A Conformational B-cell Epitope on the C-terminal End of the Extracellular Part of Human Thyroid Peroxidase∗

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To investigate the B-cell autoimmune epitopes on human thyroid peroxidase (TPO), we generated proteolytic peptides by enzymatic hydrolysis of TPO in non-denaturing and nonreducing conditions. The hydrolysate was chromatographed on a reverse phase column. We eluted a material immunoreactive with both a TPO monoclonal antibody recognizing a linear epitope (mAb47, amino acid 713–721) and TPO autoantibodies (aAb) from patients. The aAb immunoreactivity, but not that of mAb47, was lost after reduction. Western blots after electrophoresis without reduction showed that the aAb and mAb47 were immunoreactive with a 66-kDa band and that aAb identified a doublet at 20 kDa. For electrophoresis under reducing conditions, the 66-kDa band resolved into two peptides of 40 and 26 kDa, whereas the doublet at 20 kDa remained unchanged. None of these reduced peptides was immunoreactive with aAb, whereas the 40-kDa peptide was immunoreactive with mAb47. The 40-kDa peptide extends from amino acid 549 to 933 of TPO, and its last 192 amino acids overlap the autoimmune 20-kDa peptide. After iodine labeling, the 20-kDa peptide lost its immunoreactivity. We conclude that the C-terminal end of the extracellular part of TPO, which includes all the tyrosine residues of the 20-kDa peptide, contains at least one conformational B-cell epitope involved in autoimmune thyroid diseases.

Thyroid peroxidase (TPO)1 is a membrane-bound enzyme that faces the colloid and that acts at the apical pole of the thyrocytes. TPO catalyzes the iodination of thyroglobulin and the coupling of some iodotyrosine residues to form thyroid hormone residues. This catalysis is under thyrotropin control through its specific receptor (1, 2). Like thyroglobulin and the thyrotropin receptor, TPO is one of the main autoantigens (aAg) in autoimmune thyroid disease (AITD). However, the immune response to this sequestered aAg is not clear (for review, see Ref. 3). After TPO was identified as microsomal aAg (4, 5), many studies investigated the human immune response to this enzyme. We showed there were two autoimmune domains on the surface of the molecule (6); this was confirmed by another group (7, 8). Disappointingly, no difference was observed in autoantibody (aAb) response to TPO for patients with Graves’ disease and Hashimoto’s thyroiditis, the two well defined AITD (9).

One of the major tasks in AITD is to identify the immunodominant B-cell epitopes of the main aAg. This may help explain the mechanisms causing immunopathological states and, consequently, may provide targets for diagnosis and therapeutic strategies. TPO is a valuable model for such studies, given the preponderance of its corresponding aAb in AITD. Moreover, TPO is implicated in the physiological function of the thyroid, and aAb binding to TPO might impair thyroid hormone synthesis through cytotoxic processes (10), thus leading to hypothyroidism. Some authors (11–14) but not others (5, 15) claimed that TPO aAb inhibit the catalytic activity of the enzyme at the iodine and the aromatic sites. However, aAb from patients with thyroiditis may block the enzyme function by binding to epitopes different from the enzymatic sites, as speculated for bispecific thyroglobulin and TPO aAb (16).

Many attempts to localize and identify the main TPO B-cell autoantigens forming the immunodominant regions targeted by the pathologic aAb from patients with AITD. Various linear TPO epitopes were identified through cDNA sublibraries or recombinant bacterial proteins. Others, however, using a eukaryotic expression system to preserve the three-dimensional structure of the protein, claimed that TPO B-cell autoantigens are conformational (for review, see Refs. 17–19). Since molecular biology techniques identify only minimum peptides not truly representative of the conformational B-cell epitopes, we mapped the immunodominant region of TPO by an alternative approach: proteolytic peptides generated by enzymatic hydrolysis of the native immunopurified human TPO. Through pilot experiments, we selected endopeptidase Lys-C, which can cleave at 26 lysine residues along the amino acid sequence of TPO. We found an extracellular conformational B-cell epitope susceptible to reduction and iodination near the membrane anchorage and the spanning region of TPO.

