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EXPERIMENTAL STUDY

Epitope mapping of cytochrome P450 cholesterol side-chain cleavage enzyme by sera from patients with autoimmune polyglandular syndrome type 1

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Abstract

Objective: Autoimmune polyglandular syndrome type 1 (APS-1) is a disease associated with defects of the autoimmune regulator gene and is characterized by autoimmune lesions of several tissues, predominantly endocrine glands, with multiple autoantibodies. In this study we describe autoantigenic epitopes on cholesterol side-chain cleavage enzyme (P450scc) using sera from Finnish and Sardinian patients with APS-1, and analyze the epitope reactivities during disease follow-up.
Methods: A series of P450scc cDNA fragments were expressed in *E. coli* and tested by immunoblotting assay using the patients' sera.
Results: Epitope regions were found over the whole P450scc molecule except the last N- (amino acids (aa) 1–40) and C-termini (aa 456–521). The strongest reactivity with patients' sera was found with central and C-terminal regions of the P450scc protein. All studied patients had IgG1 subclass antibodies.
Conclusions: The results show that Finnish and Sardinian patients with APS-1 have similar, polyclonal immune reactions against P450scc, and that epitope reactivities did not change during the disease course. These results support the opinion that autoantibodies against P450scc and their epitope reactivity pattern are formed at an early stage of steroidogenic autoimmunity.

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Introduction

In autoimmune polyglandular syndrome type 1 (APS-1), also known as autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy, the most common disease components are Addison's disease, hypoparathyroidism and chronic mucocutaneous candidiasis. The disease may also include ectodermal dystrophies, ovarian atrophy, type 1 diabetes, pernicious anemia, autoimmune hepatitis and hypothyroidism (1, 2).

APS-1 is caused by a defective AIRE (autoimmune regulator) gene on chromosome 21q22.3 and over 40 mutations have been identified to date (3–5). Its highest incidences are in the isolated populations of the Finns, the Sardinians and Iranian Jews, estimated at 1 in 25 000, 1 in 14 400 and 1 in 9000 respectively (4, 6, 7). AIRE is expressed in thymus (8) and has a transcriptional activity (9, 10), indicating that it may regulate the induction and maintenance of central immune tolerance.

In the Addison's disease of APS-1, three major autoantigens are the steroid 21-hydroxylase (P450c21), the steroid 17-hydroxylase (P450c17) and the cholesterol side-chain cleavage enzyme (P450scc) (11–16). Of these P450 cytochromes, all three are expressed in the adrenal cortex, and P450c17 and P450scc are in the gonads. Also, their expression has been described in the brain and skin (17, 18). Subcellularly, P450c17 and P450c21 are localized in the endoplasmic reticulum (19, 20), while P450scc is transported into the mitochondria by an N-terminal signal peptide (21). P450scc, a protein of 521 amino acids, catalyzes the first step in the production of the steroid hormones, converting cholesterol to pregnenolone in the mitochondria (22). It is a heme-containing protein participating in interactions with cholesterol and electron transfer proteins, adrenodoxin and adrenodoxin reductase (23, 24). Sera from patients with APS-1 inhibit the conversion of cholesterol to pregnenolone by P450scc in an *in vitro* assay (14).

To understand the molecular basis of steroid-cell autoimmunity the autoantigenic regions of the P450 cytochromes need to be characterized. The autoantibody epitopes of P450c17 and P450c21 have been analyzed in patients with APS-1 (25–29), but no data are available about epitope regions of the P450scc. We have studied the autoantigenic regions on P450scc using sera from Finnish and Sardinian patients with APS-1. For epitope analysis, fragments of different length were expressed as fusion proteins and tested for antigenicity through an immunoblotting assay. Also, we studied epitope characteristics in the Finnish patients over years of follow-up and did subclass typing of the IgG autoantibodies against P450scc.

