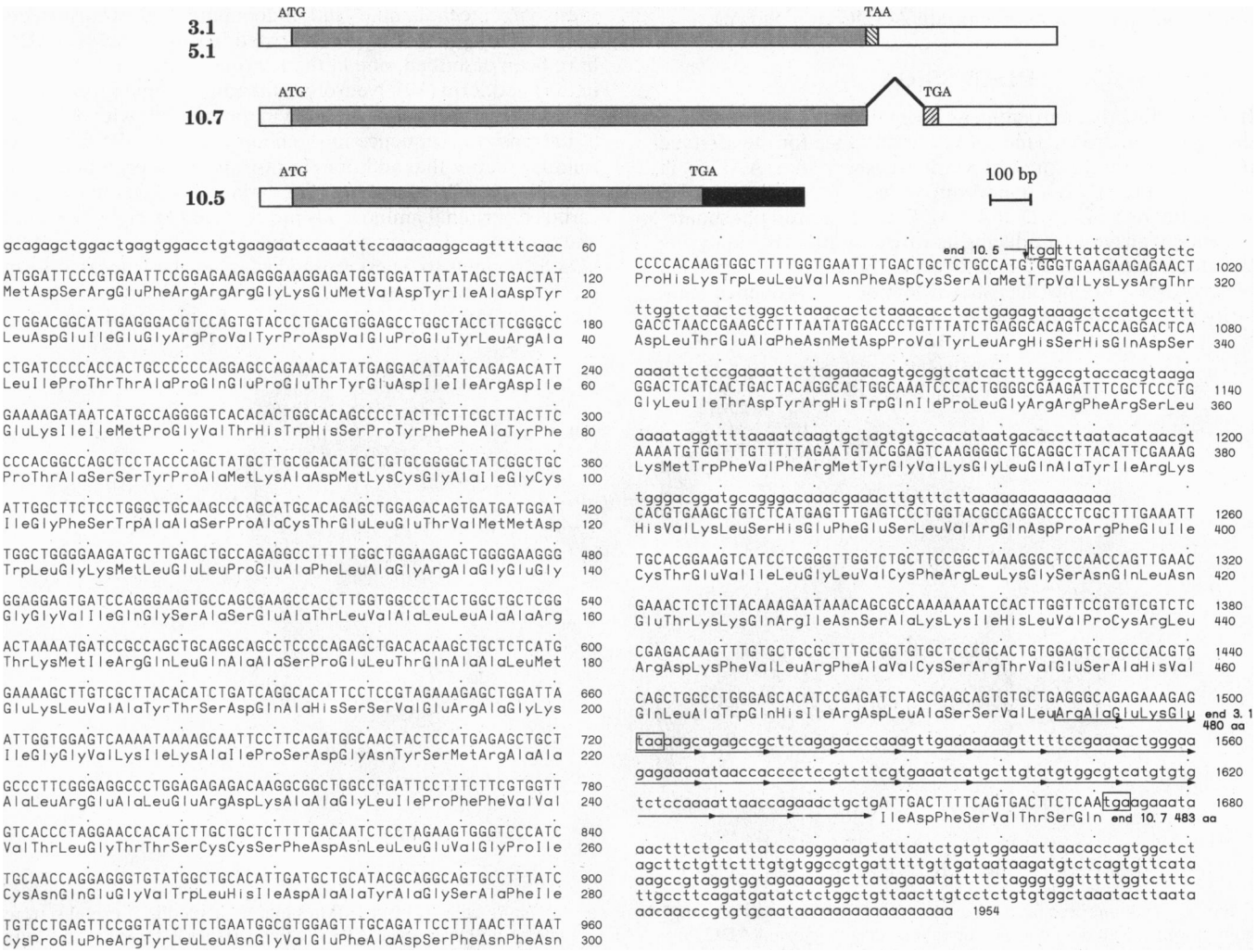

FIG. 1. Physical maps (*Upper*) and nucleotide sequences and the deduced amino acid sequences (*Lower*) of the AADC clones. Coding sequences are written in uppercase letters. Stop codons are boxed. The alternatively spliced sequence is indicated by arrows.

in-frame stop codon at position 1004 (codon 315), giving rise to a protein with a calculated molecular mass of 35 kDa. Clone 10.7 was a splice variant coding for a protein in which the five terminal amino acids in the protein coded for by clone 3.1/5.1 were replaced by eight different ones (Fig. 1).

