Localization of the Thyroid Peroxidase Autoantibody Immunodominant Region to a Junctional Region Containing Portions of the Domains Homologous to Complement Control Protein and Myeloperoxidase*

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Thyroid peroxidase (TPO) autoantibody epitopes are largely restricted to an immunodominant region (IDR) on the extracellular region of the native molecule. Localization of the IDR has been a longstanding and difficult goal. The TPO extracellular region comprises a large myeloperoxidase-like domain, linked to the plasma membrane by two smaller domains with homology to complement control protein (CCP) and epidermal growth factor (EGF), respectively. Recent studies have focused on the CCP- and EGF-like domains as the putative location of the TPO autoantibody IDR. To address this issue, we attempted to express on the surface of transfected cells native TPO in which the CCP- and EGF-like domains were deleted, either together or individually. We used a quartet of human monoclonal autoantibodies that define the TPO IDR, as well as polyclonal TPO autoantibodies in patients’ sera, to detect these mutated TPO molecules by flow cytometry. The combined CCP/EGF-like domain deletion did not produce a signal with TPO autoantibodies but did not traffic to the cell surface. In contrast, both monoclonal and polyclonal autoantibodies recognized TPO with the juxtamembrane EGF-like domain deleted equally as well as the wild-type TPO on the cell surface. TPO with the CCP-like domain deleted expressed normally on the cell surface, as determined using the polyclonal mouse antiserum. Nevertheless, this modified TPO molecule was recognized very poorly by both the human monoclonal autoantibodies and the polyclonal autoantibodies in patients’ sera. In conclusion, we have clearly excluded the juxtamembrane EGF-like domain as being part of the IDR. In contrast, a component of the CCP-like domain does contribute to the IDR. These data, together with findings from other studies, localize the TPO autoantibody IDR to the junction of the CCP-like domain and the much larger myeloperoxidase-like domain on TPO.

Thyroid peroxidase (TPO), a heme-containing glycoprotein on the thyrocyte apical membrane that plays a key role in thyroid hormone biosynthesis, is also the dominant autoantigen in human autoimmune thyroiditis (1–3). A remarkable feature of polyclonal TPO autoantibodies in the sera of all patients is that their epitopes are largely directed to a restricted area on the native antigen, termed the immunodominant region (IDR) (reviewed in Ref. 4). This epitope profile contrasts with antibodies generated in mice by immunization with purified TPO together with adjuvant, which have a wide range of epitopes on both native and denatured TPO (5). Even within the IDR, TPO autoantibodies in an individual patient maintain the same epitopic fingerprint over many years (lack of epitope spreading) (6), a phenomenon that appears to have a genetic basis (7).

The role of TPO autoantibodies in the pathogenesis of disease is debated. Some evidence supports their involvement in thyrocyte damage by antibody-mediated cellular cytotoxicity (8, 9). Perhaps more important is the influence of antibodies complexed to antigen in modulating antigen processing with enhancement or suppression of presentation of different T cell determinants (10). Identification of the TPO IDR may, therefore, provide important information for understanding the immunopathological mechanism underlying autoimmune thyroiditis and may facilitate identification of molecules for immunosuppression of disease. Consequently, there has been much effort over the past decade, utilizing different approaches, to identify the precise amino acids that comprise the TPO IDR, a difficult task because of the need to work with the native molecule and the lack of information on the three-dimensional structure of TPO.

TPO is a 933-amino acid residue molecule with a single membrane-spanning region (11–13). The major extracellular portion of the TPO molecule (amino acid residues 1–745) has high (∼42%) homology to myeloperoxidase (MPO), an intracellular enzyme whose three-dimensional structure has been determined (14). The extracellular region of TPO also contains a juxtamembrane segment (amino acid residues 741–838) with a complement control protein (CCP)-like domain and an epidermal growth factor (EGF)-like domain (13, 15) (Fig. 1). Numerous studies (for example, Refs. 16–26) over the past decade have reported human TPO autoantibody epitopes in disparate locations throughout the MPO-like region. More recently, the CCP- and EGF-like portions of TPO have received much attention as containing a conformational B cell epitope for TPO autoantibodies (15, 27, 28). The present study was undertaken to address the question of the relative importance of the CCP- and EGF-like domains in the IDR of TPO autoantibodies in human autoimmune thyroid disease.
TPO Autoantibody Immunodominant Region

MATERIALS AND METHODS

TPO Deletion Mutants—The cDNA for human TPO (11) in pcDNA3.1 was used as template for deletions. The following TPO codons were deleted from the cDNA by generating overlapping PCR fragments using Pfu (Stratagene, La Jolla, CA) (Fig. 1): (i) codons 741–794 (CCP-like domain of TPO); (ii) codons 795–838 (EGF-like domain); and (iii) codons 741–838 (both CCP- and EGF-like domains). The upstream oligonucleotide primer contained the internal ClaI site in TPO (codons 631 and 632). The downstream oligonucleotide primer included an XbaI site in the vector multiple cloning site. After ClaI and XbaI restriction, the cDNA fragments were substituted for the corresponding regions in the wild-type TPO cDNA in pcDNA3.1. The PCR-generated fragments and their cloning sites were confirmed by nucleotide sequencing.

