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Autoimmune Addison's disease

Analysis of autoantibody binding sites on human steroid 21-hydroxylase

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Human steroid 21-hydroxylase (21-OH) expressed in an in vitro translation system was found to react specifically with adrenal autoantibodies from patients with Addison's disease. The epitopes on 21-OH which reacted with autoantibodies were studied by incorporating a series of terminal and internal deletions into the 21-OH gene and analysing the expressed proteins by Western blotting. N-Terminal deletions up to amino acid 280 had no effect on autoantibody binding whereas a series of C-terminal deletions and truncations (amino acids 281–494) showed marked effects. Our results indicate that a central segment (281–379) and a C-terminal segment (380–494) of 21-OH interact to form at least one major autoantibody binding site.

21-Hydroxylase; Addison's disease; Adrenal autoantibody; Autoantibody epitope

1. INTRODUCTION

Recent studies have shown that steroid 21-hydroxylase (21-OH) is a major autoantigen in adult onset autoimmune Addison's disease [1–3]. In order to understand the molecular basis of adrenal autoimmunity it is necessary to characterise the autoantigenic epitopes on 21-OH. Consequently, we have prepared a series of deletions and truncations in the 21-OH gene, expressed the corresponding proteins and analysed their interactions with adrenal autoantibodies.

2. MATERIALS AND METHODS

2.1. *Patients*

Sera were obtained from 11 patients with adult onset autoimmune Addison's disease. All the sera contained autoantibodies to 21-OH and were part of the Addison sera panel we have described previously [1]. Sera were also obtained from 3 Hashimoto's disease patients, 3 Graves' disease patients, 3 rheumatoid arthritis patients and 9 healthy laboratory staff.

2.2. *Plasmid construction*

Various in-frame deletions and truncations of the 21-OH gene were carried out by cutting at different restriction enzyme sites. The modified genes were cloned into pYES expression vectors and expressed in vitro as described below.

The full-length 21-OH gene was cloned into pYES2 as described previously [1] and the resulting construct defined as p21-OH1 (Table I). The restriction enzyme sites used to prepare the various 21-OH gene modifications are shown in Table I and Fig. 1. In the case of construct p21-OH11, an extension coding for 12 homologous amino acids was added at the *Stu*I site (Table I).

Truncated and deleted versions of the 21-OH gene were cloned into pYES3, a derivative of the yeast expression vector pYES2 (Invitrogen). The vector pYES3 was constructed from pYES2 by cloning a linker into the *Eco*RI and *Sph*I sites of the multiple cloning site of pYES2. The unique linker, prepared by annealing two oligonucleotides (5′-AATTCCCTGAGGCCACGTGACTGACTGAGCATG-3′ and 5′-CTCAGTCAGTCACGTGGCCTCAGGG-3′) was designed to include *Sau*I and *Bbr*PI restriction sites for cloning and a series of TGA stop codons which ensured translation termination of all the truncated 21-OH genes.

2.3. *In vitro transcription/translation*

The various 21-OH gene constructs (Table I, Fig. 1) were transcribed and translated in a rabbit reticulocyte lysate system using a Promega TnT transcription/translation kit. Synthesis of proteins coded for by the various constructs was assessed by including [^{35}S]methionine in the transcription/translation reactions. The labelled proteins were then run on acrylamide gels (9%) in SDS and analysed by autoradiography.

Reactions without added [^{35}S]methionine were analysed on SDS-PAGE followed by Western blotting using 21-OH autoantibodies. In addition, Western blotting was carried out with a rabbit antibody to 21-OH (1,3,4) kindly provided by Dr. Bon-chu Chung.

2.4. *Expression in yeast*

Expression of full-length 21-hydroxylase in *Saccharomyces cerevisiae* and preparation of yeast extracts was as described previously [1].

3. RESULTS

When different constructs were expressed in the in vitro transcription/translation system in the presence of [^{35}S]methionine, labelled recombinant proteins of the expected molecular weights (32 kDa–55 kDa) were detected in every case (Fig. 2a, Table I). In addition, Western blot analysis indicated that all but 2 of the recombinant proteins (from constructs p21-OH4 and p21-OH5) reacted with rabbit antibodies to 21-OH (Table I, Fig. 2b).

