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Search for the Autoantibody Immunodominant Region on Thyroid Peroxidase: Epitopic Footprinting with a Human Monoclonal Autoantibody Locates a Facet on the Native Antigen Containing a Highly Conformational Epitope

Jin Guo, Xin-Min Yan, Sandra M. McLachlan, and Basil Rapoport

Autoantibodies to thyroid peroxidase (TPO), a thyrocyte apical plasma membrane glycoprotein, is the major target Ag in human autoimmune thyroiditis (Hashimoto’s thyroiditis), the commonest form of organ-specific autoimmunity in humans. Serum TPO autoantibodies, the primary markers of thyroid autoimmunity (1), are likely to be involved in autoantigen presentation to T cells (2, 3) and may also play a direct role in the process leading to thyroid cellular destruction (4, 5). The molecular cloning and expression of TPO autoantibody genes have made available a panel of recombinant, monoclonal human TPO autoantibodies that define a restricted, immunodominant region on TPO (6, 7). Elucidation of the structure of this immunodominant region will provide important information for further understanding the immunopathologic mechanisms leading to autoimmune thyroiditis and may facilitate strategies for immunotherapeutic intervention. Despite much investigative effort, however, the location on TPO of the autoantibody immunodominant region remains unknown.

TPO is very large (107 kDa) and, based on the known three-dimensional structure of closely related myeloperoxidase (8), is a highly complex, convoluted, globular molecule that may be likened to bowl of spaghetti. It is, therefore, not surprising that identification of conformational, possibly discontinuous, TPO autoantibody epitopes (9–11) on the native Ag has not been possible using synthetic peptides. Some polypeptide fragments of TPO are recognized by TPO autoantibodies in patients’ sera (12–25). However, the wide spectrum and large size of these fragments as well as the fact that (in the native structure) polypeptides can traverse the molecule and contribute to widely separated facets on the surface of the protein have not led to deduction of the site of the immunodominant region. Moreover, polyclonal patient serum interaction with polypeptides may reflect the minor component of TPO autoantibodies that recognize denatured, linear determinants. Other approaches have involved competition between mouse mAbs (26) or rabbit antisera (27) for human autoantibody binding to TPO. However, potential steric hindrance by IgG molecules, which are even larger than TPO, does not allow definitive localization of an epitopic region.

Identification of the TPO autoantibody immunodominant region could be attained by determination of the three-dimensional structure of crystals of TPO-autoantibody complexes. However, although the crystal structure of one TPO autoantibody Fab has been reported (28), this goal, or indeed determining the structure of uncomplexed TPO (29, 30), has to date not been achieved. In this context, definitive identification of even a single amino acid within the TPO immunodominant region would provide vital information by indicating which facet of TPO contains this region, and this individual residue could then be the landmark for future guided mutation studies to more fully characterize this region.

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2 Address correspondence and reprint requests to Dr. Basil Rapoport, Autoimmune Disease Unit, Cedars-Sinai Medical Center, 8700 Beverly Boulevard, Suite B-131, Los Angeles, CA 90048. E-mail address: rapoportb@cshs.org
3 Abbreviations used in this paper: TPO, thyroid peroxidase; MPO, myeloperoxidase; TPO-Bio, biotinylated TPO; TPO-ep-Bio, TPO epitope-protected-Bio; CHO, Chinese hamster ovary; HABA, 2-(4′-hydroxyazobenzene)-benzoic acid.

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mutagenesis studies based on the known structure of myeloperoxidase (MPO) (8). In the present study we used an epitopic footprinting approach to identify for the first time a specific TPO amino acid residue (Lys713) in the autoantibody immunodominant region.