Materials and methods

Patients

We studied serum samples from ten Finnish (nos 2–5 female, nos 1, 6–10 male; age 8–20 years) and five Sardinian (nos 11, 13 and 15 female, nos 12 and 14 male; age 7–30 years) patients with APS-1 (Table 1). All sera contained steroid-cell autoantibodies according to indirect immunofluorescence tests and were reactive against P450scc, as demonstrated by immunoblotting with 1–370 amino acid (aa) polypeptide for the Finnish (15) and the full-length P450scc for the Sardinian patients (30, 31). The diagnosis was based on clinical and laboratory criteria (32). To reveal changes in epitope reactivity over years, we analyzed 26 follow-up sera from seven of the Finnish patients. Two or three samples were taken from nos 3, 7 and 8, over 3–5 years; five or six samples from patients nos 4 and 5, over 5–10 years; and four samples from patients nos 9 and 10, over 7–9 years. Thirty-five sera from healthy persons (14 females and 15 males aged 1.5–14 years; four females and two males aged 29–48 years) served as negative controls in the antibody assays.

Construction of P450scc deletion variants

The full-length P450scc cDNA (1566 bp) (15) was used as a template to generate eleven P450scc deletion constructs using PCR. Forward and reverse primers (DNA Technology A/S, Aarhus, Denmark) were designed with flanking restriction enzyme recognition sites used in cloning to the expression vectors. The PCR was performed with a Gene Amp XL-PCR Kit (Perkin Elmer, Norwalk, CT, USA) in a final reaction volume of 100 μl containing rTth polymerase, 200 ng pUC18-P450scc template and 20 pmol forward and reverse primers. The reaction mix was subjected to 25 cycles in a temperature cycler (PTC-100; MJ Research Inc., San Francisco, CA, USA). The amplified fragments were digested with restriction enzymes, purified using the Qiagex II Agarose Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into pET19B (Novagen, Madison, WI, USA) or pQE41 (Qiagen) vectors (Fig. 1). The presence of cDNA fragments was verified by DNA restriction analysis (33) and DNA sequencing.

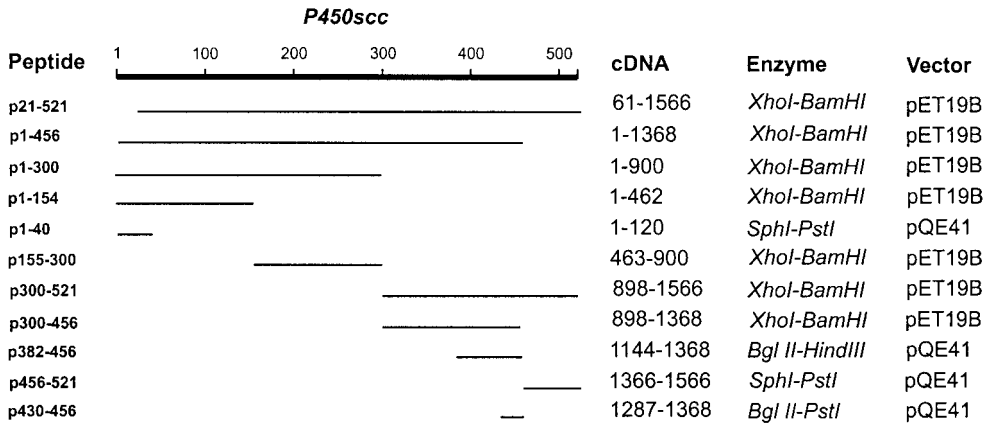
Expression of P450scc polypeptides and preincubation of sera

The pET19B plasmids were expressed in DE3-BL21, and pQE41 constructs in an M15 *E. coli* strain (Qiagen)

Table 1 Characterization of patients with APS-1.

