Identification of a Thyroxine-Containing Self-Epitope of Thyroglobulin Which Triggers Thyroid Autoreactive T Cells

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Summary

Although thyroglobulin (Tg), the thyroid prohormone, is well known as a T cell dependent autoantigen in human and experimental autoimmune thyroid disease, very little is known about the molecular basis of Tg recognition by T cells. In this paper, we have characterized the epitopes recognized by two clonotypically distinct, murine Tg autoreactive T cell hybridomas, CH9 and ADA2. In vitro iodination of a Tg preparation which was deficient in in vivo organified iodine was first used to confirm our previous observation that these T cells recognize iodination-related epitopes in the Tg molecule. Affinity chromatography of tryptic peptides derived from normally iodinated human Tg revealed that these epitopes were exclusively located in thyroxine (T4) containing peptides. Through the use of synthetic T4-containing peptides, representing the four major hormonogenic sites in Tg, we demonstrated that both CH9 and ADA2 recognize an epitope containing the T4 at position 2553 in human Tg. Sets of overlapping 5mer to 12mer peptides around this T4 showed that the most potent peptide was a 9mer beginning at Asp 2551. The T4 was shown to be a critical residue, since its replacement with any of the 20 naturally occurring amino acids produced only nonstimulatory peptides. Since the T cell hybridomas could also be stimulated by major histocompatibility complex class II positive (interferon-γ-treated) thyroid epithelial cells in vitro, and their parent T cell lines can induce thyroiditis on adoptive transfer, the T4-containing Tg sequence described here is implicated as a pathogenic epitope in murine thyroid autoimmunity.

The production of the thyroid hormones thyroxine (T4), tri-iodothyronine (T3) and reverse T3 (rT3) is dependent on the organification of iodine into thyroglobulin (Tg), the major protein product of the thyroid (1, 2). This involves thyroid peroxidase catalyzed iodination of tyrosine residues in Tg to form mono- and di-iodotyrosines and their subsequent crosslinking to form the iodothyronines T3 and T4. These mature Tg molecules are stored in a colloidal form in the lumen of thyroid follicles. Secretion of T4 and T3 involves the endocytosis and subsequent proteolysis of colloidal Tg, which releases the hormone residues for diffusion into the circulation. The recent cloning of the genes coding for Tg from several species has allowed the precise localization of the four main hormonogenic sites within the molecule (3–5). In human Tg, tyrosines at positions 5, 2553, 2567 and 2746 of the 2748 amino acid monomer may be modified to form T4 or T3 residues. Under conditions of limited iodine availability, hormonogenesis occurs preferentially at position 5 near the N-terminus (6, 7). Factors other than iodine levels which may regulate the degree of hormonogenesis at the other sites are not clear. However, some evidence suggests that position 2746 may be preferentially converted to T3 rather than T4 (8).

Tg has been known for over three decades as an autoantigen in human and experimental autoimmune thyroid disease (1, 9–11). Epidemiologic and experimental evidence has indicated that the autoantigenicity of Tg may be influenced by its iodine content. For example, raised dietary iodine levels in humans (12–14) and experimental animals (15–19) has been correlated with an increase in the incidence and severity of autoimmune thyroid lesions. In chickens, highly iodinated...
Tg has been shown to induce stronger autoantibody responses than poorly iodinated Tg (20), while in mice, inhibition of the peroxidase reaction with aminotriazole produces very poorly iodinated Tg which fails to elicit autoimmune thyroiditis in high responder CBA/J mice (21). To date, it is not clear which of the many iodination sites in Tg are responsible for these effects on Tg antigenicity or how they are mediated.

However, our recent observation that two clonotypically distinct autoreactive murine T cell hybridomas recognizes iodination-dependent epitopes in Tg (21) provided a clue to the molecular basis of Tg autoantigenicity. We now report that both of these T cells recognize a T4-containing peptide centered on the hormonogenic site at residue 2553 in human Tg, and that this epitope is expressed on the surface of mouse thyroid epithelial cells (TEC) cultured with IFN-γ.

Materials and Methods

Thyroglobulin Preparations. Mouse Tg (MTg) was prepared from pooled thyroids and human Tg (HTg) from individual post-mortem thyroids as previously described (22). The levels of organified iodine and thyroid hormone residues in Tg preparations were determined as described elsewhere (23).

Thyroglobulin Iodination. HTg of low iodine content (HTgMC, 0.08 T4 residues/mole) was iodinated with NaI by the Iodogen method (24), with reaction times of 10–80 min (transferring the mixture to fresh iodogen-coated tubes every 10 min) to produce preparations (TgMC-6) differing in their levels of organified T4 residues.