EXPERIMENTAL PROCEDURES

Murine mAb to TPO and aAb from Patients with AITD—We used eight TPO mAb, previously produced and characterized, directed to epitopes from two immunodominant antigenic regions of TPO (6). The TPO aAb were immunopurified as described (20) from sera of 40 adult patients thought to have AITD on the basis of clinical examination and selected for their high titer in TPO aAb as assessed by Dynotest (BRAHMS diagnostica, Berlin, Germany). The TPO mAb and aAb were titered for their TPO reactivity in ELISA and used at saturating dilution in this work.

Purification and Hydrolysis of TPO—TPO was immunopurified from sodium deoxycholate-solubilized microsomes from Graves’ thyroid tissue (4). Native, purified TPO (at a final concentration of 3 mg/ml) was treated with endoproteinase Lys-C (EC 3.4.21.50) sequencing grade proteins.
from *Lysobacter enzymogenes* (Boehringer Mannheim, Germany) at an enzyme to substrate ratio of 1:100 (w/w) in 50 mM NH₄HCO₃ with 10% acetonitrile for 18 h at 37 °C. Enzymatic digestion was stopped by freeze-drying the hydrolysate.

**Reverse Phase HPLC**—The lyophilized hydrolysate of TPO was re-stored with ultrapure water containing 0.1% trifluoroacetic acid and 20% acetonitrile (starting buffer). For each run, 250 µg of material was loaded onto a C-18 reverse phase 3.9 × 150 mm column (Waters, Millipore, Milford, MA) equilibrated in the starting buffer. Five min after starting, a linear gradient of 20–90% acetonitrile was applied for 60 min at 0.5 ml/min. The column was then reequilibrated with the starting buffer for 30 min. Elution of the peptides was monitored by absorbance reading at 215 nm. Fractions (0.5 ml) were collected in silicone-coated glass tubes. The fractions from runs corresponding to the same peak were pooled and freeze-dried.

**ELISA**—ELISA was used to detect the immunoactive peptides from HPLC fractions recognized by TPO mAb and aAb. Briefly, wells of Immulon II microtiter plates (Dynatech, Chantilly, VA) were filled with 100 µl of HPLC fractions adjusted to 5 µg/ml in PBS, pH 7.3, overnight at 4 °C under humidified atmosphere. The wells were then washed, overcoated with BSA, washed again, and filled with a saturating amount of TPO mAb or aAb in PBS, 0.1% Tween-20, 1% BSA. After 2 h at 37 °C, unbound antibodies were removed by extensive washing. mAb and aAb bindings were detected by an antimouse or antihuman second antibody labeled with alkaline phosphatase; *p*-nitrophenyl phosphate was the substrate. Absorbance was read at 405 nm. HPLC fraction 3–4 and native TPO were also tested in ELISA after chemical treatments. The lyophilized samples (10 µl) were restored in 50 mM NH₄HCO₃, 0.001% SDS by the same apparatus. Next, they were concentrated and dialyzed against methanol and ultrapure water by a centrifuge concentrator (Amicon, Beverly, MA). The salt-free isolated peptides were placed in PicoTag hydrolysis tubes (Waters, Millipore) and freeze-dried. The peptides were hydrolyzed in vapor phase with 6 M HCl, under vacuum, at 110 °C for 24 h. Amino acid compositions were determined from phenylisothiocyanate-derived amino acids by reverse phase-HPLC separation (Waters, Millipore). Amino acid sequences were determined by a computer program (22) that identifies a proteolytic peptide of a protein through the sequence of the protein and the amino acid composition of the peptide. We used the TPO sequence reported by Magnusson et al. (23). We also entered the apparent molecular weight of the peptides, estimated from the tricine SDS-PAGE, to calculate the amino acid percentages and to compare them with the experimental results. The program kept the best fitted peptide on the basis of a least squares method.

