0021-972X/04/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 89(4):1636–1640

doi: 10.1210/jc.2003-031161

Analysis of Antibody Reactivity against Cysteine Sulfinic Acid Decarboxylase, A Pyridoxal Phosphate-Dependent Enzyme, in Endocrine Autoimmune Disease

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The structurally related group II pyridoxal phosphate (PLP)-dependent amino acid decarboxylases glutamic acid decarboxylase (GAD), aromatic L-amino acid decarboxylase (AADC), and histidine decarboxylase (HDC) are known autoantigens in endocrine disorders. We report, for the first time, the prevalence of serum autoantibody reactivity against cysteine sulfinic acid decarboxylase (CSAD), an enzyme that shares 50% amino acid identity with the 65- and 67-kDa isoforms of GAD (GAD-65 and GAD-67), in endocrine autoimmune disease. Three of 83 patients (3.6%) with autoimmune polyendocrine syndrome type 1 (APS1) were anti-CSAD positive in a radioimmunoprecipitation assay. Anti-CSAD antibodies cross-reacted with GAD-65, and the anti-CSAD-positive sera were also reactive with AADC and HDC. The low frequency of anti-CSAD reactivity is in striking contrast to the prevalence of antibodies against GAD-65, AADC, and HDC in APS1 patients, suggesting that different mechanisms control the immunological tolerance toward CSAD and the other group II decarboxylases. Moreover, CSAD may be a useful mold for the construction of recombinant chimerical antigens in attempts to map conformational epitopes on other group II PLP-dependent amino acid decarboxylases. (*J Clin Endocrinol Metab* 89: 1636–1640, 2004)

IN INSULIN-DEPENDENT diabetes mellitus (IDDM) the 65-kDa isoform of the pyridoxal phosphate (PLP)-dependent enzyme glutamic acid decarboxylase (GAD-65), which is expressed in the insulin-producing β-cells of the pancreas, is a major target of autoantibodies (1). In a subset of patients with IDDM, antibodies against the larger isoform GAD-67, which is encoded by a separate gene (2), can also be detected (3). GAD can be assigned to the group II PLP-dependent amino acid decarboxylases, which also include aromatic L-amino acid decarboxylase (AADC) and histidine decarboxylase (HDC) (4). The three-dimensional structure of pig AADC has recently become available (5).

Autoimmune polyendocrine syndrome type 1 (APS1), also known as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (Online Mendelian Inheritance in Man no. 240300), is caused by defects of the AIRE gene (for autoimmune regulator) on chromosome 21 (6, 7). Manifestations of this syndrome include chronic mucocutaneous candidiasis, hypoparathyroidism, adrenal failure, alopecia, vitiligo, IDDM, pernicious anemia, intestinal dysfunction, and chronic active hepatitis (8). Several of these manifestations have been linked to specific autoantibodies against intracellular enzymes, including GAD-65, AADC, and HDC (9, 10), and it has been proposed that APS1 could be a useful model for organ-specific autoimmunity in general.

The rate-limiting enzyme in the taurine biosynthesis, cysteine sulfinic acid decarboxylase (CSAD) shows 50% amino acid sequence identity with GAD-65, making it the PLP-dependent decarboxylase most closely related to GAD-65 apart from GAD-67 (11, 12). For comparison, the levels of amino acid identity between GAD-65 and the other group II decarboxylases are shown in Fig. 1. CSAD consists of homodimers of approximately 55-kDa subunits (11) and is predominantly expressed in the liver and kidney (13). Recently, high levels of CSAD transcripts and enzymatic activity were found in adipose tissue (14). Brain-specific CSAD transcripts, differing in the 5′ untranslated region, have been described (12), but the level of CSAD expression in the brain is controversial (14). Expression has also been detected in other tissues such as the retina and the lactating mammary gland (15, 16).

Although evidence is lacking, it is conceivable that autoimmune reactions might contribute to retinal and renal dysfunction in a subset of IDDM patients. Autoantibodies against CSAD have been found in a rat model for hepatocellular carcinoma (17), but never, to our knowledge, in humans. In the study presented here, we investigated the presence of anti-CSAD antibodies in APS1, IDDM, and Addison's disease.