Patient no.	Sex	Age[a]	AD	HP	GF	D	HT	AH	17OH	21OH	No. of sera studied in follow-up[b]
Finnish											
1	M	13	+	+	na	−	−	−	+	+	0
2	F	15	+	+	+	−	−	−	+	+	0
3	F	8	+	+	+	−	−	−	+	+	2 (3)
4	F	20	+	+	+	−	−	−	+	−	5 (10)
5	F	13	+	+	+	−	−	−	−	−	6 (5)
6	M	19	+	+	na	−	−	−	+	+	0
7	M	12	+	+	na	−	−	−	+	+	2 (5)
8	M	17	+	+	−	−	−	−	+	+	3 (3)
9	M	11	+	+	na	+	+	−	−	−	5 (9)
10	M	13	+	+	+	−	−	−	−	−	4 (7)
Sardinian											
11	F	21	+	−	+	−	−	+	−	+	0
12	M	19	+	+	+	−	−	−	+	−	0
13	F	16	+	+	+	−	−	−	+	+	0
14	M	30	+	+	+	−	−	−	−	−	0
15	F	7	+	+	+	−	−	−	−	+	0

[a] Age of the patient (years) at the time of the first serum sample.
[b] Time interval (years) in brackets.
AD, Addison's disease; HP, hypoparathyroidism; GF, gonadal failure; D, diabetes (type 1); HT, hypothyroidism; AH, autoimmune hepatitis; 170H, 210H, Western blot results with P450c17 and P450c21 antigens; na, data not available.



Peptide	cDNA	Enzyme	Vector
p21-521	61-1566	*XhoI-BamHI*	pET19B
p1-456	1-1368	*XhoI-BamHI*	pET19B
p1-300	1-900	*XhoI-BamHI*	pET19B
p1-154	1-462	*XhoI-BamHI*	pET19B
p1-40	1-120	*SphI-PstI*	pQE41
p155-300	463-900	*XhoI-BamHI*	pET19B
p300-521	898-1566	*XhoI-BamHI*	pET19B
p300-456	898-1368	*XhoI-BamHI*	pET19B
p382-456	1144-1368	*Bgl II-HindIII*	pQE41
p456-521	1366-1566	*SphI-PstI*	pQE41
p430-456	1287-1368	*Bgl II-PstI*	pQE41

Figure 1 Schematic diagram of full-length and deletion fragments encoding human P450scc truncated protein. The numbers of cloned cDNAs, and restriction enzyme sites and vectors are given in columns.

using 0.5 mmol/l isopropylthio-β-D-galactoside in 2YT bacterial medium as described (34). The pET19B and pQE41 constructs both express polypeptides with His-tag. In addition, pQE41 gives a fusion protein with mouse dihydrofolate reductase (26 kDa) at the N-terminus of the recombinants. The correct peptide size was analyzed in 10, 12 or 8–16% gradient SDS-PAGE and stained with Coomassie Blue. For pre-incubation of patient sera, empty pET19B and pQE41 plasmids were induced under the same conditions as the recombinant plasmids, and the lysate was used in pre-incubation as described (15).

Immunoblotting assay with P450scc full-length and deletion variants

After separation by SDS-PAGE, the proteins were transferred onto nitrocellulose filters (0.45 μm) (Bio-Rad, Richmond, CA, USA), which were blocked with 3% skimmed milk in TBS-Tween-20 (0.05%) buffer for 1 h at room temperature and shaken with pre-incubated sera (1:200) for 12–16 h at +4 °C (35). The filters were subsequently exposed to anti-human IgG-alkaline phosphatase for 2 h at room temperature (1:2000; Dako, Glostrup, Denmark). A rabbit serum raised against P450scc (kindly provided by Dr O Kämpe, Uppsala University, Sweden) was used as a positive control (1:8000). As another control for the detection of polypeptides, a monoclonal antibody (1:2000) against His-tag (Mab 4D11; NeoMarkers, Union City, CA, USA) was used. Antibody reactions were detected using the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) color development system. The positivity of the reactions was estimated in three categories from + (weak reaction) to +++ (strong reaction). The control sera from healthy persons were used as negative controls without detected reactivity. Construction of plasmid vectors and expression of P450c17 (aa 266–509) and P450c21 (aa 272–503) proteins were done as described (15). The immunoblottings of P450c17 and P450c21 were performed similarly to that with P450scc.

