Directed Mutagenesis in Region 713–720 of Human Thyroperoxidase Assigns 713KFPED717 Residues as Being Involved in the B Domain of the Discontinuous Immunodominant Region Recognized by Human Autoantibodies*

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Autoantibodies (aAbs) to thyroid peroxidase (TPO), the hallmark of autoimmune thyroid disease (AITD), recognize conformational epitopes restricted to an immunodominant region (IDR), divided into two overlapping domains A and B. Despite numerous efforts aimed at localizing the IDR and identifying aAb-interacting residues on TPO, only two critical amino acids, Lys713 and Tyr717, have been characterized. Precise and complete delineation of the other residues involved in the IDR remains to be defined. By using a recombinant anti-TPO aAb T13, we demonstrated that four regions on TPO are part of the IDR/B; one of them, located between amino acids 713 and 720, is particularly important for the binding of sera from patients suffering from AITD. To precisely define critical residues implicated in the binding of aAb to human TPO, we used directed mutagenesis and expressed the mutants in stably transfected CHO cells. Then we assessed the kinetic parameters involved in the interactions between anti-TPO aAbs and mutants by real-time analysis. We identified (i) the minimal epitope 713–717 recognized by mAb 47 (a reference antibody) and (ii) the amino acids used as contact points for two IDR-specific human monoclonal aAbs TR1.9 (Pro715 and Asp717) and T13 (Lys713, Phe714, Pro715, and Glu716). Using a rational strategy to identify complex epitopes on proteins showing a highly convoluted architecture, this study definitively identifies the amino acids Lys713-Asp717 as being the key residues recognized by IDR/B-specific anti-TPO aAbs in AITD.

Thyroid peroxidase (TPO)1 is an essential membrane-bound enzyme involved in the biosynthesis of iodinated thyroid hor-...
Interestingly, different studies have pointed out the relationship between region 710 and 722 of hTPO and the B domain of the IDR, as defined with four recombinant human Fab molecules from McLachlan and Rapoport’s group (nomenclature used in this article). Chronologically, in 1991, Libert et al. (27) described a linear epitope, called C21, located between amino acid residues 710 and 722, that is able to interact with anti-TPO aAbs in the sera from patients suffering fromAITD. During the same year, Finke et al. (28) characterized the mouse mAb 47 produced by Ruf et al. (12), as recognizing a linear determinant in the region 713–721. Further studies, performed by different groups, showed that mAb 47/C21 epitope (i) characterizes in part the B domain of the IDR (12, 13) and (ii) specifically competes with the IDR/B-specific recombinant human monoclonal anti-TPO aAbs TR1.9 and T13 for binding to their cognate antigen (13, 29). These competitions have been explained, firstly, by the fact that Lys713, comprising part of the mAb 47 epitope, was shown to be a contact point for Fab TR1.9 on TPO (26). Secondly, by using the combination of phage-displayed peptide technology followed by sequence alignment between mimotopes and primary sequence of hTPO, we recently localized the discontinuous immunodominant epitope of aAb T13 and found that it is partially composed of region 713–720 of hTPO (29). Importantly, in the same study, we revealed by directed mutagenesis that this region is one major component of the epitopes recognized by human anti-TPO aAbs from patients’ sera.

Despite the fact that all these data argue in favor of a critical role of region 713–720 inside the IDR, only one of the residues, Lys713, has been described as being a part of the IDR/B; thus, precise delineation of the critical residues from region 713–720 involved in IDR remains to be elucidated. By a guided mutagenesis study of this region followed by eukaryotic cell expression of each mutant, we identified: (i) the minimal epitope recognized by mAb 47 (amino acids 713–717) and (ii) the amino acids used as contact points for human aAbs TR1.9 (Pro715 and Asp717) and T13 (Lys713, Phe14, Pro15, and Glu17). By using a rational strategy to identify complex epitopes on proteins showing a highly convoluted architecture, the present study definitively assigns the amino acids from TPO region 713–717 as being the key residues recognized by IDRB-specific anti-TPO aAbs inAITD. These data should be of great importance to rationally design therapeutic peptides able to block undesired autoimmune responses.