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Table I

Reactivity of full-length and modified human steroid 21-OH with Addison's sera and rabbit antibody

Construct	Modified 21-OH				Reaction with 21-OH antibody	
	Enzyme sites used	Amino acids deleted	Region of deletion	Mol. wt** (kDa) of expressed protein	Addison sera (proportion reacting)	Rabbit Antibody
p21-OH1	None	None	None	55	(11/11)	+
p21-OH13	*NarI/Eco*RI	15–162	N-terminal	42	(11/11)	+
p21-OH11	*Stu*I (linker)*	460–494	C-terminal	48	(5/11)	+
p21-OH2	*Stu*I	448–494	C-terminal	48	(4/11)	+
p21-OH3	*Bst*XI	418–494	C-terminal	46	(4/11)	+
p21-OH4	*Sau*I	381–494	C-terminal	43	(1/11)	−
p21-OH5	*Bbr*PI	282–494	C-terminal	32	(0/3)	−
p21-OH6	*PvuII/PvuII*	142–240	Internal	45	(5/5)	+
p21-OH10	*PvuII/Bbr*PI	142–280	Internal	39	(11/11)	+
p21-OH7	*Eco*RI/*Sau*I	165–379	Internal	34	(0/11)	+
p21-OH12	*Sau*I/*Bst*XI	382–414	Internal	48	(7/11)	+

*Oligonucleotide linker coding for 12 amino acids of 21-OH additional to the truncation created at the *Stu*I site.
**Mol. wt. of 21-OH was determined by SDS-PAGE of in vitro translation reactions.

Analysis of autoantibody binding to full-length and modified recombinant 21-OH is summarised in Table I.

Autoantibodies in all 11 Addison sera studied reacted with full-length 21-OH expressed in vitro (construct p21-OH1, Table I and Fig. 2c, lane 6). Furthermore, an N-terminal deletion of amino acids 15–162 (construct p21-OH13) had no effect on autoantibody binding (Table I). In addition, 2 deletions of amino acids (142–240 and 142–280; constructs p21-OH6 and p21-OH10) overlapping with this region did not influence autoantibody binding (Table I and Fig. 2c, lane 5).

Initial experiments with a C terminal truncation (construct p21-OH5) indicated that 3/3 sera strongly positive for 21-OH autoantibodies were unable to recognise 21-OH with amino acids 282–494 deleted (Fig. 2c, lane 4 and Table I).

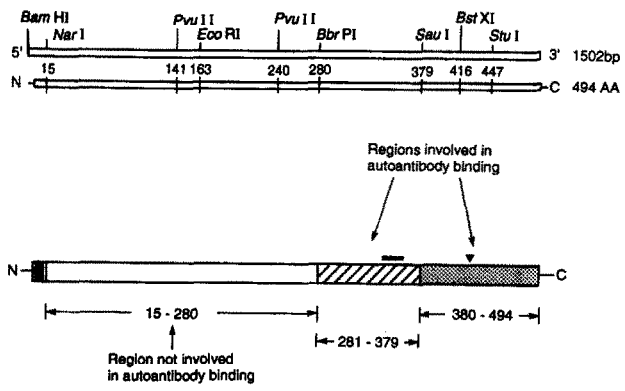

Fig. 1. The restriction enzyme map and amino acid sequence of human steroid 21-OH are shown in schematic form. ▨, Signal peptide (amino acids 1–14 are part of the *STE2* leader peptide [1]). Regions involved in autoantibody binding: ▩, segment 1; ▩, segment 2; □, region not involved in autoantibody binding; ■, presumptive substrate-binding site; ▼, cysteine at position 428 involved in haem-binding.

These studies were then extended to include all 11 sera and a series of C-terminal truncations and deletions. The results were as follows:

(a) None of the 11 sera reacted with 21-OH with amino acids 165–379 deleted (construct p21-OH7).

(b) Only 1 out of 11 sera reacted with the protein truncated at amino acid 381 (construct p21-OH4).

(c) 4 out of 11 sera reacted with 21-OH truncated at amino acids 418 or 448 (constructs p21-OH3 and p21-OH2, respectively).

(d) 5 out of 11 sera reacted with the protein truncated at amino acid 460 (construct p21-OH11). These 5 sera included all the 4 sera which reacted with 21-OH truncated at amino acid 448.

(e) 7 out of 11 sera reacted with 21-OH with amino acids 382–414 deleted (construct p21-OH12).

