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Epitope mapping of human thyroglobulin reveals a central immunodominant region

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Thyroglobulin is the thyroid hormone precursor and the major antigen frequently involved in autoimmune diseases. The primary structure of human thyroglobulin is known but the spatial structure remains largely undetermined. By using fusion protein produced in prokaryotic system we have characterized seven short immunoreactive peptides carrying at least one epitope. None of them includes hormonogenic sites, but five are concentrated in the central part of the monomeric molecule, which thus emerges as the major immunogenic region of this protein.

Expression vector; Fusion protein; Immunoscreening; Thyroglobulin; Three-dimensional structure

1. INTRODUCTION

Thyroid hormone synthesis depends mainly on the presence of a normal thyroglobulin (Tg) in the thyroid gland [1]. The primary structure of this hormone precursor was established a few years ago [2]. Many attempts to elucidate its three-dimensional structure have not yet led to complete definition. Electrophoresis in denaturing conditions, chemical and enzymatic proteolysis, electron microscopy, and isolation of a messenger RNA have established that Tg is a globular protein composed of two identical subunits. The large size of this glycoprotein has, to date, precluded crystallization and analysis by X-ray diffraction methods or by NMR technique. All data on Tg structure point to a globular structure with many disulfide bonds, except the N-terminal part which contains few hemi-cystine residues. Similarly, the hormonogenic sites were located near the two extremities of the monomer. This has been correlated with a higher degree of flexibility of these parts of the molecule. Human thyroglobulin is a glycoprotein containing about 30 carbohydrate units per molecule. The localization and the role of these oligosaccharidic chains are largely unknown. In other respects, Tg is the major antigen of the thyroid gland. About forty epitopes are suspected to be present on the molecule but their localization remains unknown [3]. Using a plasmid expression library of cDNA fragments coding for the entire hTg messenger, we have characterized an immunodominant region, far from the hormonogenic area, which may correspond to a part of the molecule which is especially exposed at the surface.

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2. MATERIALS AND METHODS

2.1. *Fusion constructs*

The ten hTg cloned cDNA representing the totality of hTG messenger were previously inserted in *Pst*I site of pBR322 plasmid except clone M6 which was a cloning in pUC18 plasmid [1]. An equimolar mixture of these ten DNA preparations was digested by *Pst*I endonuclease and the digest product was cloned in appropriate pEX plasmid [4]. pEX vector was an equimolar mixture of the three forms of this plasmid, thus allowing expression of inserted cDNA fragments in the three reading frames. An expression library was thereby constituted by transfection in the pOp2136 *E. coli* strain [7]. The clones selected during the first immunoscreening were characterized by restriction mapping. Shorter cDNA fragments were subcloned in appropriate pEX plasmid, thus allowing synthesis of shorter peptides. All constructs were confirmed by DNA sequencing [5].

2.2. *Antibodies*

Polyclonal antibodies were produced by rabbit immunization according to Waltukaitis [6] and the IgG fraction was purified by ammonium sulphate precipitation and DEAE-cellulose ion exchange chromatography. Antibodies against the surface antigens were selected by chromatoaffinity using nondegraded hTg coupled with CNBr-activated Sepharose CL4B. The nondegraded structure of hTg was verified in SDS-PAGE. The concentration of immunopurified anti-hTg was 490 μg/ml.

2.3. *Expression of fusion proteins and immunoscreening*

About 200 colonies were plated on a nitrocellulose disk, grown at 32°C on LB agar-ampicillin medium, and expression of fusion protein was induced by a 2 h incubation at 42°C which inactivated the cI857 repressor. Nitrocellulose disks were exposed to chloroform vapor for 30 min, incubated in lysis buffer (Tris 50 mM, pH 7.5, NaCl 150 mM, $MgCl_2$ 5mM, 3% BSA, DNase I 1 μg/ml and lysozyme 40 μg/ml) for 4 h, and rinsed in TBS (Tris 50 mM, NaCl 150 mM, pH 7.5). They were incubated during 2 h at room temperature with rabbit

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anti-hTg antibodies diluted 1/1000 in TBS, 3% BSA, 1% bacterial lysate (obtained from pOp2136 *E. coli* transformed with wild-type pEX plasmid). The positive immunoscreened colonies were revealed by peroxidase-conjugated anti-rabbit antibodies and 4-chloronaphthol as chromogen.

2.4. *Electrophoresis and immunoblotting*

Fusion protein was extracted by sonication from bacterial culture after induction of protein expression. SDS-PAGE was performed according to Laemmli [8], and Western blotting by using the method of Towbin et al. [9]. Immunoreactive bands were revealed as described above.