Abbreviations: AADC, Aromatic L-amino acid decarboxylase; APS1, autoimmune polyendocrine syndrome type 1; CSAD, cysteine sulfinic acid decarboxylase; GAD, glutamic acid decarboxylase; HDC, histidine decarboxylase; IDDM, insulin-dependent diabetes mellitus; PLP, pyridoxal phosphate.

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Materials and Methods

Patients and sera

Serum samples from 10 Swedish, 16 Norwegian, and 57 Finnish patients with APS1, 84 patients with IDDM, and 30 patients with Ad-

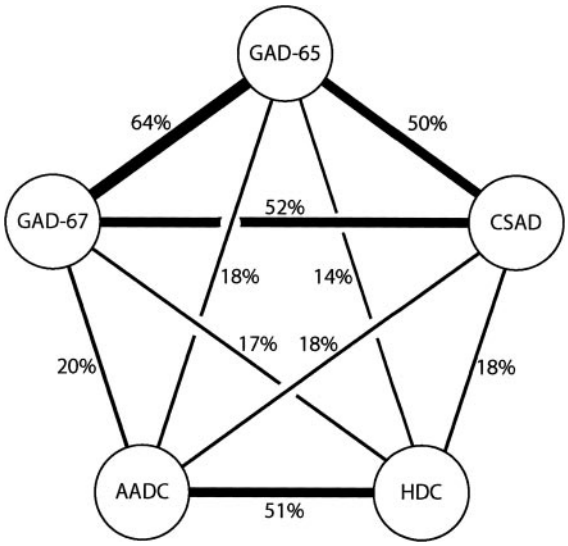

FIG. 1. Schematic diagram of the relationship between the human group II PLP-dependent amino acid decarboxylases GAD-65, GAD-67, AADC, CSAD, and HDC (GenBank accession nos. NP_000809, NP_000808, Q9Y600, NP_000781, and NP_002103, respectively). Sequence identity (in percentage) by pairwise ClustalW alignments of the full-length amino acid sequences is indicated. The *thickness* of each *line* is proportional to the degree of sequence identity.

dison's disease were analyzed (18–20). As controls, 87 healthy blood donor sera were analyzed. Data on antibody reactivity against GAD-65, AADC, and HDC were available from previous studies (9, 10, 21). Serum samples were collected with the informed consent of the subjects for the purpose of autoantibody analyses. Blood donor samples were rendered anonymous so that they could not be traced to specific individuals. The study was approved by the local ethics committee.

Sequence analyses

The expressed sequence tag database at the National Center for Biotechnology Information at http://www.ncbi.nlm.nih.gov was searched with the Basic Local Alignment Search Tool (22), using published amino acid sequences of CSAD and GAD-65 to identify full-length CSAD cDNA clones. The Mammalian Gene Collection cDNA clone 18185 (GenBank accession no. BC008561) was obtained from the UK Human Genome Mapping Project Resource Centre (Cambridge, UK). ClustalW alignments (23) were performed at www.ebi.ac.uk/clustalw.

In vitro transcription and translation

Coupled *in vitro* transcription and translation of ^{35}S-labeled antigen was performed using the TnT system (Promega, Madison, WI). Translation products were analyzed by SDS-PAGE followed by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA). ^{35}S-methionine incorporation was determined by trichloroacetic acid precipitation followed by scintillation counting.

Radioimmunoprecipitation assay

Immunoprecipitation of *in vitro*-translated CSAD was carried out in 96-well plates, essentially as described previously (24, 25). All serum samples were analyzed in duplicate. Approximately 9000 cpm of radiolabeled CSAD and 2.5 μl serum in a final volume of 50 μl were used for each reaction. An anti-CSAD reactivity index was calculated for each serum as follows: (cpm of unknown sample − cpm of negative control)/(cpm of positive control − cpm of negative control) as described (24, 25). As a positive control, we used the APS1 serum that showed the strongest reactivity in pilot experiments, and as a negative control serum from a healthy donor. An anti-CSAD reactivity index of 0.1 was set as an arbitrary upper normal limit, as this value segregated the APS1 cohort into those with clearly elevated indices and those with normal or slightly elevated values (25).