Materials and Methods

TPO and autoantibody purification, complexing, and biotinylation

Recombinant human TPO, expressed in Chinese hamster ovary (CHO) cells as an 848-aa, secreted protein (31), was affinity purified as described previously (32). TR1.9, one of four recombinant human TPO autoantibodies (expressed as a Fab) that define the immunodominant determinants on TPO (7), was affinity purified as described previously (33). This Fab was cloned by screening a combinatorial Ig gene library constructed from thymidin-infiltrating B lymphocytes. TPO-TR1.9 complexes were prepared by incubating (in a typical preparation) 2 mg of TPO with 6 mg of TR1.9 in 1.5 ml of 10 mM Tris, pH 7.0, for 2 h at room temperature. Complexing efficacy (~20 μg aliquot) was analyzed by fast protein liquid chromatography (Pharmacia LKB, Piscataway, NJ) using a Sephacryl S-200 HiPrep 16/60 column equilibrated with PBS. Subsequently, 1 mg of uncomplexed TPO and the remaining TPO-TR1.9 complex were separately dialyzed against 50 mM NaHCO₃, pH 8.5, and then incubated with EZ-Link Sulfo- NHS-LC-Biotin (Pierce, Rockford, IL) at a biotin to protein molar ratio of 100:1 for 2 h at room temperature. Free biotin was removed by five cycles of dilution and concentration in 10 mM Tris, pH 7.0, using a Centricon YM-30 (Millipore, Bedford, MA). The biotin-TPO molar ratio was determined by the 2-(4'-hydroxyazobenzene)-benzoic acid (HABA) method according to the protocol of the manufacturer (Pierce).

Dissociation of the TPO-TR1.9 complex and purification of TPO

Nonbiotinylated TPO, biotinylated TPO (TPO-Bio), and the biotinylated TPO-TR1.9 complex were each incubated for 1 h at 80°C in 6 M guanidine hydrochloride (Sigma, St. Louis, MO) and 700 μM 2-ME before fast protein liquid chromatography on a Sephacryl S-200 column equilibrated with 4 M guanidine hydrochloride. The peaks of nonbiotinylated TPO, TPO-Bio, and TPO recovered from the biotinylated TPO-TR1.9 complex (termed TPO-epitope-protected Bio (TPO-ep-Bio)) were collected, dialyzed against 10 mM Tris, pH 7.0, and concentrated to 1 mg/ml using a Centricon YM-30 (Millipore, Bedford, MA). The biotin-TPO molar ratio was determined by the 2-(4'-hydroxyazobenzene)-benzoic acid (HABA) method according to the protocol of the manufacturer (Pierce).

Tryptic digestion of TPO

TPO, TPO-Bio, and TPO-ep-Bio were treated (18 h at 37°C) with sequencing grade trypsin (Roche, Indianapolis, IN) at an enzyme/substrate ratio of 1:50 (w/w) in digestion buffer (100 mM Tris-HCl, pH 8.5) with 1% ace- tonitrile. The trypsin digestion profiles of TPO, TPO-Bio, and TPO-ep-Bio were examined by reverse-phase HPLC (see below). The presence of biotin on individual tryptic fragments was assessed by adsorption on streptavidin. Aliquots of digested TPO (20 μg) were incubated (1 h at room temperature) with 200 μl of streptavidin agarose (Pierce) prewashed twice with PBS. After microcentrifugation, the supernatants were subjected to reverse-phase HPLC, and the tryptic fragment profiles were compared with those of the same material not adsorbed with streptavidin.