RESULTS AND DISCUSSION

All the *PstI* cDNA fragments stemming from the ten cDNA clones overlapping the hTg message were cloned in pEX plasmid and constitute an expression library of human Tg. Twenty-one fusion proteins containing an hTg peptide represented the totality of this thyroid protein. The library was screened with polyclonal anti-hTg antibodies recognizing the surface of the native protein. Five different clones were selected during the first screening, and unambiguously characterized by their restriction maps (Fig. 1). Each of the positive clones was cleaved by one or more restriction enzyme and each DNA fragment was subcloned in het appropriate pEX plasmid. All the new constructions were screened under the same conditions as in the first screening, the shorter immunoreactive peptides were thereby selected in this manner. Seven 38-to 104-amino acids long immunoreactive peptides were characterized. Results are reported in Table I. Each peptide contained at least one epitope. These immunoreactive peptides were unevenly distributed on the hTg monomeric molecule. One of them was close to the N-terminus, another was near the C-terminal part of the molecule on the region presenting a homology with acetylcholinesterase, but the five other antigenic areas were concentrated in the middle of the hTg molecule. No structural or functional feature was linked to this dominant immunogenic region. The antibodies used during the immunoscreening were purified by chromatoaffinity using nondegraded hTg. We thereby selected the antibodies directed against the surface of the molecule. Moreover, it is classically admitted that the epitopes of a globular protein are located at the surface of the molecule at regions called 'protruding' [10-12]. A previous report proposed a structural model for the hTg molecule. This hypothesis stemmed from enzymatic action on the Tg molecule; the action of proteases allows one to define the areas of the Tg molecule which are accessible to these enzymes. These regions overlap with the epitope-bearing regions characterized in the present work. Noting the similarity of areas accessible to enzymatic and immunological actions, we can accept the three-dimensional model suggested, except the possibility of internal duplication which is invalidated by present knowledge of the primary structure. Surprisingly, the hormogenic peptides were not selected through immunoscreening although these sites should be accessible to external interactions. In fact, one epitope is close to the major hormonogenic site near the N-terminus. The C-terminal part of the molecule carrying the three other sites contains few cysteine residues and has been described as presenting a high level of accessibility [4]. In fact, accessibility is not the only feature acting upon hormonosynthesis. It is a complex process which requires iodine supply, peroxidase action and coupling of two tyrosine residues. The dimeric structure of the molecule is a necessary condition for hormonosynthesis, which is possibly an interchain process. Otherwise, the synthesis of fusion protein in prokaryotic system cannot restitute the totality of the epitopes of the molecule. Some

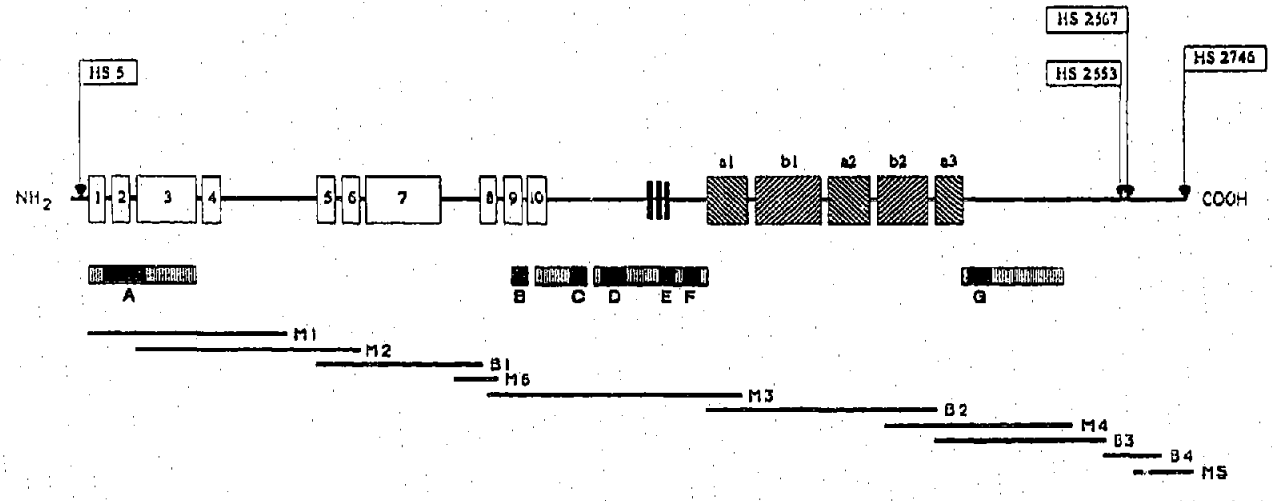

Fig. 1. hTg monomer is represented above with the three types of internal homologies previously described [2] (blank, black and hatched boxes) and with the localization of hormonogenic sites (HS) named by their position on peptide chain (arrows). The immunoreactive peptides are reported underneath by gray boxes for clones isolated during the first screening and by black boxes for shorter immunoreactive peptides characterized after subcloning (called A to G). The bottom of the figure presents the ten initial hTg cDNA clones.

Table I

Shorter immunoreactive peptides isolated after subcloning and characterized by their epitopes

Epitopes	Boundaries of cDNA fragment (5'-3')	Length of cDNA (bp)	Position on hTg cDNA (bp)	Length of peptide (aa)	Position on hTg chain (aa)
A	*Bgl*II-*Mbo*I	314	249-562	104	84-187
B	*Pst*I-*Pst*I	155	3294-3448	53	1097-1149
C	*Sma*I-*Pst*I	112	3773-3884	38	1258-1295
D	*Mbo*I-*Mbo*I	188	3936-4123	62	1313-1374
E	*Xma*I-*Ball*	162	4359-4520	54	1454-1507
F	*Ball*-*Mbo*I	160	4520-4680	53	1508-1560
G	*Alu*I-*Alu*I	220	6519-6728	69	2174-2242

features of the three-dimensional structure are restored but not all, e.g. glycosylation and dimeric constitution. It is not obvious that only sequential epitopes are characterized by this methodology [15]. Nevertheless the polyclonal antibodies produced against nondegraded hTg do specifically recognize seven peptide segments expressed in a prokaryotic system. These seven antigenic sites are essentially concentrated in the middle of the monomeric hTg molecule. Other antigenic regions may be present but our methodology does not allow their detection.

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