Immunoprecipitation of Rat Islet and RINm 5F Lysates. Immunoprecipitations of isolated [^{35}S]methionine-labeled islets of Langerhans from rats were performed to establish whether the 51-kDa protein and AADC were identical. All sera from patients with APS I precipitated a 51-kDa protein which comigrated with AADC precipitated by a specific anti-AADC serum (Fig. 2 *Left*). In accordance with previous data (9), a majority of the sera (5 of 7) also precipitated a doublet of approximately 65 kDa corresponding to GAD. Furthermore, when a lysate from RINm 5F cells was precleared with the specific anti-AADC serum, the ability of patient sera to subsequently immunoprecipitate the 51-kDa autoantigen was abolished (Fig. 2 *Right*).

Immunoprecipitation of Recombinant AADC. To confirm the identity of the 51-kDa protein, clone 3.1 was ligated into the pBK-CMV vector. After transient expression in COS cells, all APS I sera immunoprecipitated AADC (Fig. 3, lanes a, c, e, and g), while no reactivity was detected in COS cells transfected with the empty vector (Fig. 3, lanes b, d, f, and h). Identical results were obtained with the alternatively spliced cDNA clone 10.7, whereas the truncated clone 10.5 gave no immunoprecipitate at the predicted position around 35 kDa (not shown). Sera from 20 patients with IDDM of recent onset, 3 patients with stiff man syndrome, and 20 healthy blood donors did not immunoprecipitate AADC (not shown).

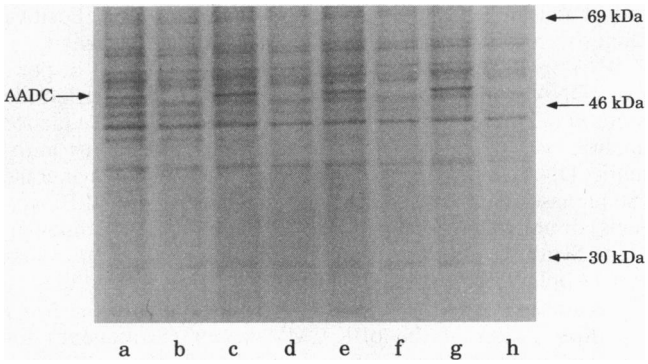

FIG. 3. Immunoprecipitation experiments with AADC transiently expressed in COS cells. COS cells containing the pBK-CMV vector with an AADC insert (lanes a, c, e, and g) or no insert (lanes b, d, f, and h) were metabolically labeled with [^{35}S]methionine, and proteins were immunoprecipitated with four different APS I sera.

DISCUSSION

It is intriguing that autoantigens in organ-specific autoimmune diseases often are enzymes of key importance for the affected organ (16). In the present work we show that AADC, in addition to GAD, is a major autoantigen in β cells in APS I. Interestingly, both GAD and AADC are pyridoxal phosphate-dependent enzymes producing neurotransmitters by catalyzing the decarboxylation of amino acids. Sequence comparison between the enzymes has shown low overall sequence similarity (17), except for short stretches of sequence identity at and around the pyridoxal phosphate-binding site, Asp-Pro-His-Lys (17). The fact that no relation between reactivities against AADC and GAD was found in individual patients and the observation that an anti-AADC antibody did not cross-react with GAD indicate that there was no cross-reactivity between GAD autoantibodies and AADC.

AADC catalyzes the decarboxylation of aromatic L-amino acids, notably L-3,4-dihydroxyphenylalanine (dopa) and 5-hydroxytryptophan (5-HT), which are intermediates in the synthesis of catecholamine and indolamine neurotransmitters (18). In *Drosophila melanogaster* two different forms of AADC have been described, one in the nervous system and the other in the hypoderm (19). Neuronal and nonneuronal forms of the enzyme have also been observed in the rat (20), with a unique 5′ untranslated sequence in the neuronal form. We have now found evidence that additional isoforms of the protein may be present in β cells, namely a splice variant in which the five final carboxyl-terminal amino acids are replaced by eight new ones and a truncated form with a calculated molecular mass of 35 kDa. It remains to be established whether the truncated form is a naturally occurring protein. At present we cannot rule out the possibility that the cDNA coding for this predicted form