Reverse-phase HPLC, amino acid composition, and sequencing

For qualitative analyses, ~20 μg of trypsin-digested TPO supplemented with trifluoroacetic acid (0.1% final concentration) was applied to a C₄ reverse-phase 3.9- × 150-mm column (Waters, Milford, MA). Unless indicated otherwise, tryptic fragments were eluted with a linear gradient of 0–100% acetonitrile and 0.1% trifluoroacetic acid for 60 min at 1 ml/min. Eluted peptides were monitored by absorbance at 214 nm. Digestion of each of the three TPO preparations (TPO, TPO-Bio, and TPO-ep-Bio) was repeated at least three times followed by reanalysis by reverse-phase HPLC. After identification of a peak of interest (see Results), larger amounts (1–2 mg) of TPO digests were subjected to reverse-phase HPLC under identical conditions. The peptide peak was collected in 1.8 ml in a 2-ml polypropylene vial (Corning, Cambridge, MA). The purity of the peak was confirmed by reinjection of 100 μl of collected material. Where indicated, the eluted peak was treated (1 h at 80°C) with 700 mM 2-ME followed by reabsorption and recovery of peptides separated by disruption of disulfide bonds. Amino acid composition (400 pmol of peptide) and N-terminal amino acid sequencing analysis (40 pmol of peptide) were determined by the Molecular Structure Facility, University of California-Davis School of Medicine.

Flow cytometry for autoantibody recognition of TPO

CHO cells stably expressing wild-type TPO (34) or a chimeric TPO-MPO molecule (G2) (35) were propagated in Ham’s F-12 medium supplemented with 10% FCS, penicillin (100 U/ml), gentamicin (50 μg/ml), and amphotericin B (2.5 μg/ml). Cells were released by light trypsinization and incubated (1 h at 4°C) with the indicated concentrations of purified, recombinant human monoclonal autoantibodies, TR1.9 and SP1.5, expressed as Fab (6, 7). Binding of TR1.9 and SP1.5 was detected with PE-conjugated monoclonal mouse anti-human κ Ab (Caltag, Burlingame, CA). As controls, cells were incubated with buffer alone or second Ab alone. Flow cytometry was performed (10,000 events) using a FACScan with CellQuest software (Becton Dickinson, San Jose, CA).

Results

Biotinylation of TPO alone and TPO-autoantibody complex

Purified TPO autoantibody TR1.9 (expressed as a Fab) has previously been reported to form an immune complex with purified TPO, as evident by immunoprecipitation of tracer quantities of [125I]TPO (7) and by gel filtration of larger quantities of unlabeled proteins (32). For maximal epitope protection, we complexed TPO with a 3- to 6-fold molar excess of TR1.9 (e.g., 1 mg of TPO and 3 mg of TR1.9). Both TPO alone and the TPO-Fab complex were then heavily biotinylated under native conditions using a biotin/protein molar ratio of ~100:1. The objective of this approach was to biotinylate many Lys residues as feasible on the surface of free TPO. However, a Lys residue(s) on TPO at the Ab-antigen interface would be protected from biotinylation (Fig. 1). The extent of biotinylation of TPO and the TPO-TR1.9 complex was determined by the HABA reaction to be typically about 15 biotins/molecule of protein. Before proteolytic digestion, it was then necessary to remove the TR1.9 Fab from the TPO-ep-Bio.

Dissociation of the TPO-TR1.9 complex was achieved by reduction as well as by denaturation in 6 M guanidine. The TPO-ep-Bio (100 kDa) was separated by gel filtration from the Fab, which dissociates under these conditions into heavy and light chains of 25 kDa each. The column was equilibrated in 4 M guanidine to avoid the possibility of Ab-Ag reassociation. The TPO peak (excluding the trailing edge overlapping with the Fab) was retained for trypsin digestion (Fig. 2A). Although not necessary because of the absence of Fab, TPO-Bio was processed in parallel (Fig. 2B).

The efficacy of removal of the Fab from the TPO-TR1.9 complex was confirmed by PAGE. Thus, despite the large excess of Fab, none remained in the TPO-ep-Bio material used in subsequent procedures (Fig. 2C, lane 6). It should be noted that glycosylated TPO stains less well with Coomassie blue than does the nonglycosylated Fab. In addition, the presence of ~15 biotin molecules on TPO, estimated to contribute about 4 kDa, is evident by the slightly slower mobility of TPO-Bio and TPO-ep-Bio vs nonbiotinylated TPO included as a control (Fig. 2C, lanes 4 and 6 vs lanes 2 and 5). The presence of biotin on TPO-Bio and TPO-ep-Bio was also confirmed by their complete removal on adsorption with streptavidin-agarose (data not shown).