Preabsorption

Serum samples diluted 1:25 in a volume of 50 μl were incubated with 1 μg of purified recombinant GAD-65 (Diamyd, Stockholm, Sweden) for 4 h at +4 C, whereafter 30,000 cpm ^{35}S-labeled *in vitro*-translated CSAD, GAD-65, or AADC was added in a volume of 50 μl. Samples were incubated overnight at +4 C, and 100 μl of 10% Fast Flow protein A-Sepharose (Amersham Biosciences, Uppsala, Sweden) was then added. After 1 h of agitation, immune complexes were washed and analyzed by SDS-PAGE followed by PhosphorImager analysis.

Results

The Mammalian Gene Collection cDNA clone 18185 (GenBank accession no. BC008561) was used as template for coupled *in vitro* transcription and translation of ^{35}S-labeled antigen. This cDNA contains an open reading frame encoding a putative protein of 493 amino acids with more than 99% identity (492/493 amino acids) with the recently published mouse CSAD sequence (13). Furthermore, the nucleotide sequence completely matches the genomic sequence of mouse chromosome 15 (data not shown). This clearly shows that the cDNA used in subsequent experiments represents mouse CSAD. ClustalW alignments showed that the deduced amino acid sequence of mouse CSAD has 52% identity with human GAD-65 and 89% identity with human CSAD. *In vitro* translation of mouse CSAD yielded a major product of approximately 55 kDa as estimated by SDS-PAGE analysis (Fig. 2A).

Analysis of serum samples from patients with APS1,

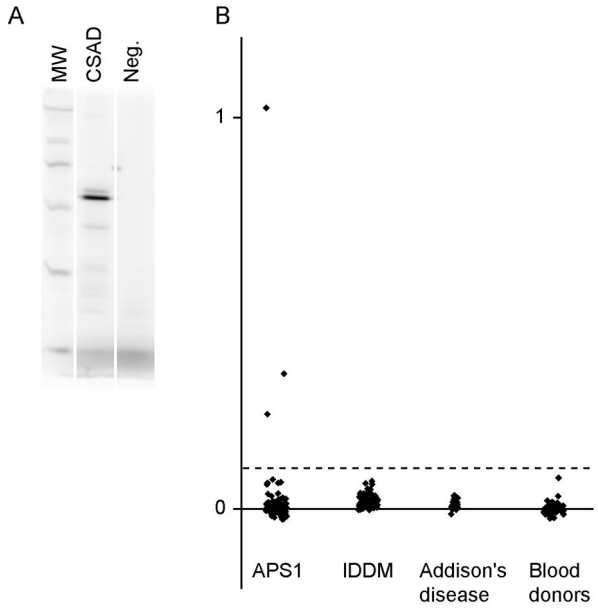

FIG. 2. A, Coupled *in vitro* transcription and translation of ^{35}S-labeled CSAD analyzed by SDS-PAGE followed by phosphorimaging. Lane 1, ^{14}C-labeled molecular weight markers corresponding to 220, 97, 66, 45, 30 and 14 kDa; lane 2, mouse CSAD *in vitro* translation product; lane 3, negative control *in vitro* translation product (no template DNA added). B, Scattergram showing the antibody reactivity (expressed as a reactivity index defined in the text) against CSAD in serum samples from patients with APS1 (n = 83), IDDM (n = 84), Addison's disease (n = 30), and healthy blood donors (n = 87). The *broken line* indicates a cutoff value of 0.1.

IDDM, and Addison's disease and from healthy blood donors showed that only three of 83 APS1 sera (3.6%), and none of the other serum samples, had antibody levels clearly above the background (Fig. 2B). Two of these patients were only weakly positive. Clinical characteristics of the three anti-CSAD-positive patients are summarized in Table 1.