Identification of IgG subclasses

IgG subclasses of the autoantibodies were determined using biotinylated anti-human IgG1, IgG2, IgG3 and IgG4 antibodies (dilution 1:10 000; Sigma Chemical Co., St Louis, MO, USA) as secondary antibodies after incubating sera on filters where p21-521 was used as the antigen. Pre-incubations with *E.coli* lysate were performed as above. Patient sera dilutions (1:200 or 1:125) were incubated with the filter for 12–16 h at +4 °C. Immunoglobulins bound to the antigens were visualized with streptavidin-alkaline phosphatase conjugate (2 μg/ml; Pierce, Rockford, IL, USA) and with NBT/BCIP.

Computer analysis

The similarity predictions of P450 cytochromes were carried out with the LALIGN program (36). A three-dimensional analysis of P450scc peptides was performed using the program RasMol Version 2.6 in Windows software (http://www.umass.edu/microbio/rasmol/makescrp.html) based on the model of bovine P450scc (37).

Results

Mapping of epitopes

The P450scc polypeptides reacted strongly with rabbit antiserum against P450scc but not with the control sera (normal rabbit and healthy human sera). Weak reactivity was observed only with p1-40 signalling peptide (Table 2). Overall, the sera of the Finnish (nos 1–10) and the Sardinian (nos 11–15) patients gave

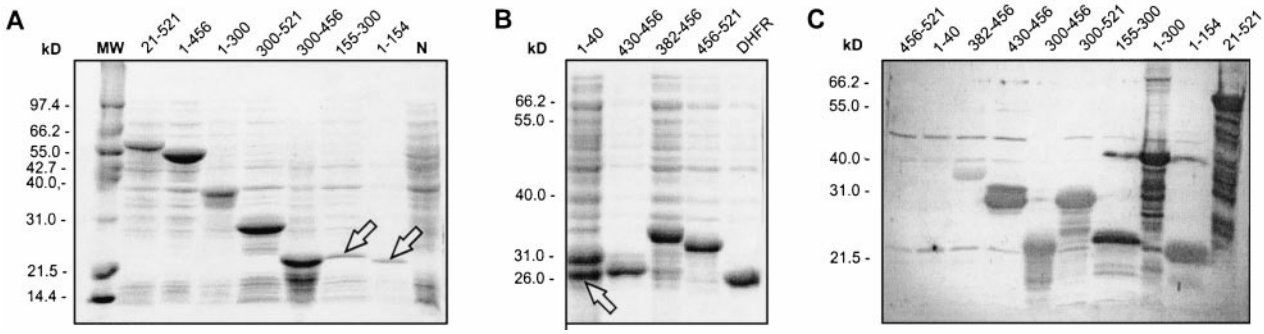


Figure 2 SDS-PAGE and immunoblotting analysis of P450scc fragments in *E. coli*. (A) Eight to 16% gradient gel used for separating P450scc polypeptides expressed in pET19B vector. MW, molecular weight markers; N, negative control (*E. coli* extract with empty pET19B vector). Arrows point to polypeptides p1-154 and p155-300. (B) Twelve percent gel used for separation of P450scc polypeptides expressed in pQE41 vector. DHFR, mouse dihydrofolate reductase (*E. coli* lysate with empty pQE41 vector). Arrow shows a double band routinely given by p1-40. (C) Immunoblot analysis of P450scc polypeptides with Sardinian patient no. 15 serum (1:200).

similar autoantibody reactivity results (Table 2). Most of the sera were positive for the peptides containing both N-terminal and central regions (p1-300, p1-456 and p21-521). However, only N-terminal p1-154 was consistently weaker than longer fragments and even negative with two patient sera. In contrast, a peptide including the central region (p155-300) gave strong positive results with all samples with the exception of one serum (no. 11), which had a low titer of anti-P450scc antibodies to the all peptides studied, and reacted well only with the almost complete P450scc fragment p21-521. Four APS-1 patients with antibodies against P450c17 or P450c21 but without antibodies to P450scc did not react with any of P450scc peptides tested (data not shown).