Difference between the trypsin fragment profiles of TPO and epitope-protected TPO

In preliminary experiments we determined the optimal conditions for trypsinization of nonbiotinylated, uncomplexed TPO. Reverse-phase HPLC on an acetonitrile gradient revealed complete digestion of the protein with a large spectrum of released fragments (Fig. 3A). The basis for the epitope-mapping approach used in the
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present study was that the tryptic fragment profile was subtly altered following biotinylation of TPO (Fig. 3B), presumably because biotinylation of Lys (K) residues on the surface of the native molecule inhibited trypsin action at these sites. Because Arg (R) residues are not biotinylated, these sites, in addition to nonbiotinylated K residues, would remain available to trypsin. High affinity Ab binding to TPO would prevent biotinylation of a K residue(s) within its epitope. This epitope protection would, therefore, restore the trypsin site lost when the Ag was biotinylated in the absence of Ab (Fig. 1).

Comparison of tryptic fragment profiles of the three forms of TPO (TPO, TPO-Bio, and TPO-ep-Bio) revealed a number of peaks that appeared or disappeared. However, very few appeared after biotinylation of the TPO-Fab complex (Fig. 3C) and disappeared again upon biotinylation of the TPO-Fab complex (Fig. 3C). The same profiles were confirmed on more than five biotinylations and digestions. In addition, biotinylation of this peptide was confirmed by its N and C termini and an internal biotinylated K residue. To confirm this expectation as well as to identify precisely the amino acids comprising this polypeptide, we collected the 38 min peak after injecting a much larger quantity (2 mg) of trypsin-digested TPO-Bio and using a more shallow acetonitrile gradient to maximize separation from adjacent peaks. Rejection of an aliquot of the collected material confirmed the purity and elution time (38 min) of the peak (Fig. 5A). However, the amino acid composition of this polypeptide did not correspond to any tryptic fragment predicted by computer analysis of the TPO amino acid sequence. The likely reason for these inconclusive data was evident on amino acid sequencing, which revealed multiple (two or three) N termini. Either multiple polypeptides were eluting at the identical location or the peak contained disulfide-linked polypeptides formed upon refolding of the denatured and reduced TPO.

To distinguish between these two possibilities we reinjected the 38 min peak (derived from trypsin digestion of another 2 mg of TPO-Bio) following reduction with 2-ME. The elution of multiple peaks indicated the presence of disulfide-linked polypeptides. These peaks were collected after maximizing separation on a more shallow acetonitrile gradient (Fig. 5B). Only the dominant peaks (1 and 2) contained peptides, as determined by amino acid composition analysis. Unlike before reduction, amino acid composition and its N and C termini and an internal biotinylated K residue. To confirm this expectation as well as to identify precisely the amino acids comprising this polypeptide, we collected the 38 min peak after injecting a much larger quantity (2 mg) of trypsin-digested TPO-Bio and using a more shallow acetonitrile gradient to maximize separation from adjacent peaks. Rejection of an aliquot of the collected material confirmed the purity and elution time (38 min) of the peak (Fig. 5A). However, the amino acid composition of this polypeptide did not correspond to any tryptic fragment predicted by computer analysis of the TPO amino acid sequence. The likely reason for these inconclusive data was evident on amino acid sequencing, which revealed multiple (two or three) N termini. Either multiple polypeptides were eluting at the identical location or the peak contained disulfide-linked polypeptides formed upon refolding of the denatured and reduced TPO.