**EXPERIMENTAL PROCEDURES**

**TPO and anti-TPO Autoantibodies**—The hTPO, purified (greater than 95% pure) from thyroid glands, was obtained from HyTest Ltd (Turku, Finland). Production and purification of the mouse mAb 47 (12) and the recombinant human Fab TR1.9 (29) were previously described. Anti-TPO mAb 6H7 was purchased from Abcys S.A. (Paris, France). Rabbit polyclonal anti-TPO was prepared in the laboratory by injection of purified hTPO (HyTest Ltd.) in a New Zealand white rabbit, followed by two boosts. Each injection contained 600 µg of antigen in 1 ml of saline solution and 1 ml of Freund’s incomplete adjuvant. The human recombinant aAb T13 was expressed as IgG or Fab by using the baculovirus/insect cell system as described (30). Human Fab T13 was purified on a protein G affinity column and the concentration determined by measuring the absorbance at 280 nm, El1% of 1.56.

Human aAb T13 (0.5 µg), expressed as Fab or full IgG, was electroforesed under non-reducing conditions on 10% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane (Amersham Biosciences). The Fab T13 expression was checked by Western blotting, using a peroxidase-conjugated anti-human Fab- or Fc-specific Ab (Sigma, dilution 1:1,000) and the ECL detection system (Amersham Biosciences). The Fab T13 specificity was determined by ELISA. Wells were coated with 1 µg/ml of TPO in 100 mM NaHCO3, pH 9, overnight at 4 °C. Plates were washed with 0.05% Tween 20 in PBS-T (PBS-T), pH 7.3, and blocked with 2% nonfat dry milk in PBS-T (saturation buffer) for 1 h at 37 °C. After washing, aAb T13, expressed as Fab or the full IgG, molecule, was incubated with 1% nonfat dry milk in PBS-T (incubation buffer) for 1 h at 30 min at 37 °C. After washing, a peroxidase-conjugated anti-human Fab Ab (Sigma, dilution 1:1,000 in the incubation buffer) was added, and the plates were incubated for 1 h at 37 °C. After washing three times, the enzyme activity was detected with a 4 mg/ml 2-phenylenediamine solution containing 0.03% (v/v) hydrogen peroxide in 0.1% citrate buffer, pH 5.0. After 20 min, the reaction was stopped by adding 50 µl of 1 M H2SO4 to each well, and the resulting absorbance was measured at 490 nm (A490).

**Kinetic Parameters of anti-TPO Autoantibody Binding Assessed by BIACORE Analysis**—Surface plasmon resonance (SPR) analysis was performed at 25 °C with a 30 µl/min flow rate in HBS-EP (10 mM HEPES buffer, pH 7.4, 0.5 mM EDTA, 0.005% Biacore surfactant P20, 150 mM NaCl), using a BIACORE 2000 instrument (Biacore AB, Uppala, Sweden). To measure the association and dissociation rate constants (ka and kd, respectively), and the dissociation equilibrium constant (Kd = ka/kd) for the binding of mAb 47, IgG, or Fab T13, and Fab TR1.9, human TPO was covalently immobilized at 1500 RU on the flow cell of a CM5 sensorchip by the amine coupling kit provided by the manufacturer. A second flow cell was subjected to the same chemical treatment without the protein and used as reference. Five concentrations of anti-TPO Ab were injected during 180 s over the two flow cells, followed by a dissociation step of 400 s and a regeneration step (10 µl of 0.1 M HCl) between each concentration analysis. All the sensograms were corrected by subtracting the signal from the reference flow cell and were globally fitted to a 1:1 Langmuir isotherm using BLAevaluation version 3.2 software. Irrelevant mAb and Fab were used as analyte without giving any binding signal. Experiments at different flow rates showed an absence of mass transport and rebinding effects.

**Peptide Synthesis and Immunoadsorption on Cellulose Membrane-bound Peptides**—The general protocol for Spot parallel peptide synthesis was described previously (31). A set of 14-mer synthetic peptides corresponding to the region 709–722 of hTPO and its fourteen alanine analogs were synthesized by the Spot method. The membrane-bound peptides were probed by incubation of 2 µg/ml of mAb 47, Fabs T13, or TR1.9. Antibody binding was detected by using an alkaline phosphatase-conjugated anti-mouse Ig (Sigma, dilution 1:1,000), followed by addition of the phosphatase-labeled 5-bromo-4-chloro-3-indolyl phosphate and thiazolyl blue tetrazolium (Sigma), which gives a blue precipitate on peptides having bound the antibody. The membrane was re-used after a regeneration cycle. The intensity of the spots was evaluated with the ScionImage software.