Interestingly, the most N-terminal part of the protein containing the mitochondrial signalling sequence (p1-40) was negative for all APS-1 sera studied. Similar to the signalling sequence, the most C-terminal peptide fragment (p456-521) also appeared to be negative with all 15 sera. Peptides containing C-terminal region p300-521 were clearly positive with all sera, although the reactivity was slightly weaker with three of them. Considering that the most C-terminal peptide was completely negative, a more truncated peptide, p300-456, was expected to give similar results to that with p300-521; however, this fragment had generally weaker reactivity than p300-521. Relatively weak or negative reactions were also obtained with two shorter peptides, p382-456 and p430-456, from the C-terminal region of the protein. Taken together, the results indicate that, except for the N- and C-terminal parts, the autoantibody epitopes are distributed all over the P450scc protein. The strongest reactivity was in the central (aa 155–300) and C-terminal (aa 300–521) regions of the protein, but the epitopes were also present on N-terminal peptides (aa 1–154). Characteristically, the length of the expressed peptide fragment usually correlated with the strength of the autoantibody reactivity.

Also, we analyzed the serum reactivity of the Finnish and Sardinian patients to two other Addison's disease autoantigens, P450c17 and P450c21. Nine patients were found to have anti-P450c17 antibodies

Table 2 Summary of immunoblotting results with APS-1 sera using recombinant polypeptides of P450scc. The intensity of immunoblotting was graded +++, strong; ++, moderate; +, weak. Nos 1–10 are the Finnish, nos 11–15 the Sardinian patients.

	Patients no.																
aa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	**APS sera** (proportion reaction)	**Rabbit antisera**[a]
21–521	++	+++	+++	+++	+++	+++	+++	++	+++	++	++	++	++	+++	+++	15/15	+
1–456	++	+++	+++	+++	+++	+++	+++	++	+++	++	+	++	++	+++	+++	15/15	+
1–300	++	+++	+++	+++	+++	+++	+++	++	+++	++	+	++	++	+++	+++	15/15	+
1–154	–	++	+++	++	–	+++	+++	++	++	++	+	++	+	+	++	13/15	+
1–40	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0/15	+/–
155–300	++	++	++	+++	+++	+++	+++	++	+++	++	–	++	++	+++	+++	14/15	+
300–521	++	++	+++	+++	+++	++	+++	++	+++	+	+	+	++	++	++	15/15	+
300–456	+	+	+	++	++	++	+++	–	+++	–	–	+	+	–	+	11/15	+
382–456	–	+	+	+	+	++	+	–	–	–	–	+	+	–	+	9/15	+
456–521	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0/15	+
430–456	–	–	+	++	–	+	+	–	–	–	–	–	++	–	++	6/15	+

[a]Rabbit antisera: +, positive, +/– weak positive.

and another nine sera contained anti-P450c21 autoantibodies (Table 1). A slight cross-reactivity of anti-P450scc rabbit control antiserum was also observed with P450c17 protein (data not shown).

Epitope mapping with sera obtained during disease follow-up

Using 11 P450scc polypeptide constructs in immunoblots we did not reveal any changes in epitope reactivity over 3–10 years in sera from the Finnish APS-1 patients. However, weakening of the antibody reactivity over time was common (data not shown).

IgG subclasses of the autoimmune sera

We used anti-human IgG1, IgG2, IgG3 and IgG4 antibodies to determine the IgG subclass specificity of the anti-P450scc autoantibodies. In all 15 patients studied, the autoantibodies against P450scc belonged to the IgG1 subclass as no IgG2, IgG3 or IgG4 reactivities were detected (data not shown).