Preparation of Tg Peptides. HTg was reduced and alkylated with iodoacetamide in 8 M urea (25, 26), dialyzed to remove the urea, digested with TPCK-Trypsin (Sigma Chemical Co., Poole, UK) at an enzyme:substrate ratio of 1:50 in 0.1 M ammonium bicarbonate for 4 h. The digest was lyophilized and redissolved in 20 mM sodium phosphate, pH 7.4 to 1 mg/ml and passed through an affinity matrix consisting of rabbit anti-T4 anti-bodies (Miles, Stoke Poges, UK) coupled to tosyl activated Sepharose 4B (26). Unadsorbed peptides (T4-) were collected. Adsorbed peptides (T4+), which most likely represented T4-containing peptides (T4+) were recovered by elution with 0.1 M ammo mium bicarbonate for 1 h. The affinity-purified T4+ fraction was dialyzed into HBSS containing 20% FCS. Following centrifugation (200g, 10 min), the cell pellet was resuspended and washed two times with HBSS/10% FCS. The cells were then suspended in culture medium (RPMI/10% FCS) containing sodium iodide (1 IAM), hydrocortisone (10⁻⁴ M), bovine insulin (100 IU/ml), bovine transferrin (5 µg/ml) and HEPES (5 mM) as additional supplements. Because of clumping it proved difficult to accurately count the TECs before culture. Therefore, cells prepared from 10–12 thyroids were used to seed one complete 96-well flat-bottomed microtiter plate (200 µl/well). After 1 d of culture, nonadherent cells were removed by extensive washing and the adherent cells recultured in complete medium containing 1% FCS. After 2 d culture, some wells received rat IFN-γ (100 U/ml). At day 5, cells were washed three times with T cell hybridoma culture medium and Tg cells (10⁴/well) added for 24 h. Cell-free supernatants were then tested for IL-2 as described above. These TEC culture conditions were found to be optimal for the induction of I-A²MHC class II molecules on the TEC surface by IFN-γ, as assessed by standard immunohistochemical techniques with specific antibodies (not shown). Microscopic examination of the TEC monolayers before addition of hybridoma cells showed them to be free of mononuclear cells.

Results

Autoreactive Tg-specific Cloned T Cells Recognize Iodination-Dependent Epitopes of Tg. We have previously reported that...
two clonally distinct Tg-specific, I-A\(^{k}\)-restricted autoreactive T cell populations, represented by the hybridomas CH9 and ADA2 (and the parent clone of ADA2, called MTg9B3), only recognize Tg which is sufficiently iodinated (21). These clones have also been shown to have distinguishable epitope specificities by their differential responsiveness to a panel of Tgs prepared from different species (28). Both could be cross-stimulated by human Tg, but recognized bovine Tg weakly or not at all (28).

To further evaluate the critical role of iodination in recognition of Tg by these T cells, the iodogen method was used to generate iodothyrosine and iodothyonine residues in a poorly iodinated human Tg preparation (TgMC). Different incubation conditions were used to prepare samples (TgMC1-6) which differed in their total iodine content and their T4 levels. The response of MTg9B3 to these Tg preparations is illustrated in Fig. 1. In contrast to the lack of response to the poorly iodinated TgMC, MTg9B3 cells responded well to in vitro iodinated preparations TgMC1-5. The TgMC6 preparation failed to stimulate MTg9B3 cells. Similar results were obtained with the hybridomas ADA2 and CH9 (not shown). The lack of response to the most heavily iodinated preparation (TgMC6), found with all the cells, suggests that excessive iodination in some way blocks processing and presentation of the critical epitope.

CH9 and ADA2/MTg9B3 Recognize Thymoxine-Containing Tg Tryptic Peptides. To test the importance of T4 residues to the antigenic site(s) recognized by CH9 and ADA2/MTg9B3, human Tg was reduced and alkylated followed by trypsin digestion and affinity chromatography of the tryptic peptides on an anti-T4 column to produce T4-depleted (T4\(^{-}\)) and T4-enriched (T4\(^{+}\)) peptide preparations. As previously reported (1), CH9 responded as well to tryptic peptides as to native Tg or reduced/alkylated Tg, whereas ADA2 (and MTg9B3, not shown) responded less well to the trypsin digest (Fig. 2). Affinity chromatography effected a complete compartmentalization of the stimulatory peptides for both T cell populations into the T4\(^{+}\) peptide pool. Although peptides lacking T4 may have non-specifically adsorbed to the column to become included in the T4\(^{+}\) peptide pool, this result indicated that the epitope(s) is associated with a hormonogenic site (as opposed to a sequence containing iodothyrosines, or dehydroalanine produced incidentally during peroxidase-catalyzed Tg modification [1, 34]).