**Blocking ELISA Experiments**—The wells were coated with 1 µg/ml TPO in 100 mM NaHCO3, pH 9.6, overnight at 4 °C. Plates were washed and blocked with saturation buffer for 1 h at 37 °C. After washing three times, mouse mAb 47 or 6H7 was incubated at two concentrations (50 and 5 µg/ml) in incubation buffer for 2 h at 37 °C. Washing was performed and then human Fab T13 or TR1.9 was incubated at a concentration giving an A490 of 1.5. After 1 h at 37 °C and washing, a horse- peroxidase-conjugated anti-human Fab Ab (Sigma, dilution 1:1,000 (Sigma), was used to detect the binding of human Fab on TPO by incubation 1 h at 37 °C. Washing (5 ×) was performed, and then the reactivity was revealed as described above. The percent inhibition was calculated by comparing the binding of human Fab with or without inhibitor.

**Guided Mutagenesis and Stable Expression of Wild-type and Mutated TPO Complementary DNA**—The full-length wild type (wt) and mutated TPO in the region 713–720 (fully mutated TPO713–720) were previously cloned in the pCDNA5/FRT expression vector from the Flp-In™ system (29). The eight amino acids in the region 713–720 were replaced by alanine mutants (TPO713–720). The full-length wild type (wt) and mutated TPO (TPO514–517) were previously cloned in the pcDNA5/FRT expression vector from the Flp-In system (29). The eight amino acids in the region 713–720 were replaced by alanine mutants (TPO514–517). All mutants were constructed by overlap extension PCR as described previously (32). The final PCR products were cloned into the full-length TPO cDNA by using the unique restriction endonuclease sites Clal and EcoN1. All sequences were verified by the dideoxynucleotide termination method (33). Then, the 4T1 mouse system was used to generate CHO cells. In 4T1 cells, a CHO line cells expressing wt or mutated TPO as we previously described (29). Cloning and expression of TPO fully mutated in the regions 713–720 (TPO713–720) or 506–514 (TPO506–514) were previously described (29).

**Flow Cytometry Analysis of Wild-type and Mutated TPO Expressed on the Surface of Stably Transfected CHO Cells**—Stably transfected CHO cells were scraped, rinsed, and pelleted (5 min, 900 rpm, 4 °C) in PBS supplemented with 2% heat-inactivated fetal calf serum (FACS buffer). The cells were resuspended and incubated with 200 µl of buffer containing 5% fetal calf anti-TPO Ab or 2 µg/ml of mAb 47.
for 45 min at 4 °C. The cells were washed three times and incubated in 200 μl of FACS buffer with 10 μg/ml of fluorescein-conjugated anti-rabbit (Sigma) or anti-mouse IgG (Rockland, Gilbertsville, PA) for 45 min at 4 °C in the dark. As control, the cells were incubated with second Ab alone. After washing three times with FACS buffer, the cells were analyzed (10,000 events) on an EPICS cytofluorometer (Beckman-Coulter, Fullerton, CA).

Membrane Protein Extraction from CHO Cells—Stably transfected or wt CHO cells were washed three times with PBS and scraped at 4 °C. Membrane protein extraction was performed as previously described (29). After centrifugation at 1,000 rpm for 5 min at 4 °C, membrane proteins were solubilized by adding 500 μl of cold lysis buffer (PBS containing 0.5% Triton X-114 and a protease inhibitor mixture tablet/10 ml of lysis buffer [Roche Applied Science], maintained at 4 °C) per 10⁶ cells, and incubated for 30 min on ice (mixed by vortexing every 10 min). After centrifugation at 800 rpm for 8 min at 4 °C, the supernatants containing the membrane proteins were recovered and incubated for 5 min at 33 °C to allow the separation of the detergent from the aqueous phase. After centrifugation at 800 rpm for 8 min at 22 °C, the upper aqueous phase, containing soluble TPO, was removed and concentrated to ~10 mg/ml using an Ultrafree<sup>®</sup>-4 Centrifugal Filter Unit (Millipore Corp., Bedford, MA). All protein concentrations were evaluated by the BCA protein assay reagent (Pierce).

Binding of Solubilized Membrane Proteins Containing Wild-type or Mutated TPO to Human Fab Assessed by BIACORE Analysis—Human Fab T13 or TR1.9 were covalently immobilized at 9,600 RU or 3,600 RU, respectively, on flow cells 2 and 3, respectively, of a CM5 sensorchip-activated surface with EDC/NHS (amine coupling kit from Biacore AB). Flow cell 1 was used as the reference control surface. Each solubilized membrane protein extract, containing wt or mutated TPO, was diluted in HBs-EP buffer to the same final total protein concentration (1 mg/ml) and loaded onto flow cells 1, 2, and 3 in a single injection of 90 μl of FACS buffer with 10 μM HCl. To control the binding stability of the immobilized Fab T13 and TR1.9, each membrane protein analysis was preceded and followed by an injection of solubilized membrane protein extract containing wt TPO under the same conditions. As a negative control, a preparation of membrane proteins obtained from non-transfected CHO cells was diluted to the same final protein concentration and injected over the flow cells. Three independent experiments were performed.

