The insulin A-chain epitope recognized by human T cells is posttranslationally modified

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The autoimmune process that destroys the insulin-producing pancreatic β cells in type 1 diabetes (T1D) is targeted at insulin and its precursor, proinsulin. T cells that recognize the proximal A-chain of human insulin were identified recently in the pancreatic lymph nodes of subjects who had T1D. To investigate the specificity of proinsulin-specific T cells in T1D, we isolated human CD4+ T cell clones to proinsulin from the blood of a donor who had T1D. The clones recognized a naturally processed, HLA DR4–restricted epitope within the first 13 amino acids of the A-chain (A1–13) of human insulin. T cell recognition was dependent on the formation of a vicinal disulfide bond between adjacent cysteine residues at A6 and A7, which did not alter binding of the peptide to HLA DR4. CD4+ T cell clones that recognized this epitope were isolated from an HLA DR4+ child with autoantibodies to insulin, and therefore, at risk for T1D, but not from two healthy HLA DR4+ donors. We define for the first time a novel posttranslational modification that is required for T cell recognition of the insulin A-chain in T1D.
self-antigens (16). To our knowledge, there is only one report of human CD4+ T cells that recognize an epitope formed by posttranslational modification. Subjects who have rheumatoid arthritis have T cells that react to a glycosylated collagen epitope (17). Here, we sought to define the specificity of proinsulin-specific CD4+ T cells in T1D. We show for the first time that T cell recognition of the first 13 amino acids of human insulin A-chain requires posttranslational modification of adjacent cysteine residues at A6 and A7.

RESULTS AND DISCUSSION
Identification of the insulin A-chain 1–13 epitope
17 proinsulin-specific CD4+ T cell clones were isolated from the blood of a donor who had established T1D (18). The insulin A1–13 epitope was identified using an overlapping panel of 15-mer peptides. First, the clones were cultured with eight peptide pools, each containing three to four peptides, and covering the entire sequence of proinsulin. 5 of 17 clones recognized a peptide within pool 8 (Fig. 1 A); four were studied further. The three peptides in pool 8 were tested separately, and a single peptide comprising the last two amino acids of the C-peptide and the first 13 amino acids of the A-chain of insulin (KRGIVEQCCTSICSL) stimulated all four clones (Fig. 1 B). Moreover, individual peptides comprising the first 13 and 15 amino acids of the A-chain of insulin (Table S2, available at http://www.jem.org/cgi/content/full/jem.20051251/DC1) also stimulated the clones with equivalent dose response (Fig. 1 C). Furthermore, insulin and proinsulin stimulated the clones equally. The response to clinical grade recombinant insulin was HLA DR dependent (Fig. 1 D). Hence, the clones recognize a minimal epitope comprising the first 13 amino acids of the insulin A-chain.
A-chain of human insulin. To confirm that the T cell clones recognized an epitope that was derived from native human insulin, their response to human islet lysate was tested. The clones proliferated in a dose-dependent manner to islet, but not spleen, lysate from the same donor (Fig. 1 E). Hence, the clones recognized a naturally processed and presented epitope in the first 13 amino acids of the A-chain of human insulin.

Mapping the fine HLA restriction of the insulin A1–13 epitope

Experiments with HLA isotype–specific antibodies showed that proliferation of all clones was blocked by anti-HLA DR (L243) mAb (Fig. 2 A). Next, EBV-transformed B cells from a patient who had bare lymphocyte syndrome (BLS) that were transfected with different HLA–β genes were used as APCs. Cells that were transfected with DRB1*0401, DRB1*0404, or DRB1*0405 presented the A1–13 epitope, whereas cells that were transfected with DRB4*0101 (DR53), DQB1*0201 (DQ2), or DQB1*0302 (DQ8) did not (Fig. 2 B). Hence, the A1–13 epitope can be presented by HLA DRB1*0401, 0404, and 0405. It is well established that HLA DR4 confers susceptibility to T1D (2, 3). HLA DR4 has been associated with high-affinity and high-titer insulin autoantibodies (6) that typically are found in young individuals who are at high risk for developing T1D. Achenbach et al. (6) reported that these antibodies bound to the middle of the A-chain (A8–10/13), a region that is contained within the A1–13 CD4+ T cell epitope described here. Antibodies can increase antigen uptake and processing (19). Therefore, insulin autoantibodies that bind to the A-chain of insulin could enhance the presentation of the A1–13 epitope to CD4+ T cells, and, in turn, augment autoantibody production and explain, in part, the high titers of insulin antibodies in HLA DR4+ subjects.

The requirement for cysteine residues in T cell recognition

The requirement for the three cysteines in the A1–13 epitope was determined by testing peptides in which serine was substituted for each cysteine (Table S2). Replacing either of the adjacent cysteines at A6 or A7 with serine completely abolished the peptide’s capacity to stimulate the T cell clones. Oxidized cysteine at A6 and A7, but not antigen processing, is required to elicit responses of T cell clones. (A) Serine for cysteine substitutions. Insulin-specific T cell clones were cultured in the presence of 50–0.05 μM peptide (see Table S2). KR-A1-13 peptide (open squares), S-6 (filled triangles), S-7 (filled diamonds), S-11 (filled circles), or murine KR-A1-13 (open circles). (B) Effect of TCEP on responses to the A1-13 epitope. S-11 peptide (1 μM) was treated with TCEP at the concentrations shown (filled squares). Controls were comprised of either PHA (1.25 μg/ml) and IL-2 (2.5 U/ml; filled circles) or were without antigen (open squares). (C) Effect of APC fixation. HLA-DRB1*0404–transfected BLS cells were fixed cultured with RP-HPLC purified S-11 peptide and T cell clones (filled circles), or with unfixed BLS cells (open circles). Fixed cells with solvent alone (filled squares), or unfixed cells with solvent alone (open squares) were included. The mean ± SEM of triplicate wells is shown.