Expression of TPO and Detection by Flow Cytometry—COS-7 cells were cultured in 10-cm diameter dishes in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and standard antibiotics. Plasmids (10 μg) for the wild-type TPO, the three different TPO deletion mutants, and the empty vector were transiently transfected into the COS-7 cells using FuGENE 6 (Roche Molecular Biochemicals) according to the method of the manufacturer. Two days after transfection, cells were resuspended by mild trypsinization and used for flow cytometry. The following antibodies were used in a final volume of 0.1 ml: (i) polyclonal TPO antiserum (dilution 1:500) pooled from 5 BALB/c mice immunized with purified recombinant human TPO together with complete Freund’s adjuvant (30); (ii) 4 recombinant human monoclonal autoantibodies to TPO expressed as Fab (TR1.8, TR1.9, WR1.7, and SP1.5; 10 μg/ml) (31); and (iii) 11 sera (dilution 1:50) from patients with thyroid autoimmunity known to contain TPO autoantibodies.

Antibody binding was detected with mouse anti-human κ phycoerythrin-conjugated monoclonal antibody or goat anti-mouse heavy plus light chain phycoerythrin-conjugated monoclonal antibody (both from Caltag, Burlingame, CA). Assays also included cells incubated with buffer alone or second antibody alone. Flow cytometry was performed (10,000 events) using a FACScan with CellQuest Software (BD PharMingen).

Competition by Monoclonal TPO Autoantibodies for Serum Antibody Binding to TPO—The competition assays were performed as previously described (30, 31). In brief, the polyclonal mouse antiserum to TPO (see above) was incubated with [125I]TPO for 1 h at room temperature (~15,000 cpm, total volume 200 μl). Competition for this binding to [125I]TPO was performed by including a pool (10^-6 m each) of four recombinant human monoclonal TPO autoantibodies expressed as Fab (TR1.8, TR1.9, WR1.7, and SP1.5) (30, 31). These four Fab define the TPO autoantibody immunodominant region. In the same assay, we included human serum with TPO autoantibodies (pool of sera from eight patients). Preliminary experiments were performed to determine the dilution of the mouse and human sera to achieve binding values of ~15% in the absence of the monoclonal TPO autoantibodies. Such dilution is necessary to obtain maximal inhibition of TPO binding by a saturating concentration of monoclonal autoantibodies. Immune complexes were precipitated using Pansorbin (Calbiochem) for the human sera and Sac-Cel (IDS, Boldon, Great Britain) for the mouse sera (30), as previously described in detail. Nonspecific binding (~3% of total cpm) was subtracted to calculate the percentage of specific binding.

RESULTS

Deletion of Both CCP- and EGF-like Domains in TPO—To determine directly whether the TPO autoantibody IDR was contained, wholly or in part, in the CCP- and EGF-like domains of TPO, we transfected COS-7 cells with TPO cDNA in which both of these domains (amino acid residues 741–838) were deleted (Fig. 1, Del-CCP/EGF). In this construct, the major MPO-like domain (residues 1–740) was linked to the membrane-spanning and intracellular components of TPO. For detection of the autoantibody IDR, we performed flow cytometry with four recombinant monoclonal human autoantibodies, expressed as Fab, that define this region (31). A representative example of these experiments, using one of the monoclonal human TPO autoantibodies (TR1.9) is shown in Fig. 2A. Because not all transiently transfected COS-7 cells express TPO on the surface, we expressed our data as the percentage gated in M2 rather than mean fluorescence. None of the monoclonal autoantibodies (TR1.8, TR1.9, WR1.7, and SP1.5, each to one quadrant of the IDR) produced a specific signal when tested with cells transfected with the TPO mutant (Fig. 2B). In contrast, as positive controls in the same experiments, the monoclonal autoantibodies readily recognized the wild-type TPO on the cell surface. As expected, no signal was observed with any of these antibodies when tested on cells transfected with the empty vector.