**Sequence Determination**—The amino acid sequence of the 20-kDa peptide was determined on material electroeluted from tricine SDS-PAGE as above and then blotted on a PVDF membrane in a Prospin cartridge (Applied Biosystems, Foster City, CA). The NH₂-terminal sequence was analyzed in an Applied Biosystem Procise Sequencer at the Pasteur Institute (Paris, France).

**Peptide Labeling and Radioimmunoassay**—Peptides from HPLC fraction 3–4 were labeled with ¹²⁵I-Na by the chloramine-T method. Briefly, 10 µg of material was mixed with 5 µl of ¹²⁵I-Na (500 µCi) and 10 µg of chloramine T in 200 mM sodium phosphate buffer pH 7.2. After 1 min, the reaction was stopped by adding 20 µg of Na₂S₂O₅. The labeled peptides were separated by gel filtration through a Superdex 75 column (Pharmacia Biotech Inc., Uppsala, Sweden) equilibrated with PBS, pH 7.3, containing 0.1% BSA and 0.02% NaN₃. The fractions collected from the column were analyzed for their ¹²⁵I-peptide content by Tricine SDS-PAGE in nonreducing condition (5,000 cpm/fraction). The gel was then scanned with a phosphoimager (FujiXBas1000, Japan) equipped with a Tina 2.09 computer program (Raytest, Courbevoie, France). The column fractions were tested for...
immunoreactivity by a solid phase radioimmunoassay (6). Briefly, Startubes (Nunc, Roskilde, Denmark) were coated with purified TPO aAb or mAb47, overcoated with BSA, and incubated with 100,000 cpm of 125-I-peptides. After extensive washing, the radioactive material bound to the tube was counted.

**Protein Assay**—The protein contents of the native TPO preparation and of the HPLC fractions of hydrolyzed TPO were estimated by Picotag amino acid analysis (Waters, Millipore) as above.

**RESULTS**

**Proteolytic Peptides from TPO**—To obtain relevant peptides, we cleaved native TPO with endoproteinase Lys-C. The hydrolysis products were submitted to reverse phase-HPLC. The elution profile resolved into seven major peaks and many minor ones, with a total protein recovery of 50% (Fig. 1). Peak 0 was free of protein; it was due to an air bubble entrapped during the injection. For convenience, fractions corresponding to peaks 3 and 4 were pooled and named fraction 3+4. Nonhydrolyzed, native TPO eluted as one major peak corresponding to fraction 7 at the end of the acetonitrile gradient (data not shown). The peptides in the HPLC fractions were further analyzed by Tricine SDS-PAGE. In native conditions, various bands were obtained from fractions 2 to 6 (Fig. 2A). The most heterogeneous fraction was fraction 3+4, yielding bands from 3.5 to 66 kDa. Fraction 1 showed no band, suggesting that its peptides were less than 3.5 kDa. Fraction 7 and, to a minor extent, fraction 6 showed a major band at 110 kDa, corresponding to poorly hydrolyzed TPO. Submitted to reduction, most of the bands from the fractions remained unchanged (Fig. 2B). A few were modified, thus yielding bands with lower molecular mass, e.g. the 66-kDa band in fraction 3+4 yielded a 40- and a 26-kDa band. These modifications resulted from the presence, in native conditions, of TPO peptides linked by disulfide bridges.