CH9 and ADA2/MTg9B3 Recognize Closely Related Epitopes of Human Tg Containing T4 at Residue 2553. The results portrayed in Fig. 2 indicated that the epitopes recognized by CH9 and ADA2 involved one or more of the four hormonogenic sites in Tg (1, 2), or were located close to one of these sites. The amino acid sequences of these 4 sites in human Tg are shown in Fig. 3. To determine which, if any, of these T4 residues was involved in forming the epitopes for CH9 and ADA2/MTg9B3, overlapping sets of 12-amino acid poly-
peptides centered on each of the four hormonogenic sites in the primary sequence of human Tg were synthesized. These peptides were then tested for their ability to stimulate CH9 and ADA2 cells. The results (illustrated in Fig. 4) clearly demonstrated that both CH9 and ADA2 recognize an epitope involving the T4 residue at position 2553. The sequence surrounding this residue corresponds to a potential T cell epitope based on two predictive algorithms (35, 36). Replacement of T4 in the sequence with any of the 20 naturally occurring amino acids (including tyrosine) abrogated the capacity to stimulate both CH9 (Fig. 5) and ADA2 (not shown), indicating that this T4 is a critical residue for both T cell hybridomas.

To delineate the boundaries of this epitope, we challenged the T cell hybridomas with a series of 5 to 12 mer peptides centered on the T4 at position 2553. T4-containing peptides of 8 amino acids or less were all non-stimulatory for either T cell hybridoma at concentrations up to 1.25 μM (not shown), as were peptides beginning at Asp 2552 (see Fig. 4). This indicates that the smallest fully effective epitope is a T4-containing 9mer. At present we cannot rule out the possibility that a smaller peptide could stimulate at much higher concentrations. Fig. 6 shows example dose curves for both CH9 and ADA2 stimulated with different 9 to 12mer peptides and summarizes the relative stimulatory capacity of these peptides in terms of the concentration required to achieve half-maximal stimulation (ED50). Deletion of Ala 2559 from the C-terminus of the peptide abrogated their ability to stimulate CH9, but only diminished their activity with ADA2 by 20–30-fold. Elongation of the peptides C-terminal to Ala 2559 consistently diminished the stimulatory capacity for both T cells, whereas addition of residues N-terminal to Asp 2551 had no appreciable effect on antigenic potency. These observations, coupled with previous data using a panel of Tgs from different species (28), indicate that CH9 and ADA2 recognize the same T4-containing epitope in Tg, but have subtly different structural requirements for optimal stimulation. Comparison of the published primary sequences of human (3), rat (4), and cow (5) Tg in this region revealed that the maximally stimulating 9mer (boxed in Fig. 7) is identical in all three species.

Mouse Thyroid Epithelial Cells Cultured with IFN-γ Express the Epitope Recognized by CH9. To determine whether the epitope described above can be expressed by thyroid cells, we cocultured CH9 with primary cultures of syngeneic TEC, without added Tg. TEC precultured with IFN-γ, using conditions which induce MHC class II expression (not shown) and increase Tg release (1), were able to stimulate IL-2 production by CH9 cells (Fig. 8). The level of response was generally low, but similar to that triggered by comparable numbers
Figure 6. Responses of CH9 and ADA2 to T4-containing peptides of different length representing the hormonogenic site at position 2553 of human Tg. (A and B) Representative dose-curves of CH9 and ADA2 responses to the peptides defined in C. (C) The responses of CH9 and ADA2 to the T4-containing peptides shown are represented as the concentration required to stimulate a half-maximal response (ED50). Peptides of 5, 6, 7, or 8 amino acids containing the T4 residue were all nonstimulatory (not shown). Results represent mean 3H-thymidine incorporation by triplicate cultures of CTLL cells. NT = not tested.

Table of Peptide Concentration (nM)

| Peptide | Sequence | ED50 (nM) | CH9 | ADA2 |
|---------|----------|-----------|-----|------|
| 2       | E H S T  | D D T4 A S F S R | >1250 | 30   |
| 7       | E H S T  | D D T4 A S F S S | >1250 | >500 |
| 3       | H S T D  | D D T4 A S F S A | 6    | 2.5  |
| 8       | H S T D  | D D T4 A S F S R | >1250 | 48   |
| 12      | H S T D  | D D T4 A S F S S | >1250 | >500 |
| 4       | S T D D  | T4 A S F S R A L | 100  | 72   |
| 9       | S T D D  | T4 A S F S R A L | 14   | 3.4  |
| 13      | S T D D  | T4 A S F S R A L | >1250 | 85   |
| 16      | S T D D  | T4 A S F S R A L | >1250 | >500 |
| 5       | T D D D  | T4 A S F S R A L E | 52 | 23   |
| 10      | T D D D  | T4 A S F S R A L E | 34 | 14   |
| 14      | T D D D  | T4 A S F S R A L E | 8   | 2.2  |
| 17      | T D D D  | T4 A S F S R A L E | >1250 | 70   |
| 6       | D D D D  | T4 A S F S R A L E N | 70 | 32   |
| 11      | D D D D  | T4 A S F S R A L E N | 75 | 32   |
| 15      | D D D D  | T4 A S F S R A L E N | 62 | 25   |
| 18      | D D D D  | T4 A S F S R A L E N | 12 | 7.7  |
| 1       | P L R Q G | G G G G G G G G G G | >1250 | NT   |