To test whether anti-CSAD antibodies cross-react with GAD-65, we performed a competition experiment in which preincubation of serum samples with excess recombinant human GAD-65 was followed by immunoprecipitation analysis of ^{35}S-labeled, *in vitro*-translated CSAD (Fig. 3). Parallel immunoprecipitations of ^{35}S-labeled GAD-65 and AADC with and without GAD-65 competition were carried out as controls. After preabsorption with excess unlabeled GAD-65, the anti-CSAD reactivity was decreased in all three anti-CSAD-positive sera, most obviously in the serum with the strongest anti-CSAD reactivity. As expected, preincubation of patient sera with unlabeled GAD-65 also efficiently blocked subsequent immunoprecipitation of ^{35}S-labeled GAD-65. Anti-AADC reactivity was not affected by preabsorption with GAD-65.

Data on autoantibody reactivity against GAD-65, AADC, and HDC were available for the APS1 patients, including the three patients who were positive for anti-CSAD antibodies (9). A comparison of the antibody reactivity patterns for all 83 APS1 sera against these four different enzymes is illustrated in Fig. 4.

Discussion

Using a candidate approach, we have identified CSAD as a minor target of autoantibodies in APS1. To our knowledge, this is the first report of anti-CSAD antibody reactivity in humans. Despite its close structural relation to GAD-65, CSAD does not seem to have any relevance as an autoantigen in IDDM, as 84 IDDM serum samples tested were negative. Anti-CSAD antibodies do not appear to be associated with any of the known autoimmune manifestations of APS1, and mucocutaneous candidiasis was the only manifestation present in all three patients. A preabsorption experiment with recombinant human GAD-65 indicated that at least part of the anti-CSAD reactivity may be attributed to the presence of cross-reactive anti-GAD-65 antibodies. The finding that anti-CSAD antibody reactivity was found in APS1 patient sera only, and the observed cross-reactivity with GAD-65, may reflect the propensity of APS1 patients to develop anti-GAD antibodies directed against different epitopes than IDDM patients do (26).

It is notable that all three anti-CSAD-positive patient sera also reacted with all the other three PLP-dependent decarboxylases GAD-65, AADC, and HDC. The comparison of antibody reactivity against the different group II PLP-dependent amino acid decarboxylases in the 83 APS1 patients also showed that none of the patients who were anti-GAD-65/anti-HDC double-positive were anti-AADC negative. The limited number of samples makes a statistical interpretation difficult, but it may be speculated that this latter finding is related to coexpression of AADC with GAD-65 and HDC in different endocrine cell types.

TABLE 1. Clinical manifestations present in the three patients with APS1 positive for antibody reactivity against CSAD

Patient no.	Anti-CSAD reactivity index	Sex	APS1 manifestations
1	1.02	Female	Ca, HP, GI
2	0.35	Female	Ca, HP, AD, HG, Al, GI, H
3	0.24	Male	Ca, AD, Al, H

Ca, Mucocutaneous candidiasis; HP, hypoparathyroidism; GI, intestinal dysfunction; AD, adrenal failure; HG, hypogonadism; Al, alopecia; H, hepatitis.

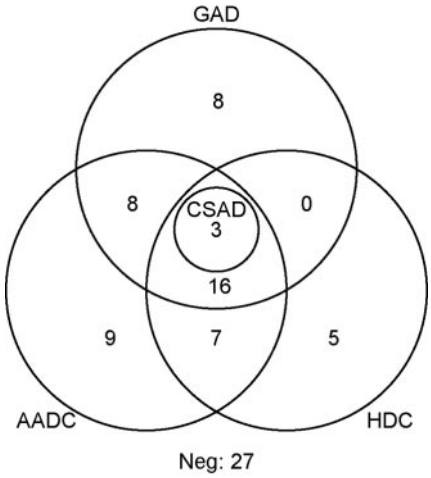

FIG. 4. Venn diagram illustrating the overlap of antibody reactivity against *in vitro*-translated CSAD, GAD-65, AADC, and HDC in serum samples from the studied patients with APS1 (n = 83). The *numbers* indicate how many patients fall into each subset. Neg, Serum samples negative for all three autoantibodies.