**Immunoreactivity of TPO mAb and aAb with TPO Peptides**—By ELISA, we searched for relevant peptides containing TPO epitopes in the HPLC fractions. Fractions were tested in native conditions with mAb and aAb directed to potential TPO autoepitopes. Native, nondegraded TPO served as control to ensure all TPO antibodies were in saturating conditions (Fig. 3). Fractions 6 and 7 were the most frequently recognized by the panel of antibodies. Among the remaining fractions, fraction 3+4 was the most immunoreactive with TPO mAb47 and aAb. To identify the immunoreactive peptides, we tested the HPLC fractions by Western blot with mAb47 and aAb as specific reagents. From native Tricine SDS-PAGE, mAb47 (Fig. 4A) and aAb (Fig. 4B) both identified a broad band at 66 kDa in fraction 3+4, and aAb identified a doublet at 20 kDa. Both reagents revealed a band at 110 kDa in fractions 6 and 7. The mAb47 and aAb immunoreactive band at 66 kDa shifted to 40 and 26 kDa when fraction 3+4 was separated in Tricine SDS-PAGE under reducing conditions (see Fig. 2), and only the 40-kDa bands remained reactive with mAb47 (Fig. 4C). In contrast, these bands and the aAb immunoreactive doublet from fraction 3+4 were no longer revealed by aAb in reducing conditions (Fig. 4D). The 110-kDa band of TPO was revealed by mAb47 in reduced fractions 6 and 7, whereas only the 110-kDa band of fraction 7 was revealed by aAb. To confirm that recog-
inition of aAb depends on the antigenic conformation, we tested fraction 3 in ELISA after reduction and alkylation of the coated material. As a control, native TPO was tested in the same way. After treatment, fraction 3 was not recognized by aAb (Fig. 5A), whereas the autoreactivity of the native TPO decreased slightly (Fig. 5B). In contrast, mAb47 was slightly more reactive on treated than untreated materials.

Identification of the Immunoreactive Peptides—The 40- and 20-kDa bands reactive with the mAb47 and aAb, respectively, were electroeluted from fraction 3 after they were run in Tricine SDS-PAGE under reducing conditions. The amino acid composition was determined for these peptides, and a computer program localized the best fitted fragments in the entire TPO amino acid sequence. Table I shows the experimental and calculated amino acid percentages for the two TPO peptides. The optimized errors were 1.97 and 2.35% for the 40- and 20-kDa peptides, respectively. The two peptides were within the 550–923 and 744–922 C-terminal amino acid sequences of the TPO, respectively (Fig. 6). Taking into account the localization of the theoretical lysine peptides, we deduced that the 40-kDa peptide encompassed 12 noncleaved lysine peptides from amino acid 549 to 933 at the C-terminal end of the TPO and that the last five lysine peptides from amino acid 742 to 933 overlapped the 20-kDa peptide.

Localization of the Autoimmune Epitopes—The 20-kDa peptide encompassed an extracellular part of the molecule followed by the transmembrane region and an intracytoplasmic region. Consequently, the autoimmune epitopes could be situated inside and/or outside the thyrocyte. Considering that the 20-kDa peptide contained three tyrosine residues in the extracellular part of the TPO molecule, we tested the immunoreactivity of TPO aAb for the peptide after modification of the tyrosine.

**Fig. 4.** Western blot patterns of TPO mAb47 and aAb reactivities with the peptides from the HPLC fractions. The TPO peptides electrophoresed on Tricine SDS-PAGE were electrotransferred onto a PVDF membrane and tested with TPO mAb47 (A and C) and aAb (B and D). Western blots were done with samples used in native conditions (A and B) or after treatment with β-mercaptoethanol (C and D). The numbers of the HPLC fractions and the molecular mass standards are the same as in Fig. 2. Arrows indicate the immunoreactive bands.
residues by iodination. After $^{125}$I labeling and gel filtration of the peptides from HPLC fraction 3+4, the 66- and 20-kDa labeled peptides were in fractions 4 and 10, respectively (Fig. 7B). The mAb47 recognized the 66-kDa $^{125}$I-labeled peptide. In contrast, TPO aAb recognized none of the peptides modified by iodination (Fig. 7A). To ascertain that the loss of TPO aAb reactivity to the iodinated 66- and 20-kDa peptides resulted from the modification of the tyrosine residues and not the oxidative stress of the chloramine-T method, we tested by ELISA the TPO aAb and mAb47 immunoreactivity of the HPLC fraction 3+4 after treatment by the chloramine-T method with and without iodide. The TPO aAb immunoreactivity for the HPLC fraction 3+4 decreased only when the peptides were iodinated (Fig. 8A). In the absence of iodide, the oxidative stress of chloramine-T did not abolish the autoimmune epitopes on the peptides. As expected, the linear epitope recognized by the mAb47 containing no tyrosine residue was not affected by the iodination procedure. The treatment of native TPO by the chloramine-T method, with or without iodide, slightly affected the immunoreactivity of TPO aAb but not that of mAb47 (Fig. 8B).