Figure 7. Comparison of the amino acid sequences of rat, human, and cow Tg around the hormonogenic site at position 2553 (2555 in cow Tg). The minimal 9mer sequence recognized by CH9 and ADA2 is boxed, as is the N-glycosylation site at position 2562 in human and rat Tg which is missing in cow Tg.

Figure 8. The epitope recognized by the T cell hybridoma CH9 can be expressed by IFN-γ-treated syngeneic thyroid epithelial cells (TEC). Three experiments demonstrating the activation of CH9 by TEC precultured with (or without) IFN-γ, to induce MHC class II expression, are shown. Responses to mouse Tg (MTg) presented by comparable numbers of splenic antigen presenting cells are shown for comparison. Responses were assessed as described in Fig. 2.

Discussion

In this paper we have shown that two independently derived autoreactive mouse T cell hybridomas both recognize (albeit slightly differently) the same T4-containing epitope in Tg. Although we have shown that the T4 residue is critical for recognition by these hybridomas, it is not yet clear whether it directly interacts with the T cell receptor or the I-Ak molecule (or both). This is a particularly intriguing question when one considers the extremely large molecular size of T4 compared with the rest of the 9mer peptide (molecular weight of 763 out of a total 1627 for the entire peptide). Competition studies and molecular modeling will be required to address this point.

A comparison of the amino acid sequences around this T4, at position 2553 in human Tg, with those around the equivalent site in rat and bovine Tg (the mouse Tg sequence is not available) provides interesting information regarding the recognition of this autoantigenic site by CH9 and ADA2 (Fig. 7). As previously reported (29), both CH9 and ADA2 were
generated as autoreactive mouse Tg-specific T cells which cross-reacted well with human and rat Tg, but were only weakly responsive to bovine Tg (and other members of the Artiodactyla family). However, the sequence containing the minimal stimulatory epitope defined above (boxed in Fig. 7) is identical in all three species. This observation suggests that sequences outside of the minimal epitope also play a critical role in generating stimulatory I-A\(^{\nu}\)/peptide complexes. Since position 2555 in bovine Tg has been shown to be one of the major hormonogenic sites (5), it is unlikely that the lack of reactivity of our T cells to this species of Tg is due to the lack of a T4 residue at this position (the bovine Tg used had normal T4 levels). A more plausible hypothesis is that residues outside the epitope influence the processing of Tg by antigen presenting cells. An alternative, but not mutually exclusive, explanation would be that the naturally processed Tg fragment is larger than the minimal epitope and that residues included in this fragment influence either the formation or activity of I-A\(^{\nu}\)/peptide complexes. The information in Fig. 7 presents two testable explanations for the low potency of bovine Tg. The replacement of Ser 2549 and Thr 2550 in human Tg with Asp and Ser, respectively, in bovine Tg might prevent MHC binding of a peptide containing these residues or remove/create critical proteolytic cleavage sites. Similar considerations could be given to the Gln for Asn substitution at position 2562; this substitution also removes an N-glycosylation site (known to be glycosylated in human Tg [37]) which could influence intracellular processing events.

The ability of IFN-\(\gamma\) stimulated TECs to activate the hybridoma CH9 to elicit thyroiditis in CBAmice (21), indicate that T cell recognition of the T4-containing epitope of Tg described in this paper can have pathological consequences. A striking feature of the autoantigenic T cell epitope described in this paper is that it is generated from Tg in the target organ by physiologically important post-translational mechanisms involved in the hormone-forming function of the gland. The production of this epitope will depend on the relative availability of iodine in the gland: under conditions of iodine deficiency, very little will be formed due to hormonogenesis occurring primarily at the N-terminal site (6, 7). Since some evidence suggests that circulating Tg may be poorly iodinated, particularly in neonates (44-46), T cell tolerance to the T4-containing epitope may not be effectively established. This could account for the ease of induction of both experimental tolerance (47, 48) and autoimmune thyroiditis (49) in mice. It may also help explain the ability of thyroid stimulating hormone (TSH) to induce tolerance to Tg in mice (48), since TSH administration has been reported to stimulate increases in the iodine content of circulating Tg (50). Although the importance of T4-containing T cell epitopes in human thyroid autoimmunity is unknown, our findings could provide a molecular basis for the link between dietary iodine levels and spontaneous thyroid disease, both in man (12-14) and in animals (15-19).
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