Computer analysis

According to computer prediction analysis, the mature forms of human and bovine P450scc amino acid sequences are 73.4% identical. A three-dimensional structural model for bovine P450scc has been proposed (37). According to that model, the N-terminal (aa 1–154) and central (aa 155–300) regions of the homologous human protein are located almost entirely on the surface. The C-terminal part of the protein contains two α-helixes (aa 300–345), which lie inside the molecule, a third α-helix (aa 348–362) and the following β-sheets (aa 362–380) that reside on the surface. Amino acids 394–423 represent extended strands and are partly located in the inner molecule, whereas the 429–455 sequence represents mainly β-sheets on the outside of the molecule. The most C-terminal end (aa 456–521) of P450scc is located in the center of the molecule, except for the last ten amino acids that lie on the molecule's surface.

Discussion

In this study we describe linear autoreactive B-cell epitope regions on the P450scc, using sera from Finnish and Sardinian patients with APS-1. The expression of several P450scc deletion constructs enabled us to reveal autoantibody-reacting P450scc epitopes over the whole molecule with the most immunogenic regions on the P450scc to be the central region and C-terminus. No differences were found in the immunological reactivity between the Finnish and the Sardinian sera, and most of the sera had autoantibodies directed to multiple epitopes spread over the protein. Only one serum, no. 11, showed limited reactivity, that could result from immunosuppressive treatment of the patient with azathioprine (31).

The P450scc N-terminal signal peptide (p1-40) was not immunogenic, indicating that the induction of an autoimmune reaction may require a mature posttranslationally processed protein. However, signalling peptides are conserved among proteins, and this might be one reason for such lack of immunogenicity. Also, we did not find epitopes in the highly conserved C-terminal end of the P450scc (aa 456–521) containing the heme-binding site. Comparison of the P450c21 epitope maps (25, 27, 28) with our results suggests that the N-terminus of the mature protein is more immunogenic in the P450scc than in the P450c21. In the P450c17 and the P450c21, most of the antigenic regions reside at the end of the C-terminus (25–28).

A three-dimensional structural model has been proposed for bovine P450scc (37). Analysis of the P450scc molecule structure provided strong evidence of correlation between surface peptide location and antigenic regions. Accordingly, the N-terminus and the central region lie mainly on the surface of the molecule, which correlates closely with the reactivity of patient sera with p1-154 and p155-300 fragments. As the p300-456 fragment had stronger reactivity with patient sera than did the p382-456 fragment, it is plausible that this is mostly because of the region between aa 300 and 382, which is on the protein surface. Furthermore, the conserved region at the end of the C-terminus (aa 456–521), that was negative in our immunoblot results, lies in the inner area of P450scc.

We also wanted to find whether epitope spreading occurs in APS-1. Analyzing consecutive sera taken over a 3–10 year follow-up of seven Finnish patients, we observed the epitope pattern remained stable. In all the patients, we detected P450scc autoantibodies of the IgG1 subclass, although we cannot entirely exclude the presence of low levels of autoantibodies of other IgG subclasses. These findings together suggest that in APS-1 the autoantibodies against P450scc could be formed at an early stage of the autoimmune attack, associated with a strong propensity towards quick epitope spreading with restricted IgG1 isotype development. To prove this possibility, more patients with initial stages of APS-1 should be immunologically followed up.

We conclude that patients with APS-1 have autoantibodies to multiple linear epitopes from aa 40 in the N-terminus up to aa 456 on the C-terminus of the P450scc, but the strongest immunogenicity is directed towards the central region (aa 155–300) and towards the C-terminus (aa 300–521). Although the predominant AIRE mutations are different among Finnish (R257X) and Sardinian (R139X) APS-1 patients (4, 6, 7), the patients seem to have similar P450scc epitope region pattern. No epitope spreading in P450scc protein appears to occur over the course of disease.

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