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**FIGURE 1.** Localization of an epitope on an intact, native molecule by protection from biotinylation of superficial lysine (K) residues. For simplicity, only a linear epitope is depicted. However, the same principle would apply to a conformational, discontinuous epitope. A, Among a number of trypsin sites in a polypeptide segment (R and K residues, shown by small arrows), a K residue (C) is present within the epitopic site (black heavy line). Trypsin digestion at these sites generates four fragments, including fragments X and Y that contain the epitope (all or in part). B, Upon biotinylation of the protein in the absence of Ab, the K residue within the epitope is biotinylated, and this trypsic site is eliminated, resulting in the generation of three (rather than four) tryptic fragments. The X and Y fragments are lost, but are replaced with a new fragment (Z) containing the biotinylated K residue. C, The formation of an Ag-Ab (or Fab) complex before biotinylation blocks the lysine biotinylation site. Therefore, the lysine in the epitope will again be subject to trypsin digestion. However, before trypsin digestion it is necessary to dissociate the Ag-Ab complex and to purify the Ag, although the latter no longer is required to be native. Once again, trypsin would generate four fragments, including X and Y, and fragment Z (B) would be lost. Comparing the digestion profiles of the three antigenic materials on reverse-phase HPLC, either the newly created fragment Z (B) or the lost fragments X and/or Y (A) could be isolated for amino acid composition and sequencing. The lysine involved in the Ab epitope would, therefore, be identified.

**FIGURE 2.** Separation by gel filtration of biotinylated, epitope-protected TPO from TR1.9 Fab. A, Reduction and denaturation of the TPO-TR1.9 complex dissociates TPO-ep-Bio (100 kDa) from the Fab (50 kDa), which also dissociates into 25-kDa heavy and light chains. This mixture (1 ml) was applied to a Sephacryl 200 column equilibrated in 4 M guanidine, and 1-ml fractions were collected. The leading and trailing peaks represent TPO and the TR1.9 heavy and light chains, respectively. The TPO peak (except for the trailing edge overlapping with the Fab) was retained for trypsin digestion. Absorbance was measured at 254 nm. B, TPO biotinylated in the absence of TR1.9 (TPO-Bio) was treated identically (reduction and denaturation) and applied to the same column. C, Efficacy of separation of TPO from the TR1.9 Fab. PAGE (10% gel) was performed under denaturing and reducing conditions, followed by staining with Coomassie blue. Lane 1, M, markers. Lanes 2 and 3, TPO (1 μg) and TR1.9 (~3 μg), respectively. These proteins, included as controls, were nonbiotinylated, uncomplexed, and not subjected to gel filtration. The concentrations of TPO and TR1.9 were estimated by their extinction coefficients of 17.9 and 13.5 (280 nm), respectively. Lane 4, TPO-Bio; TPO biotinylated and recovered following gel filtration (B). Lane 5, TPO-TR1.9; the TPO-Fab complex before biotinylation and not subjected to gel filtration. Lane 6, TPO-ep-Bio; epitope-protected, biotinylated TPO recovered following gel filtration (A). Note the absence of contaminating Fab in this preparation as well as its slightly larger size relative to the nonbiotinylated TPO (lanes 2 and 5). Also note that glycosylated TPO stains less well with Coomassie blue than does the nonglycosylated Fab.
CONFORMATIONAL TPO AUTOANTIBODY EPITOPE

FIGURE 3. Effect of biotinylation of TPO alone or complexed with autoantibody on the TPO tryptic fragment profile. Qualitative reverse-phase HPLC analyses of tryptic digests were performed for nonbiotinylated TPO (A), TPO biotinylated in the absence of autoantibody (TPO-Bio; B), and TPO biotinylated after complexing with human TPO autoantibody TR1.9 expressed as a Fab (TPO-ep-Bio; C). Biotinylations were performed on native, conformationally intact TPO molecules, after which they were purified by gel filtration under denaturing and reduced conditions (see Materials and Methods). The ordinate represents absorbance at 214 nm. The arrow in B indicates a peptide eluting at 38 min following digestion of TPO-Bio that is not present in nonbiotinylated TPO and that disappears after biotinylation of the TPO-Fab complex (C). This profile was observed on more than five injections from five separate biotinylations.