RESULTS

Production, Functionality Analysis, and Kinetic Binding Parameters of Human Fab T13—The anti-TPO aAb T13, previously produced as full IgG<sub>1</sub> (30), was cloned and expressed as human Fab by using the baculovirus/insect cell system as described (34). Human Fab T13 was purified by protein-G affinity chromatography (purity greater than 95%, data not shown) and then analyzed on 10% SDS-PAGE (Fig. 1A). We observed a 50-kDa band corresponding to human Fab T13, only detectable by the peroxidase-conjugated anti-human Fab Ab but not by the peroxidase-conjugated anti-human IgG specific Ab (Fig. 1A, lane 3 versus lane 1). Human T13 expressed as full IgG<sub>1</sub> could be detected by both secondary Abs (Fig. 1A, lane 2 versus lane 4). Approximately 0.5 mg of purified Fab T13 was obtained per liter of SF9 insect cell culture supernatant. The functionality of Fab T13 was checked by ELISA. The results clearly show that human Fab T13 was able to bind to hTPO, in a dose-dependent manner, as was the full IgG<sub>1</sub> T13 (Fig. 1B).

Affinity Measurements of Human Fabs T13, TR1.9, and mAb 47 Binding to TPO—By using BIACORE technology (Fig. 2, A–D), we determined the binding kinetics for the interaction between Fab T13, full IgG<sub>1</sub> T13, Fab TR1.9, or mAb 47 and hTPO immobilized on the sensorchip. The kinetic parameters of aAb T13 binding, produced as Fab (Fig. 2A) or full IgG<sub>1</sub> (Fig. 2B), were measured after separate injections of five different concentrations of purified aAb on TPO. We found affinities (K<sub>d</sub>) for IgG<sub>1</sub> and Fab T13 of 0.19 ± 0.01 nM and 0.80 ± 0.02 nM, respectively, using the same global fitting model, with a higher association rate constant for aAb T13 expressed as whole IgG<sub>1</sub> versus Fab and quite similar dissociation rate constants (Fig. 2E). The difference observed between the association rate constants is probably due to a stronger avidity of the full IgG<sub>1</sub> (two paratopes) in comparison with the Fab T13 (one paratope). In a previous study (30), we reported an affinity value for aAb T13 immobilized by an anti-human IgG Fc specific Ab, whereas those in lanes 3 and 4 were detected with a peroxidase-conjugated anti-human Fc specific Ab, less than 10⁻⁷ M (13). In our experiment (Fig. 2E), this mAb strongly interacted with TPO and immobilized on the BIACORE sensor chip and showed high affinity binding with K<sub>d</sub> = 64.10 ± 0.02 × 10⁻³ M⁻¹ s⁻¹ (Fig. 2E). In previous studies, using Scatchard analysis, the affinity of mAb 47 could not be calculated, probably because this mAb binds with a low affinity to TPO (K<sub>d</sub> less than 10⁻⁷ M) (13). In our experiment (Fig. 2E), this mAb strongly interacted with TPO immobilized on the BIACORE sensor chip and showed high affinity binding with K<sub>d</sub> = 0.11 ± 0.001 nM, which is similar to that obtained with human Fabs T13 and TR1.9 using the same binding protocol. One explanation for such a difference in the mAb 47 affinity value could be that the Scatchard analysis was performed with soluble and highly convoluted TPO, whereas real-time analysis used a covalently immobilized TPO.

Evidence That Human Fabs T13 and TR1.9, as Well as mAb 47, Interact with the Region 713–720 of Human TPO—Mouse mAb 47 has been used as reference to describe the B domain of the IDR and often in competition with human anti-TPO aAb (13, 15, 29, 35). Previous data localized the mAb 47 epitope between amino acids 713 and 721 by using TPO protein fragments expressed in