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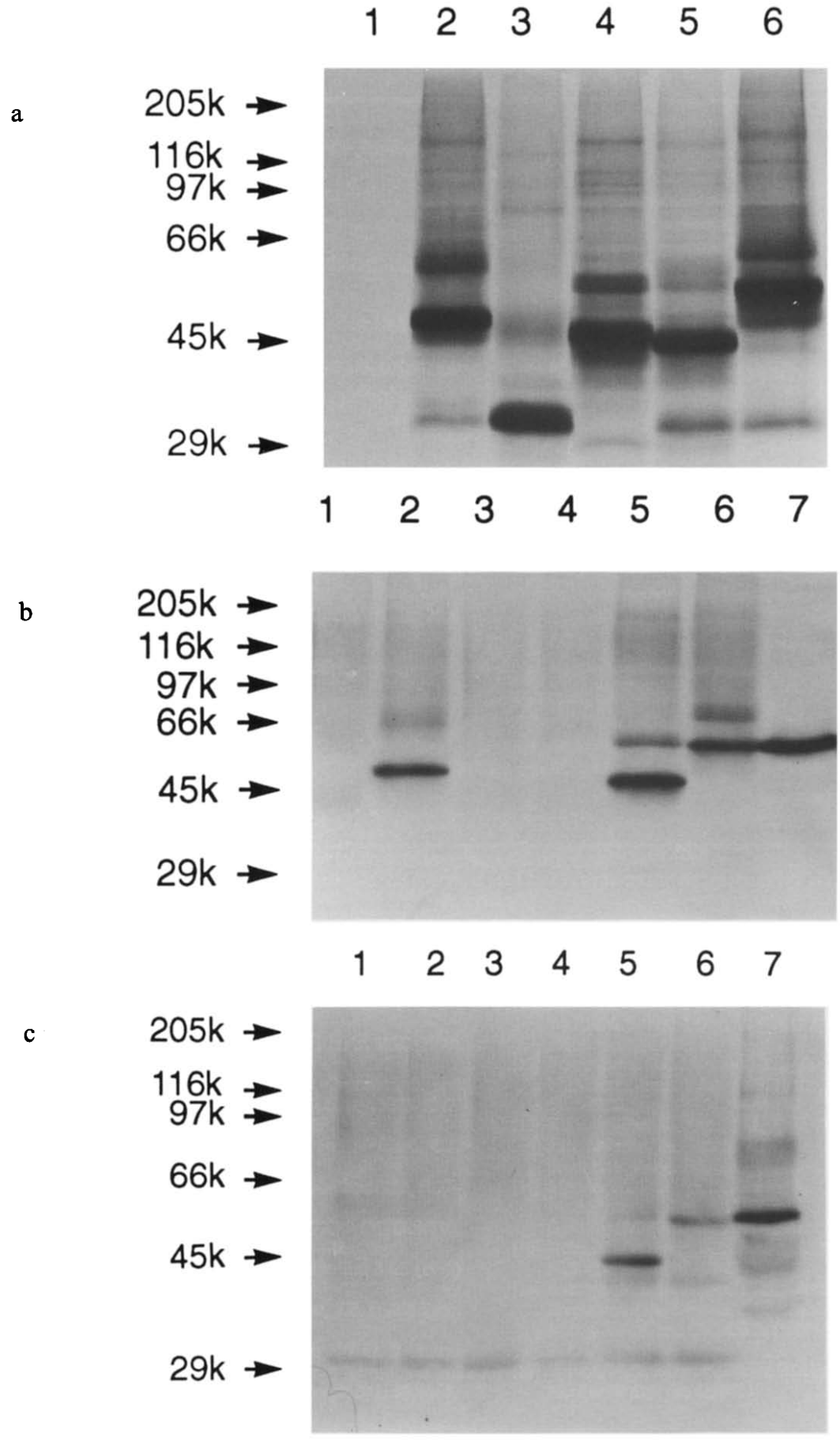
Fig. 2. Analysis of full-length and modified human steroid 21-OH produced using an in vitro translation system. (a) [^{35}S]Methionine-labelled reactions analysed by SDS-PAGE and autoradiography. pYES3 (vector only) (lane 1), p21-OH2 (amino acids 448–494 deleted) (lane 2), p21-OH5 (amino acids 282–494 deleted) (lane 3), p21-OH6 (amino acids 142–240 deleted) (lane 4), p21-OH4 (amino acids 381–494 deleted) (lane 5), p21-OH1 (full-length) (lane 6). (b) Reactions analysed by SDS-PAGE and Western blot with rabbit anti-human 21-OH antibody. pYES3 (vector only) (lane 1), p21-OH2 (lane 2), p21-OH4 (lane 3), p21-OH5 (lane 4), p21-OH6 (lane 5), p21-OH1 (lane 6), p21-OH1 expressed in yeast (lane 7). The ECL system (Amersham International) was used to detect antibody reactivity. (c) Reactions analysed by SDS-PAGE and Western blot (ECL system) with pooled serum from 4 Addison's patients. Results obtained when Western blotting was performed with 11 individual Addison's sera are summarised in Table I. pYES3 (vector only) (lane 1), p21-OH2 (lane 2), p21-OH4 (lane 3), p21-OH5 (lane 4), p21-OH6 (lane 5), p21-OH1 (lane 6), p21-OH1 expressed in yeast (lane 7). No specific reactions were observed with normal rabbit serum, normal human sera or sera from 3 Hashimoto's, 3 Graves' or 3 rheumatoid arthritis patients (data not shown).