Two possible explanations existed for the inability of the monoclonal human autoantibodies to recognize TPO with the CCP/EGF-like domains deleted. Either the TPO autoantibody IDR was completely contained within the CCP/EGF-like domains or, alternatively, the consequence of the deletion was failure of the mutated TPO molecule to normally fold and/or traffic to the cell surface. To distinguish between these two possibilities we used a polyclonal murine antiserum with widely diverse epitopes extending beyond the autoantibody IDR (30). As with the monoclonal human autoantibodies, this serum failed to recognize cells transfected with TPO with the CCP/EGF-like domains deleted (Fig. 2, A and C). These data indicated failure of the mutant TPO to traffic to the cell surface, rather than loss of the TPO autoantibody IDR.

Deletion of the EGF-like Domain in TPO—Because deletion of a smaller segment, rather than the entire CCP/EGF-like segment, could be compatible with cell surface expression, we deleted the juxtamembrane EGF-like domain alone (Fig. 1, Del-EGF). After transient transfection of COS-7 cells, flow cytometry was performed with the four monoclonal autoantibodies to the TPO immunodominant region (TR1.8, TR1.9, WR1.7, and SP1.5). All four recognized Del-EGF on the cell surface relative to the background signal observed with normal human IgG. One representative experiment is shown in Fig. 3A. There was no difference between monoclonal autoantibody recognition of Del-EGF and the wild-type TPO. No specific signals were evident with cells transfected with the empty vector. Data from three experiments are summarized in Fig. 3B.

Consistent with equal monoclonal autoantibody recognition of Del-EGF and wild-type TPO, the polyclonal mouse serum to TPO, with a much broader range of epitopes, produced similar signals on flow cytometry using cells expressing Del-EGF and the wild-type TPO (Fig. 3C). These data clearly demonstrate that the EGF-like domain on TPO is not a component of the IDR.

Deletion of the CCP-like Domain in TPO—Finally, we studied TPO with the CCP-like domain deleted (Fig. 1, Del-CCP). The same four monoclonal autoantibodies that define the TPO immunodominant region (TR1.8, TR1.9, WR1.7, and SP1.5)
were used in flow cytometry following transient transfection of COS-7 cells with the cDNA for TPO, and the CCP- and the EGF-like domains deleted (Del-CCP/EGF). As controls, cells were also transfected with the cDNA for wild-type TPO or the empty vector (vector). A, examples of flow cytometric data contributing to panels B and C. Data are shown for the human monoclonal autoantibody TR1.9 and the polyclonal mouse antiserum to TPO (see “Materials and Methods”). Because transient transfection does not produce TPO expression in all cells, mean or median fluorescence values cannot be used. Therefore, we expressed the data as the percentage of cells attaining an arbitrary level of fluorescence above the non-expressing cells; the M2 gate is indicated. Flow cytometric data in subsequent figures utilized the same M2 gate. B, flow cytometry using four recombinant human monoclonal autoantibodies expressed as Fab (TR1.9, TR1.8, WR1.7, and SP1.5, 10 μg/ml; see “Materials and Methods”). Background binding was assessed using purified normal human IgG. Data shown for the M2 gate are the mean ± S.E. of values from three separate experiments. C, flow cytometry using a polyclonal mouse antiserum to TPO (pool of five mice immunized with purified TPO together with complete Freund’s adjuvant; dilution 1:50). Background binding was assessed using normal mouse IgG. The percentage of cells within the M2 gate is shown as the mean ± S.E. of values from three separate experiments.

Fig. 3. Deletion of the EGF-like domain in TPO. COS-7 cells were transfected with the cDNA for TPO with the EGF-like domain deleted (Del-EGF). As controls, cells were also transfected with the empty vector (vector) with the cDNA for wild-type TPO. A, flow cytometry using four recombinant human monoclonal autoantibodies expressed as Fab (TR1.9, TR1.8, WR1.7, and SP1.5, 10 μg/ml; see “Materials and Methods”). Background binding was assessed using purified normal human IgG. B, summary of data from three separate experiments, such as shown in Fig. 3A. Data are expressed as the percentage of cells in the M2 gate. Bars indicate the mean ± S.E. of values obtained in the three experiments. C, flow cytometry using a polyclonal mouse antiserum to TPO (dilution 1:50). Background binding was assessed using normal mouse IgG. The percentage of cells within the M2 gate are shown as the mean ± S.E. of values from three separate experiments.
served with cells transfected with the empty vector. In contrast to Del-CCP, the human monoclonal autoantibodies all gave a strong signal when tested with COS-7 cells expressing the wild-type TPO. Data from a representative experiment are shown in Fig. 4A, and the values from three experiments are summarized in Fig. 4B.