![Fig. 1. Functional analysis of human Fab T13. Expression and functional analyses were performed as described under "Experimental Procedures." A, each sample (0.5 μg) was charged under non-reducing conditions on a 10% SDS-polyacrylamide gel and analyzed by Western blotting. Lanes 1 and 2, purified human Fab T13. Lanes 2 and 4, purified human IgG1 T13. After transfer, proteins in lanes 1 and 3 were detected with a peroxidase-conjugated anti-human Fc specific Ab, as well as those in lanes 3 and 4 were detected with a peroxidase-conjugated anti-human Fab. B, the functionality of aAb T13 was analyzed by ELISA. The binding of human aAb T13, expressed as Fab or full IgG<sub>1</sub>, on hTPO is shown.](http://www.jbc.org/content/39060/1/39060/F1.large.jpg)
an hTPO cDNA sub-library (28). We performed a blocking ELISA experiment to assess the ability of Fabs T13 and TR1.9 to interact with region 713–721 of hTPO recognized by mAb 47. The binding of the human Fabs T13 and TR1.9 to hTPO was inhibited in a dose-dependent manner by mAb 47 but not by the control anti-TPO mAb 6H7 (Fig. 3). These data indicate that the mAb 47 and both human Fabs, but not the mAb 6H7, bound in close proximity on the hTPO molecule causing steric hindrance.

Spot Peptide Analysis Identifies Residues 713–717 Recognized by mAb 47 but Fails to Define Fabs T13- and TR1.9-interacting Residues—Using TPO protein fragments expressed in an hTPO cDNA sub-library, the mAb 47 epitope was found to be restricted to region 713–721 (28). Spot analysis of 20-mer overlapping peptides frameshifted by five residues, covering the entire sequence of the MPO- and CCP-like domains of TPO, revealed that peptides from region 701–732 with the motif 713KFPEDFES720 are recognized by mAb 47 (data not shown).

To determine the critical amino acids involved in the recognition of mAb 47 to TPO, 15 peptides corresponding to the amino acid sequence of region 709–722 of hTPO (wt sequence and 14 peptides resulting from an alanine scan) were synthesized on a cellulose membrane. As shown in Fig. 4 mAb 47 strongly reacted with the wt 709–722 peptide, whereas the alanine mutations in positions Lys713, Pro715, and Glu716 totally abolished the binding of mAb 47 to the mutated peptides. Furthermore, the mutation of residues Phe714 and Asp717 partially decreased (inhibition of 11 and 24%, respectively) the binding of mAb 47 to the peptides as determined by comparison of spot intensities indicated on the left side of Fig. 4. Taken together, these results indicate that the minimal epitope recognized by mAb 47 is assigned to residues between amino acids 713 and 717, with the motifs Lys713, Pro715, and Glu716 essential for antibody binding. On the other hand, no reactivity with the wt peptide was observed with Fabs T13 or TR1.9 by using the spot technology (data not shown), confirming that interaction between TPO and these aAb fragments is more complex than that between mAb 47 and its linear epitope.

Flow Cytometry Analysis of CHO Cells Expressing Full-length TPO Mutants Confirms That the 713–717 Region Corresponds to the Minimal Epitope of mAb 47 but Still Fails to Identify T13- and TR1.9-interacting Residues—Using the Flp-In™ system (29), we produced eight single amino acid mutants...
between positions 713 and 720 of hTPO and a mutant called TPOKPE-SAR with a triple mutation, where residues Lys713, Pro715, and Glu716 (described above as essential for the binding of mAb 47) were replaced by residues Ser713, Ala715, and Arg716. To compare the TPO expression levels obtained for each mutant on the surface of stably transfected CHO cells, flow cytometry experiments were performed with a rabbit polyclonal anti-TPO. As indicated in Fig. 5A, similar cytometry patterns showing strong binding of the rabbit polyclonal anti-TPO serum were obtained for all mutant TPO-transfected CHO cells in comparison with wt TPO-transfected cells, whereas no binding was observed with non-transfected cells. On the contrary, when mAb 47 was tested by flow cytometry, its binding to CHO cells transfected with mutants TPOK713L, TPOP715L, TPOE716L, and TPOD717L, and to mutant TPOKPE-SAR was totally abolished (Fig. 5B). No specific signal was observed either with the non-transfected cells. These data definitively confirm that the minimal epitope of mAb 47 lies between Lys713 and Asp717 and demonstrate the functional role of these amino acids sub- stitutions in region 713–720 of hTPO.