FIGURE 4. The tryptic fragment eluting at 38 min on reverse-phase HPLC is biotinylated. TPO was biotinylated (TPO-Bio) and digested with trypsin (see Materials and Methods). Aliquots of 50 μg were subjected to reverse-phase HPLC either directly following trypsin digestion (upper tracing) or following trypsin digestion and subsequent adsorption on streptavidin-agarose beads (lower tracing). The fragment eluting at 38 min (arrow) is clearly among those removed by streptavidin.

N-terminal sequencing unequivocally identified peak 2 (Fig. 5B) to comprise a 30-aa polypeptide (TPO residues 704–733). As predicted, the polypeptide was flanked by tryptic cleavage sites (N terminus immediately downstream of R703 and the C terminus at R733). Most important, to confirm epitope protection, TPO-Bio residues 704–733 should contain a biotinylated K at position 713. Indeed, extended N-terminal sequencing identified all amino acid residues from 704–714 except for residue 713, which appeared as a strong, unrecognized signal. The polypeptide eluting at peak 1 could not be clearly identified because of contamination with the peak 2 polypeptide.

Flow cytometric evidence that K713 is part of the TPO autoantibody immunodominant region

TPO residue K713, protected by monoclonal autoantibody TR1.9, is one of a cluster of amino acids (aa 713–720) replaced with the homologous MPO residues in a chimeric TPO-MPO molecule (G2) (35). In this chimera, in which MPO residues SYPRDFVN replace TPO residues KFPEDFES, there is a K713S substitution in the TPO molecule. In principle, therefore, TPO-MPO chimera G2 should lack at least one (K713), and perhaps more, of the amino acid residues in the TR1.9 epitope (16–20 aa residues for a typical polypeptide epitope). Human autoantibodies to the immunodominant region do not recognize MPO (36). We, therefore, used flow cytometry to compare TR1.9 binding to intact CHO cells expressing on their surface either wild-type TPO or TPO-MPO chimera G2. In addition, we tested binding to these cell lines of another human monoclonal autoantibody, SP1.5, whose epitope does not overlap with that of TR1.9 yet still lies within the autoantibody immunodominant region. With the wild-type TPO, TR1.9 binding closely paralleled that of SP1.5 over a wide range of Ab dilutions (Fig. 6A). In contrast, TR1.9 recognized TPO-MPO chimera G2 less well than did SP1.5 (Fig. 6B). These data provide supporting evidence that TPO residue K713 comprises part of the TR1.9 epitope and, therefore, identifies an amino acid residue in the TPO immunodominant region.

Discussion

The approach that we used to identify for the first time a specific amino acid in the TPO immunodominant region developed from an observation made during preliminary digestions of TPO using the proteolytic enzyme lysyl endopeptidase (Lys-C), which cleaves polypeptides at the C terminus of lysine (K) residues. In contrast to near complete digestion (assessed by reverse-phase HPLC) at the 24 K residues in TPO, digestion of biotinylated TPO yielded fewer fragments, indicating that biotinylation at K residues inhibited proteolytic digestion (data not shown). We reasoned that trypsin, cutting also at more common (i.e., 62) R residues, would provide a more varied profile of smaller peptides, thereby facilitating detection of epitope protection at biotinylated K residues (see Fig. 1).