a
1 2 3 4 5 6
205k
116k
97k
66k
45k
29k
b
1 2 3 4 5 6 7
205k
116k
97k
66k
45k
29k
c
205k
116k
97k
66k
45k
29k

4. DISCUSSION

Our results show that 21-OH expressed in the in vitro transcription/translation system binds 21-OH autoantibodies.

All sera tested reacted with the protein modified by N-terminal deletions: between amino acids 15–162, 142–240 and 142–280 (Table I and Fig. 2c, lane 5). This indicates that amino acids 15–280 are not important for autoantibody binding to 21-OH.

None of the sera tested reacted with 21-OH modified by deletion of amino acids 165–379 (Table I). As this deletion overlaps (from amino acids 165–280) with the deletions which are not required for autoantibody binding (see above), it could be concluded that the segment between amino acids 281 and 379 (segment 1, Fig. 1) is important for autoantibody binding.

However, only one serum reacted with the 21-OH protein truncated at amino acid 380. In the case of the other sera, varying stretches of the C-terminal portion (amino acids 380–494; segment 2, Fig. 1) were also required for autoantibody binding (Table I). This suggests that amino acids in segment 2 are also important for autoantibody binding. As none of the sera reacted with segment 2 in the absence of segment 1 (see above) and all but one of the sera needed segment 2 for binding, it seems likely that segment 2 co-operates with segment 1 in forming at least one major autoantibody binding site. As 2 distinct regions of 21-OH co-operate to form this site, it would appear to be conformational in nature. In the case of the one serum which reacted with segment 1 without the need for a contribution from segment 2, the autoantibodies may be directed to a linear epitope in segment 1 or a conformational epitope in segment 1 which does not depend on segment 2.

One of the recombinant 21-OH proteins which reacted with some of the autoantibodies did not react with rabbit 21-OH antiserum. Furthermore, the rabbit antiserum bound to one of the modified proteins which was not recognised by autoantibodies (Table I) indicating that the antiserum contained antibodies to different epitopes than those recognised by the autoantibodies.

The nature of autoantibody binding sites tends to be conformational; for example, in the case of the thyroid autoantigens thyroglobulin and thyroid peroxidase [5–7]. Although they appear conformational, the major autoantibody binding sites on 21-OH are sufficiently robust to reform after electrophoresis in SDS under reducing conditions. This probably reflects the special renaturing treatment of the blotted proteins which we used prior to interaction with autoantibody [8]. As the adrenal autoantibodies react with reduced 21-OH, the formation of the major autoantibody binding sites does not appear to be dependent on disulphide bridge integrity.

It is of interest that the 2 segments of 21-OH shown to be important for autoantibody binding are also important for 21-OH enzyme activity. In particular, the region 342–358 within segment 1 contains the substrate-binding site [9] and the cysteine at 428 (within segment 2) is a part of the haem-binding site (Fig. 1) [10].

Our results with 21-OH can be compared with a very recent report on autoantigenic epitopes on glutamic acid decarboxylase (GAD) a major autoantigen in diabetes [11]. In the case of GAD a central region and a C-terminal region of the molecule are involved in forming 2 major conformational epitopes. Interestingly, the C-terminal region of GAD is also important for enzyme activity [11].

Overall our studies indicate that while the N-terminal half of 21-OH is not involved in autoantibody binding; a central and a C-terminal region interact to form at least one major conformational autoantibody binding site.

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