**DISCUSSION**

We localized a new immunodominant region within amino acids 742–848 at the C-terminal end of the extracellular part of TPO. This region is deprived of potential sites of glycosylation.

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**FIG. 6.** Localization of the TPO peptides in the entire amino acid sequence of TPO (23). The amino acid sequences of the 40- and 20-kDa peptides best fitted with the experimental results are underlined and double underlined, respectively. The lysine residues (K) and the N-terminal amino acid numbers of the theoretical peptides are in **bold characters**. Arrows show the tyrosine residues (Y) on the 20-kDa peptide. The mAb47 binding site and the putative membrane spanning region are **boxed**. Single amino acid code is used (see Table I).

**TABLE I**

| Amino acid | 40-kDa peptide | 20-kDa peptide |
|------------|----------------|----------------|
| Asx (D/N)  | 8.17           | 10.95          |
| Thr (T)    | 4.47           | 6.92           |
| Ser (S)    | 10.92          | 7.20           |
| Glx (E/Q)  | 15.48          | 12.68          |
| Pro (P)    | 3.81           | 5.48           |
| Gly (G)    | 12.69          | 10.09          |
| Ala (A)    | 5.25           | 7.20           |
| Cys (C)    | 0.00           | 0.00           |
| Val (V)    | 3.86           | 4.90           |
| Met (M)    | 2.80           | 1.73           |
| Iso (I)    | 5.82           | 3.17           |
| Leu (L)    | 9.79           | 10.37          |
| Tyr (Y)    | 1.75           | 1.44           |
| Phe (F)    | 4.16           | 4.32           |
| His (H)    | 2.91           | 2.59           |
| Lys (K)    | 2.39           | 3.17           |
| Arg (R)    | 5.93           | 7.78           |
| Tryp (W)   | 0.00           | 0.00           |

*In parentheses are the single amino acid codes. Asx = Asp (D) + Asn (N) and Glx = Glu (E) + Gln (Q). Cys (C) and Tryp (W) are not taken into account.*

* Amino acid composition (in percent) as determined from phenylisothiocyanate-derived amino acids by reverse phase-HPLC separation.

+ Amino acid composition (in percent) corresponding to the best fitted peptide identified by the computer program (22) in the entire amino acid sequence of TPO (23).
and contains 11 cysteine residues, some of which form disulfide bridges implicated in the three-dimensional structure of the evidenced autoepitopes. TPO was previously used to map the interaction of a panel of 13 mouse mAbs to 4 antigenic regions of TPO; TPO aAb from patients with Graves’ or Hashimoto’s disease were directed predominantly against two of the 4 regions (6). Interestingly, all but one of the epitopes in these two regions were conformational. The linear epitope (recognized by mAb47) was resistant to denaturation, and further studies localized the corresponding sequence at amino acids 713–721 (24). Human mAb were produced to conformational epitopes in two overlapping regions on the surface of native TPO that were recognized by about 80% of TPO aAb in individual patient’s sera (25). We reported that treatment of TPO by denaturing agents inactivated most of the aAb reactivity whereas treatment with a reducing agent completely abolished the autoimmune recognition (20). We therefore generated peptides from our conventional preparation of native TPO. Enzymatic hydrolysis, peptide separation, and antibody binding were done without heating and reducing agents to maintain the native conformation of the generated peptides.