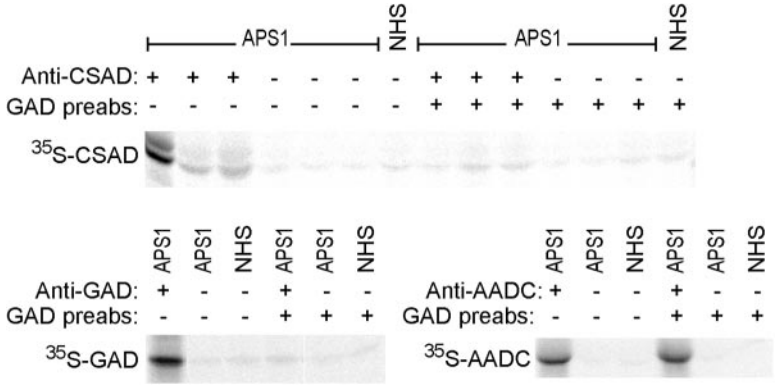

FIG. 3. Immunoprecipitation of ^{35}S-methionine-labeled *in vitro*-translated CSAD, GAD-65, and AADC in the absence (−) or presence (+) of excess recombinant GAD-65 followed by SDS-PAGE and subsequent phosphorimaging. APS1 sera that were positive (+) or negative (−) for antibodies against CSAD, GAD-65, and AADC in the 96-well radioimmunoprecipitation assay, and normal human serum (NHS) were analyzed.

It is an intriguing fact that autoantigens in organ-specific autoimmune disorders often are intracellular enzymes, and it has been speculated that this may be related to unique immunogenic properties (27, 28). The observed low prevalence of anti-CSAD antibodies is in striking contrast to the prevalence rates of antibodies against GAD-65, AADC, and HDC, which in previous studies at our laboratory have been found in 37, 51, and 37% of APS1 patients, respectively (9, 10). Defects of the AIRE gene thus only rarely cause a break of immunological tolerance to CSAD, suggesting that the frequent loss of tolerance against the related enzymes GAD-65, AADC, and HDC is in fact not due to structural features but perhaps rather due to differences in tissue distribution or other factors influencing antigen presentation and maintenance of immunological tolerance.

Autoantibodies directed against intracellular antigens in autoimmune diseases are generally believed not to be directly pathogenic themselves, but merely to reflect the activation of autoreactive T helper cells, and the destruction of target cells is supposed to be mediated by cytotoxic T cells. Although much more difficult to perform than studies of autoantibody responses, progress has been made in recent years in studies of autoreactive T cells in organ-specific autoimmunity (29, 30). Our increasing knowledge of autoantibody specificities in APS1 may form a basis for future studies of T cell reactivity against the different groups of structurally related autoantigens in this disorder, to clarify the relation between B cell and T cell autoreactivity against defined antigens, and to elucidate the possible existence of cross-reactive T cell epitopes.

In conclusion, CSAD has been shown in this study to be a novel but rare target of autoantibodies in APS1. Despite the close structural relation of CSAD with GAD-65, no reactivity was detected in IDDM sera. These findings extend our knowledge of autoantibody specificities in APS1, and CSAD may provide a useful neutral partner in construction of recombinant chimerical antigens in attempts to map the conformational epitopes on other group II PLP-dependent amino acid decarboxylases (31).

Acknowledgments

Received July 8, 2003. Accepted December 22, 2003.

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This work was supported in part by the Swedish Medical Research Council, the Torsten and Ragnar Söderberg Foundation, the Petrus and Augusta Hedlund Foundation, the Swedish Medical Society, the Claes Groschinsky Memorial Foundation, the Lennander Foundation, and the Agnes and Mac Rudberg Foundation.

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