In an attempt to demonstrate that region 713–720 of hTPO contains amino acids involved in the binding of the human Fabs T13 and TR1.9, we incubated these Fabs with CHO cells expressing wt or mutated TPO. Each membrane protein preparation was injected over the human Fabs T13 or TR1.9. The controls (Fig. 6, B), containing wt or mutated TPO over the immobilized Fab T13 or TR1.9. The $k_d$ variation ($\Delta k_d$) values were calculated as the difference between the dissociation rate of fully mutated TPO713–720 or point mutated TPO (TPOmut) and wt TPO. The dissociation rates observed with the wt TPO protein preparation were $8.22 \pm 0.23 \times 10^{-4}$ s$^{-1}$ on immobilized T13 and $0.75 \pm 0.01 \times 10^{-4}$ s$^{-1}$ on immobilized TR1.9. The dissociation rates observed with TPO713–720 preparation were $16.5 \pm 0.3 \times 10^{-4}$ s$^{-1}$ on immobilized T13 and $4.58 \pm 0.04 \times 10^{-4}$ s$^{-1}$ on immobilized TR1.9. The controls (Fig. 6E), mutations in region 506–514 of hTPO (TPO506–514), did not increase the dissociation rate of human Fabs. Interestingly, several site-specific mutations directly increased the dissociation rates measured for Fabs T13 and TR1.9 ($\Delta k_d$imal/imal)–30% greater than 10%). Residue Pro715 strongly contributed to the binding of Fab T13 to region 713–720 of hTPO ($\Delta k_d$imal/imal $= 46.8 \pm 2.2\%$), whereas the Pro715 residue, Lys713, Pro715, and Glu716, which had a ratio between 12% and 14% were found to be involved to a lesser extent in Fab T13-TPO interaction. On the other hand, residue Asp717 was found to be the main contributor to Fab TR1.9 binding to region 713–720 ($\Delta k_d$imal/imal $= 65.4 \pm 1.8\%$, Fig. 6E), whereas Pro715 residue, participated to a lesser extent to the binding of Fab TR1.9 to hTPO, with a ratio of 26.9 $\pm 0.9\%$ (Fig. 6E).

A three-dimensional model of the MPO-like domain of hTPO (Fig. 7) shows that residues Lys713, Pro715, and Glu716 protrude outside the molecule, whereas residues Phe714 and Asp717 are buried inside TPO. As shown in Fig. 7, the IDR/B architecture in region 713–720, recognized by human aAbs, is mainly formed by a group of amino acids composed of the aromatic Phe714 and the negatively charged Asp717 residues and also a protruding amino acid cluster, comprised of the Pro715 residue and the charged residues Lys713 and Glu716 whose side chains are accessible to the solvent.

**Discussion.** In this study we demonstrated, by using wt or mutated TPO extracted from stably transfected CHO cells followed by a real-time analysis (BIACORE technology), that amino acids Lys713 to Asp717 correspond to one major part of the IDR/B recognized by human anti-TPO aAb and represent the minimal epitope for mAb 47, described as a reference murine antibody for the B domain of the TPO IDR.
Numerous studies published during the past two decades have shown that the IDR recognized by human aAb on the surface of hTPO is highly discontinuous (28, 36-38). Indeed, hTPO presents a highly convoluted architecture composed of three distinct modules designated as MPO-, CCP-, and EGF-like domains (1). This characteristic makes the identification of amino acids taking part in the IDR extremely difficult. Many unfruitful efforts using the directed mutagenesis approach have been made to determine the amino acid residues in contact with human anti-TPO aAb (1, 23-25). Two recent studies showed that the (i) tyrosine residue in position 772 belongs to the IDR (22) and (ii) regions 353-363, 377-386, 713-720, and 766-775 of hTPO form an immunodominant binding surface for human anti-TPO aAb (29). Studies of the 766-775 region of hTPO, which contains Tyr\textsuperscript{772}, took advantage of the fact that the CCP- and EGF-like domains of hTPO can be expressed together by eukaryotic cells, in the absence of the MPO-like domain (15, 39). Since, the major part of the IDR on hTPO is located in the MPO-like domain (29, 40), we decided to stably express on the CHO cell surface the full-length wt or mutated hTPO by combining two technological approaches (directed mutagenesis followed by the Flp-In™ expression system). Flow cytometry analysis performed with these transfected CHO cells clearly showed that the minimal epitope to which mAb 47 binds is localized between amino acids Lys\textsuperscript{713} and Asp\textsuperscript{717}. This cellular approach confirmed the data obtained by the Spot tech-