As for TPO with both CCP- and EGF-like domains deleted (see above), the very weak signal observed with Del-CCP could be because of impaired folding and/or transport of the modified molecule to the cell surface. Dramatically, however, and in contrast to Del-CCP/EGF, the polyclonal mouse antiserum to TPO recognized Del-CCP very well, producing a signal only slightly less than with the wild-type TPO (Fig. 4C). The dissociation between monoclonal autoantibody and polyclonal mouse antiserum recognition of the same Del-CCP cells clearly establishes that deletion of TPO amino acid residues 741–794 eliminates or obscures the TPO autoantibody, IDR.

The slightly lower recognition by the polyclonal mouse antiserum of Del-CCP relative to the wild-type TPO would be consistent with this antiserum having epitopes both within and without the human autoantibody IDR (30). To confirm this observation, we used a pool of the four monoclonal TPO autoantibody Fab to the IDR (10^–8 M each) to compete for the mouse serum binding to [125I]TPO. This pool of Fab only partially inhibited, by about 40%, the mouse antiserum binding to TPO (Fig. 5A). In contrast, the Fab inhibited TPO binding by a pool of eight sera from patients with autoimmune thyroid disease to a much greater extent (88%). The polyclonal mouse antiserum, therefore, recognizes a broader range of epitopes on the TPO surface than the human autoantibodies (Fig. 5B) and is hence less affected than the autoantibodies by deletion of the CCP-like region on TPO.

Recognition of TPO Deletion Mutants by Polyclonal TPO Autoantibodies in Patients’ Sera—Definitive proof that the TPO autoantibody IDR was either deleted or obscured in Del-CCP would be reproduction of the human monoclonal autoantibody data with polyclonal TPO autoantibodies in the sera of patients with autoimmune thyroid disease. We, therefore, performed flow cytometry using 11 randomly selected sera with TPO autoantibodies. As anticipated, all sera recognized COS-7 cells transiently transfected with the cDNA encoding wild-type TPO (Fig. 6A). As observed with the monoclonal human autoantibodies, the polyclonal TPO autoantibodies barely recognized Del-CCP yet interacted with Del-EGF to the same extent as with wild-type TPO. As a positive control for the level of Del-CCP expression, the polyclonal mouse serum to TPO with epitopes both within and outside the IDR gave a signal with Del-CCP only slightly less than with the wild-type TPO and with Del-EGF (Fig. 6B).

**DISCUSSION**

TPO is a highly unusual member of the peroxidase family in that it is a membrane-associated protein with its large functional region orientated toward the exterior of the thyroid follicular cell. TPO is also the primary enzyme involved in thyroid hormone synthesis, a process involving iodination of thyroglobulin stored extracellularly in the thyroid follicular lumen. Autoantibodies to the extracellular region of TPO are a sine qua non of active autoimmune (Hashimoto’s) thyroiditis and are also present in the majority of patients with Graves’ disease, which together are the most common autoimmune diseases affecting humans. Polyclonal TPO autoantibody epitopes in all patients are largely restricted to one facet of the native molecule, the IDR with an epitopic ‘fingerprint’ that remains constant over many years and clusters in families (5, 31).

Understanding the basis for this remarkable phenomenon requires, as a first step, identification of the precise TPO amino acid residues affecting an autoantibody(s) that remains constant over many years and clusters in families (5, 31).
acids that comprise the IDR. This task has, however, been exceptionally difficult and has led to much data with disparate conclusions (for example, Refs. 16–26). The primary reason for this difficulty is that the epitopes comprising the IDR are highly conformational and almost certainly discontinuous (reviewed in Ref. 32). In addition, a number of points are frequently misunderstood, including the following. (i) Recognition of a TPO polypeptide fragment by autoantibodies in the majority of patients’ sera is insufficient to establish that this fragment is part of the immunodominant region. Serum autoantibodies are polyclonal, and a signal can be obtained from the minority of autoantibodies with epitopes outside the IDR. (ii) Competition for autoantibody binding to native TPO by immune sera generated in animals may involve steric hindrance by very large molecules rather than direct overlap of epitopic sites. Competition with much smaller, monomeric and monoclonal Fab is more likely to yield reliable conclusions. (iii) Based on the closely homologous MPO three-dimensional structure (14), shown empirically by chimeric MPO-TPO studies to be similar to that of TPO (33), numerically distant amino acids in the TPO primary sequence may be closely associated in the native molecule and vice versa.