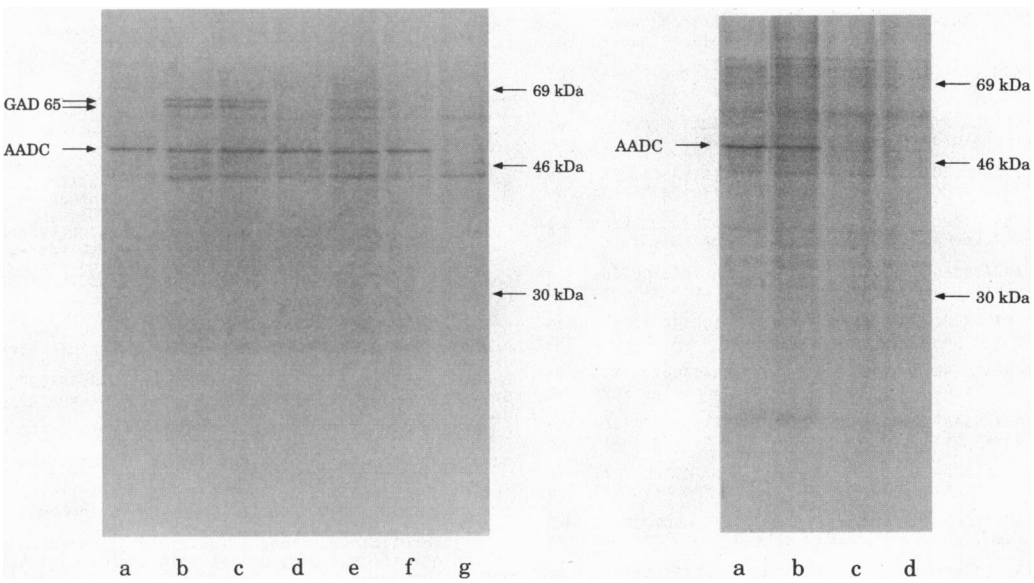

FIG. 2. Immunoprecipitation experiments with rat islets of Langerhans and RINm 5F cells. (*Left*) [^{35}S]Methionine-labeled islet proteins were immunoprecipitated with a specific rabbit serum against AADC (anti-AADC) (lane a), with sera from APS I patients (lanes b–f), and with serum from a healthy blood donor (lane g). (*Right*) Sequential immunoprecipitations of [^{35}S]methionine-labeled RINm 5F cell proteins were performed with the anti-AADC serum (lane a) followed by an APS I patient serum (lane c), and with the APS I serum (lane b) followed by the anti-AADC (lane d).

represents a cloning artifact. Our findings may explain the reported biochemical data suggesting the existence of AADC isoforms with preference for dopa and 5-HT (21). Apart from being present in the β cells and other cells capable of amine precursor uptake and decarboxylation, including the enterochromaffin cells of the intestine, AADC is also found in the liver and kidney (22, 23). Its presence in the liver and enterochromaffin cells of the intestine may explain the hepatitis and malabsorption seen in APS I patients. Interestingly, tyrosinase has recently been reported to be an autoantigen in autoimmune vitiligo, which is part of the APS I syndrome (24). This enzyme, which is the rate-limiting enzyme in melanin synthesis, oxidizes dopa to dopa quinone. We found no reactivity against AADC in a small group of patients with IDDM. However, subgroups with such reactivity may exist—e.g., IDDM patients with neuropathy, liver affection (25), or other organ-specific autoimmune manifestations.

The functional importance of neurotransmitters produced by the pyridoxal phosphate-dependent enzymes GAD and AADC in pancreatic β cells is unclear. It has been suggested that GABA, as part of the GABA cycle, may represent means by which the cell can utilize glutamate as a reserve energy source (26). Dopamine and serotonin produced by AADC could be involved in the regulation of insulin synthesis and secretion (27). It may also be speculated that AADC may decarboxylate unknown regulator molecules (28).

GAD and AADC are both intracellular enzymes. GAD is mainly found in synaptic-like small vesicles (SLMV; ref. 29), whereas AADC is located in the cytosol (30). Since antibodies are directed against antigens on the cell surface, it is unclear how autoantibodies against the intracellular enzymes GAD and AADC could be participating in the pathogenesis of IDDM. Peripheral-blood mononuclear cells from patients with IDDM have been demonstrated to proliferate *in vitro* on exposure to GAD (31, 32), indicating a role of cell-mediated immunity. Even though it has been suggested that the initial lesion is likely to be T-cell mediated and the presence of autoantibodies merely reflects β-cell destruction, as markers of the disease, it cannot be ruled out that autoantibodies do take part in the pathogenesis of IDDM.

It has recently been observed that amino acids 250–273 of GAD 65 show sequence similarity with amino acids 28–50 of the coxsackievirus B protein P2-C (33, 34). It was proposed that, as a result of this molecular mimicry, cross-reactive T-cell proliferation will occur, leading to autoimmune destruction of the β cells. However, we have not been able to find any evidence of molecular mimicry between AADC and any known microorganism upon search of protein data bases.

The fact that AADC and GAD, two important enzymes for the generation of neurotransmitters, act as autoantigens in the islets of Langerhans underlines the neuron-like nature of the β cells. In the central nervous system the blood–brain barrier seems to protect these enzymes from an immune attack, except in the rare cases of stiff man syndrome, where autoantibodies against GAD are present in the cerebrospinal fluid (35). When, as in the β cell, AADC or GAD is not protected by any barrier, autoaggressive responses may develop more easily.

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