An important feature of the approach used in the present study is that biotinylation (in the presence or the absence of Ab) was performed on the native protein. Hydrophilic K residues are more frequent on the protein surface and from the extent of TPO biotinylation achieved (biotin/TPO molar ratio of ~15/1), it appears that at least 15 of the 24 K residues are on the surface of TPO. Once biotinylated, maintaining the native form is no longer necessary; indeed, denaturation facilitates complete proteolytic digestion.
Other than the use of chimeric TPO-MPO molecules (which failed to identify the immunodominant region) (35, 37), most previous attempts to identify TPO autoantibody epitopes have examined polyclonal autoantibody interactions with polypeptide fragments (12–25) with the inherent problem of the highly conformational nature of TPO autoantibody epitopes. However, based on the putative three-dimensional model of TPO, an r- pep fragment (TPO aa residues 742–848) (23), although not defining a precise epitope, may lie in the vicinity of K713 located in the present study. The recent use of polyclonal rabbit antisera to TPO peptides in competition studies has located a TPO autoantibody epitope (TPO aa residues 599–617) believed to be part of the immunodominant region (27). However, this epitope lies on the opposite pole of the 100-kDa Ag to K713. Further studies are necessary to understand the relationship between this epitope and K713. Conceivably, each could define the extreme perimeters of the immunodominant region on one face of the molecule; TR1.9 defines only a portion of this region.

It should be emphasized that human mAb are the optimal tools for identifying the immunodominant region (35, 37), most previous attempts to identify TPO autoantibody epitopes have examined polyclonal autoantibody interactions with polypeptide fragments (12–25) with the inherent problem of the highly conformational nature of TPO autoantibody epitopes. However, based on the putative three-dimensional model of TPO, an r- pep fragment (TPO aa residues 742–848) (23), although not defining a precise epitope, may lie in the vicinity of K713 located in the present study. The recent use of polyclonal rabbit antisera to TPO peptides in competition studies has located a TPO autoantibody epitope (TPO aa residues 599–617) believed to be part of the immunodominant region (27). However, this epitope lies on the opposite pole of the 100-kDa Ag to K713. Further studies are necessary to understand the relationship between this epitope and K713. Conceivably, each could define the extreme perimeters of the immunodominant region on one face of the molecule; TR1.9 defines only a portion of this region.

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It should be emphasized that human mAb are the optimal tools for identifying the immunodominant region (35, 37), most previous attempts to identify TPO autoantibody epitopes have examined polyclonal autoantibody interactions with polypeptide fragments (12–25) with the inherent problem of the highly conformational nature of TPO autoantibody epitopes. However, based on the putative three-dimensional model of TPO, an r- pep fragment (TPO aa residues 742–848) (23), although not defining a precise epitope, may lie in the vicinity of K713 located in the present study. The recent use of polyclonal rabbit antisera to TPO peptides in competition studies has located a TPO autoantibody epitope (TPO aa residues 599–617) believed to be part of the immunodominant region (27). However, this epitope lies on the opposite pole of the 100-kDa Ag to K713. Further studies are necessary to understand the relationship between this epitope and K713. Conceivably, each could define the extreme perimeters of the immunodominant region on one face of the molecule; TR1.9 defines only a portion of this region.
K713 is the only residue (or one of a few residues) common to both the TR1.9 component of the immunodominant region and the mAb 47/C21 epitope. With ~20 contact amino acids within an epitope, it is clear that alteration of one, or a few, residues (as in TPO-MPO chimera G2) may produce only subtle changes in Ab affinity. Our previous inability to detect gross changes in monoclonal autoantibody binding to TPO-MPO chimeric molecules points to the limitation of mutagenesis studies and emphasizes the value of the epitopic protection approach used in the present study. It is also apparent from the complexity of the three-dimensional structure of MPO (and hence TPO; Fig. 7) that the short segment defined by mAb 47 represents only a linear component in a larger, possibly discontinuous, epitope. For this reason, too, it is not surprising that the human monoclonal autoantibodies to TPO fail to recognize 66- to 200-aa polypeptides generated in a TPO cDNA fragment library (6) (S. McLachlan, J. Guo, L. Farilla, and B. Rapoport, unpublished observations).

In conclusion, using an epitope protection approach, we provide the first identification of an amino acid residue (K713) comprising part of an epitope within the TPO immunodominant region. Localization of this focal residue localizes the facet on the large, highly complex TPO molecule that contains the immunodominant region and provides the basis for rational guided mutagenesis studies to more fully characterize this region.

References