![Flow cytometry analysis of the binding of rabbit polyclonal Ab and murine mAb 47 to wild-type or mutated TPO expressed on the surface of stably transfected CHO cells.](image)
FIG. 6. Several amino acids in region 713–720 contribute to the binding of human Fab T13 and Fab TR1.9 on human TPO as evidenced by real-time analysis. Solubilized CHO membrane proteins containing wt or mutated TPO were diluted to obtain the same final total protein concentration and separately injected over Fab T13 or Fab TR1.9 previously immobilized on the flow cell of a CM5 sensorchip (BIACORE 3000 technology). Representative sensorgrams of the binding of CHO membrane protein extracts containing wt TPO or fully mutated TPO<sup>713–720</sup> versus a non-transfected cell membrane protein preparation (Mock) to Fab TR1.9 (A) and Fab T13 (B) are respectively displayed. Dissociation curves of the binding of the various mutant preparations to Fab TR1.9 (C) and Fab T13 (D) were fitted, and the dissociation rate constants (<i>k_d</i>) measured by using BIAevaluation version 3.2 software. These data are expressed in the histogram (E), which displays the ratio R of (<i>Δk_d (mutant)/Δk_d (wt)</i>) as a percentage, where <i>Δk_d (mutant) = (k_d (TPO<sup>mut</sup>) − k_d (TPO<sup>wt</sup>))</i> and <i>Δk_d (wt) = (k_d (TPO<sup>713–720</sup>) − k_d (TPO<sup>wt</sup>))</i>. The solubilized membrane extract containing wt TPO was injected twice, before and after the mutants, to control the stability of each human Fab. The data were verified in three independent experiments with three different protein extractions.
Immunodominant Amino Acids in Region 713-720 of Human TPO

Controversial studies assigned the mAb47/C21 epitope (amino acid residues 713–721) sometimes as part of the IDR (12, 26, 35) and sometimes not (11, 25, 41). After several years of questioning, all recent data emphasize the importance of the position Phe714 and Asp717 on TPO for the binding of the human anti-TPO aAb (26, 29, 30). By direct footprinting, Guo et al. (26) identified the Lys713 residue as a contact point for human monoclonal aAb TR1.9, which recognizes a part of the IDR/B. Surprisingly, in our present study, replacement of Lys713 by Leu713 did not decrease the dissociation rate of Fab TR1.9. Three explanations can be given. (i) Lys713 is directly involved in the association rate and does not affect the TR1.9 dissociation rate we measured in our BIACORE experiment. (ii) The protocol used for real-time analysis used in this study is not suitable for highlighting a direct influence of Lys713 on the binding of TR1.9 to hTPO. (iii) More drastic mutation, namely, shortening the amino acid side chain length (i.e. replacement of Lys by a Val or an Ala residue) is probably necessary to affect TR1.9 antibody binding. Moreover, an epitope on a large protein involves ~20 contact amino acid residues. Mutation of only one antigenic residue (such as Lys713) may therefore, have only a minor effect on the binding affinity of the Fabs, as we indeed observed with TR1.9. Consistent with our present finding, Guo et al. (26) showed that mutation of a more extensive region (residues 713–720) only partially affected the binding of TR1.9. Therefore, although Lys713 is a contact residue for TR1.9, our present data indicate that it contributes very little, if at all, to TR1.9 affinity.

Single amino acid substitutions in region 713–720 of hTPO moderately reduced human aAb affinity and did not decrease the binding of the human aAbs to hTPO by flow cytometry; such results depend, however, on the antibody used. We must keep in mind that (i) the influence of an individual amino acid on the interaction with an anti-TPO antibody can be slightly different and sometimes modest for a given region, even if all these antibodies are characterized as being able to bind this region, as previously described (26, 28, 29), and (ii) human anti-TPO aAb such as T13 and TR1.9 recognize a discontinuous epitope involving multiple regions on hTPO, in contrast to mAb 47, which is directed against a linear sequence 713–717. Therefore, we devised a new protocol, combining membrane protein extraction from eukaryotic cells with real-time analysis, to assess the influence of the kinetic parameters on the interaction between human aAbs and their cognate antigen expressed on CHO cells. The TPO concentration, in membrane protein extracts, is very difficult to estimate. For this reason, we focused on the dissociation rate constant ($k_d$), which is independent of the analyte concentration (the wt or mutated TPO in our study) but represents the interaction force between two proteins. Our results highlight the fact that at least four amino acids (Lys713, Phe714, Pro715, and Glu716) for Fab T13 and two (Pro715, Asp717) for Fab TR1.9 contribute to the binding of these human aAbs to region 713–720 of hTPO. The amino acid residue Pro715 is the only amino acid whose presence increases the dissociation rate for both Fabs T13 and TR1.9. Due to the importance of this amino acid in the folding of proteins, we cannot formally exclude the fact that a mutation in this position would induce a slight change in the three-dimensional structure of region 713–720 of hTPO, thus explaining its effects on the binding of aAbs T13 and TR1.9 to TPO. We propose that residue Pro715 could play a role in structuring the antigenic loop but without directly interacting with aAbs T13 and TR1.9.