The most interesting peptides were recovered from HPLC fraction 3+4, which eluted with a 41–43% acetonitrile gradient. This percent of acetonitrile does not change antibody recognition since fraction 7, eluted with 52% acetonitrile, retained almost all the native TPO autoreactivity in ELISA. The 26-kDa peptide, which is linked to the 40-kDa peptide by one or more disulfide bridges, showed no TPO antibody reactivity per se and, consequently, was not further investigated. We chose to localize on the TPO amino acid sequence, the 40- and the 20-kDa peptides, which reacted with the mAb47 and aAb, respectively. A computer program (22) revealed they were in the C-terminal end of the known TPO sequence (23). The 40-
Mapping TPO Autoepitopes

A large proportion of the epitopes recognized by aAb require the correct three-dimensional structure of TPO, but mapping these regions is a considerable challenge. This is the first report describing a TPO region that contains at least one conformational epitope recognized by aAb. Further investigations of this region should determine the specificity of the corresponding aAb.

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A region within amino acids 657–767 harbors a major and frequently used autoepitope (30). The last 26 amino acids of this region overlap our 20-kDa peptide. An autoepitope is within residues 783–933 (33), and there are various autoepitopes along the TPO amino acid sequence, including the C-terminal amino acids 709–933 (34). All these autoepitopes, however, are immunoreactive under denaturing and reducing conditions. On the other hand, limited tryptic digestion, as part of the purification procedure of TPO, generates various peptideic fragments (35). One of the tryptic cleavage sites is close to or on the luminal side of the apical membrane. The possible presence of a major autoepitope around this cleavage site precludes the use of trypsinized TPO or truncated recombinant TPO for aAb clinical testing. More recently, eight TPO mutants whose creation was guided by the crystal structure of myeloperoxidase, a closely related molecule, were used (36). These mutations were strategically located to alter the surface of the TPO molecule. Unfortunately, the eight mutageneses did not affect the aAb recognition of the molecule. The mutation nearest the C-terminal end of the molecule was within TPO amino acids 722–727, i.e. exactly 15 amino acids upstream from the 20-kDa peptide region. By default, this result adds to the validity of our determination and provides evidence for the involvement of this TPO region in autoimmune recognition.

kDa peptide extends from amino acid 549 to 933 and overlaps the 20-kDa peptide by its last 192 amino acids. This overlapping probably explains why these two peptides displayed similar hydrophobicity and, consequently, eluted in the same HPLC fraction. The sequence of the 20-kDa peptide was confirmed by direct sequencing of the five NH₂-terminal amino acids. The 40-kDa sequencing failed to provide reliable information, but the mAb47 reactivity revealed that the reported mAb47 linear sequence (24) was in the deduced sequence. More precisely, the autoimmune epitopes was localized in the C-terminal part of the molecule immediately before the transmembrane region, i.e. from amino acid 742 to 848. Effectively, the three tyrosine residues on the 20-kDa peptide were in this part of the molecule, and the localization of the peptide abolished the conformational epitopes recognized by TPO aAb.

Most attempts to identify and locate the B-cell epitopes on TPO were made on recombinant TPO fragments that obviously did not always adopt the same structure as their native counterparts in intact TPO (for review, see Refs. 18–19). Identification of such linear epitopes was questioned because B-cell epitopes, unlike T-cell epitopes, are usually conformational i.e. highly dependent on the three-dimensional structure of the protein (26). Thus, to explain the auto-reactivity of linear epitopes, it was proposed that short peptide fragments of TPO may be part of larger discontinuous epitopes (27). At variance with molecular biology studies (28–32), we observed no auto-reactivity in small peptides with low molecular weight. Auto-reactive bands were very scarce, and their immunoreactivity was very faint despite the large excess of aAb. Hydrolysis at lysine residues may have damaged some autoepitopes including C2 and C21, as described by Vassart (28, 29), which are within TPO amino acids 590–622 and 709–721, respectively, and which consequently map in the 40-kDa peptide region (549–933). However, as expected, the mAb47 epitope (713–721), which is virtually identical to the C21 epitope, was evidenced in the 40-kDa peptide but not in the 20-kDa peptide. This introduced an additional autoepitope of interest outside the C2 and C21/mAb47 epitopes at the C-terminal end of the TPO molecule.

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