The CCP/EGF-like domain of TPO is a very interesting candidate for containing, at least in large part, the autoantibody IDR. It is the most unique component of TPO, without any comparable segment in MPO (a soluble molecule not associated with the cell membrane). Indeed, considerable recent evidence has supported the concept that the CCP/EGF-like domain contains TPO autoantibody epitopes (15, 27). Tyrosine 772 is particularly implicated as being involved in the IDR (28). Although elegant, these studies have a number of limitations, having been performed with polypeptide fragments that are not necessarily in the native conformation, involving detection with polyclonal autoantibodies or competition with intact murine monoclonal antibodies (not monoclonal human autoantibodies and not Fab).

In the present study, we have directly determined the interaction between human monoclonal TPO autoantibodies (expressed as Fab) with the native antigen expressed on the cell surface. Deletion of the entire CCP/EGF-like domain (amino acid residues 741–838) resulted in a TPO molecule that did not traffic normally to the cell surface. However, deletion of only the CCP-like domain (amino acid residues 838–938) as being part of the TPO IDR. It is the most unique component of TPO, without any comparable segment in MPO (a soluble molecule not associated with the cell membrane). Indeed, considerable recent evidence has supported the concept that the CCP/EGF-like domain contains TPO autoantibody epitopes (15, 27). Tyrosine 772 is particularly implicated as being involved in the IDR (28). Although elegant, these studies have a number of limitations, having been performed with polypeptide fragments that are not necessarily in the native conformation, involving detection with polyclonal autoantibodies or competition with intact murine monoclonal antibodies (not monoclonal human autoantibodies and not Fab).

In the present study, we have directly determined the interaction between human monoclonal TPO autoantibodies (expressed as Fab) with the native antigen expressed on the cell surface. Deletion of the entire CCP/EGF-like domain (amino acid residues 741–838) resulted in a TPO molecule that did not traffic normally to the cell surface. However, deletion of only the CCP-like domain was informative. Our data demonstrate that the four human monoclonal autoantibodies that define the TPO IDR, as well as polyclonal TPO autoantibodies in patients’ sera, all bind to TPO-Del-EGF expressed on the cell surface as well as to the wild-type TPO. On this basis, we can definitively exclude the juxtamembrane EGF-like domain (amino acids 795–838) as being part of the TPO IDR.

Individual deletion of the CCP-like domain (amino acid residues 741–794) also yielded interesting and informative data. Del-CCP (like Del-EGF) expressed normally on the cell surface,
Lys713, (29). One amino acid, IDR The MPO prosequence-like domain does not contribute to the autoan-
tracellular region of native (not denatured) TPO (reviewed in Ref. 32). Human TPO autoantibodies interact with the ex-
and to epidermal growth factor (EGF-like) (13, 15). Human TPO autoantibodies interact with the ex-
membrane) are two smaller juxtamembrane domains with homology to the
121 are homologous to the MPO prosequence, and the largest
homology with myeloperoxidase (MPO) corresponds to the large MPO subunit. Not present in MPO (a soluble protein not tethered to the plasma mem-
brane) are two smaller juxtamembrane domains with homology to the complement control protein (CCP-like) and to epidermal growth factor (EGF-like) (13, 15). Human TPO autoantibodies interact with the extracellular region of native (not denatured) TPO (reviewed in Ref. 32).

TPO autoantibodies bind to the implicated CCP/EGF-like domain of TPO. This library is rich in yielding TPO autoantibodies when screened on purified, intact TPO (34, 35). In contrast, only two Idr TPO autoantibodies were obtained with purified CCP/EGF-like polypeptide, and only on low stringency screening. These data suggest that a major component of the IDR lies within the MPO-like region of TPO. Other evidence for the importance of the MPO-like do-
main in the IDR is that C-terminal-truncated TPO molecules lacking the CCP/EGF-like domains of TPO (synthesized as cell-free translates) were recognized by human autoantibodies (36). Finally, in the present study, the similar effect of the CCP-like domain deletion on binding of TPO autoantibody Fab (monoclonals) and intact autoantibody IgG (patients’ sera) suggests that loss of part of the IDR played a greater role than steric hindrance in reducing autoantibody recognition. All these data, taken together, suggest that the TPO autoantibody IDR contains portions of the MPO- and CCP-like domains and, therefore, bridges these two domains. A conceptual diagram of this interaction is depicted in Fig. 7.

In conclusion, we have analyzed the contribution to the TPO autoantibody, IDR, of the unique CCP- and EGF-like domains of TPO that link the MPO-like domain to the plasma mem-
brane. We have clearly excluded the juxtamembrane EGF-like domain as being part of the IDR. In contrast, a component of the CCP-like domain does contribute to the IDR. These data, together with information from other studies, localize the TPO autoantibody IDR to the junction of the CCP-like domain and the much larger MPO-like domain on TPO.

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