Our results, shown in Fig. 3, are in accordance with the fact that mAb 47 strongly inhibits the binding of Fab T13 to hTPO but to a lesser extent than the binding of Fab TR1.9 to hTPO. Indeed, region 713–720, constituting one part of the Fab T13 discontinuous epitope, possesses four amino acids common to the mAb 47 epitope, whereas the epitopes recognized by mAb 47 and Fab TR1.9 have only three residues in common (26 and the present study). This observation is perhaps a clue to understanding why it has been so difficult up to now to determine...
whether, by using the human aAb TR1.9, the region 713–720 of hTPO is part of the IDR or not (11, 25, 26).

Recently, in an attempt to identify the TPO immunodominant epitope, several mimotopes that mimic the discontinuous epitope recognized by the T13 aAb were selected (29). These mimotopes were grouped into three families on the basis of their sequence homology. Four T13-specific mimotopes of family 1, obtained by biopanning on TPO from four phage-displayed libraries, largely matched with region 713–721 of TPO (29) and carry most of the residues found to interact with Fab T13, Fab TR1.9 or mAb 47 in the present study. Of interest, the motif LXPEDX (where X represents any amino acid), identified by alanine scanning as bearing critical residues for family 1-mimotope binding to T13 (29), mimics the Pro715 and Glu716 major anchor residues for human Fab T13; amino acids Pro715 and Asp717, characterized as contact points for human TR1.9 Fab; and Pro715, Glu716, and Asp717, essential residues for IDR/B-specific mAb 47 (Fig. 4). Two other residues, Lys713 and Phe714, were found to interact with Fab T13 by real-time analysis and with mAb 47 by flow cytometry studies on TPO-mutated CHO cells in the present study, whereas only amino acid Lys713 was identified as a TR1.9-interacting residue by footprinting (26). These two latter residues do not belong to the mimotope’s critical motif LXPEDX (29). However, a precise observation of two out of the four family 1 mimotopes (KLLPEDSRTYHTV and KLFPEDDEMRTETQR), which match in the region 713–720 (29), allowed us to identify a lysine residue in both mimotopes and a phenylalanine residue in the second mimotope just before the proline, as expected given the wt sequence of hTPO. Taken together, these observations reinforce our data demonstrating that residues Lys713, Asp717 correspond to a major part of the IDR/B recognized by human anti-TPO aAb.

Finally, these data further emphasize the importance of region Lys713, Asp717 for TPO recognition by human aAbs. Indeed, we previously observed that the binding of human anti-TPO aAbs from the serum of patients suffering from AITD is strongly affected when region 713–720 is fully mutated (29), suggesting that peptides encompassing amino acids 713–720 are predominantly presented to the immune system during onset of AITD. The resulting anti-TPO aAb response could be even more restricted and directed against amino acids in region 713–717 of hTPO (present data).

It is now strongly believed that viral infections can trigger autoimmune diseases when they occur in predisposed patients, either by inducing virus-mediated cell damage of target organs or by molecular mimicry, thus activating an immune response not only against a viral peptide but also against the aAg encompassing the same amino acid sequence (42–46). For example, it has been shown that the primary sequence of the 65-kDa isoform of glutamic acid decarboxylase (GAD65) contains a PEVVEKK motif which is (i) part of the discontinuous epitope recognized by a well-described human-derived mAb MICA3 and (ii) also present in the primary sequence of protein 2C from Coxackie B4 virus (CBV) (47). Such results favor the hypothesis, now well documented (48), that CBV infection may trigger type 1 diabetes by the process of viral mimicry with subsequent cross-reactivity with a critical epitope of GAD65. Importantly, by performing a BLAST sequence alignment between the motif 715KPFED717, which we found to be a critical epitope, and all virus sequences in the database, we identified one perfect sequence homology with the amino acids 450–454 of human metapneumovirus (hMPV), a single-stranded RNA virus, which causes lower respiratory infection disease in children (49). Thus, a relationship between hMPV infection and AITD should be investigated.
Immunodominant Amino Acids in Region 713-720 of Human TPO

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Directed Mutagenesis in Region 713-720 of Human Thyroperoxidase Assigns Residues as Being Involved in the B Domain of the Discontinuous Immunodominant Region Recognized by Human Autoantibodies

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