Figure 3. Oxidized cysteine at A6 and A7, but not antigen processing, is required to elicit responses of T cell clones. (A) Serine for cysteine substitutions. Insulin-specific T cell clones were cultured in the presence of 50–0.05 μM peptide (see Table S2). KR-A1-13 peptide (open squares), S-6 (filled triangles), S-7 (filled diamonds), S-11 (filled circles), or murine KR-A1-13 (open circles). (B) Effect of TCEP on responses to the A1-13 epitope. S-11 peptide (1 μM) was treated with TCEP at the concentrations shown (filled squares). Controls were comprised of either PHA (1.25 μg/ml) and IL-2 (2.5 U/ml; filled circles) or were without antigen (open squares). (C) Effect of APC fixation. HLA-DRB1*0404–transfected BLS cells were fixed cultured with RP-HPLC purified S-11 peptide and T cell clones (filled circles), or with unfixed BLS cells (open circles). Fixed cells with solvent alone (filled squares), or unfixed cells with solvent alone (open squares) were included. The mean ± SEM of triplicate wells is shown.
cell clones (Fig. 3 A), whereas replacing the A11 cysteine with serine had no effect. The analogue peptide (S-11), with serine substituted for cysteine at position A11, was used in subsequent experiments. A peptide corresponding to the sequence of mouse insulin A1–13 (KRGIVDQCCTSICSL) was ~10-fold less potent than was the human homologue. The absolute requirement of the adjacent cysteines at A6 and A7 for T cell recognition suggested that the epitope recognized by the T cell clones may require oxidative modification of the cysteines. To evaluate this possibility, the S-11 peptide was exposed to the disulfide-reducing agent, TCEP (Tris (2-carboxyethyl) phosphine hydrochloride). Reduction abolished the ability of S-11 to stimulate the clones (Fig. 3 B), which was consistent with oxidation-dependent modification of the adjacent cysteines required for T cell recognition. To determine if modification of the cysteines occurred spontaneously in culture medium or during antigen processing, we tested the capacity of paraformaldehyde-fixed and unfixed APCs to present the peptide to the T cell clones (Fig. 3 C). Fixation did not alter T cell reactivity. Therefore, we concluded that T cell recognition of the A1–13 epitope depended on the presence of oxidized cysteine residues at A6 and A7, a modification that did not require antigen uptake and intracellular processing.

Analysis of oxidized cysteine residues at A6 and A7
To determine the nature of the oxidized cysteine residues, the S-11 peptide was incubated in culture medium and the components were separated by reversed-phase (RP)–HPLC. A single fraction stimulated proliferation of the T cell clones (Fig. 4 A). No fractions stimulated proliferation when the S-6 peptide was used in similar experiments (unpublished data). Analysis of the active fraction by mass spectrometry (MS) showed that it contained a single species that was 2 Da tons smaller than predicted for the parental S-11 peptide (Fig. 4, B–E). Tandem MS/MS analysis revealed that this

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**Figure 4.** The A1–13 epitope contains a vicinal disulfide bond between cysteine A6 and A7. (A) Isolation of the modified peptide. The absorbance at 214 nm (line) and proliferation of an insulin-specific T cell clone (bars) in response to each fraction (1/400 dilution) is shown. MALDI-QTOF mass spectrometry was used to analyze the parental S-11 peptide at low (B) and high (C) resolution. The active fraction (fraction 7) from the serum-modification experiment at low (D) and high (E) resolution is shown.
loss of 2 Dltons arose at the adjacent cysteines (Table S3, available at http://www.jem.org/cgi/content/full/jem.20051251/DC1). This indicated that the epitope contained a vicinal disulfide bond (i.e., a disulfide bond between the adjacent A6 and A7 cysteines). This is the only modification that would eliminate two protons to result in a 2-Dlton decrease in mass. The vicinal disulfide bond formed within minutes, and treatment of the modified S-11 peptide with dithiothreitol reduced the vicinal disulfide bond and increased the mass by 2 Dltons (unpublished data). Using MS, we found no evidence of cysteinylation, sulphation, or S-nitrosylation of these residues. There was no evidence, by MS, for a vicinal disulfide bond in the recombinant proinsulin that was used to stimulate T cells.

Previous studies suggested that the oxidation state of insulin-derived peptides may play a role in immunogenicity (20). The first 14 amino acids of the A-chain of bovine insulin (GIVEQCCASVCSLY) are recognized by murine (20) and human T cells (21) after immunization. Nonetheless, T cell epitopes containing vicinal disulfide bonds have not been reported previously. The vicinal disulfide bond between A6 and A7 occurs during refolding of insulin in vitro (22). This suggests that the nonnative vicinal disulfide bond that formed between cysteines A6 and A7 predominates under oxidatively permissive conditions in vitro for the synthetic peptide, and after presentation of exogenous recombinant and native (pro)insulin.

Binding and modeling of modified and unmodified insulin A1–13 peptide to HLA DR4
The S-11 peptide containing a vicinal disulfide bond (KR.GIVEQC-CTSISL) and the serine-substituted homologue (KR.GIVEQSSTSISL) had similar binding affinities for HLA DRB1*0404 (0.024 μM and 0.030 μM, respectively, Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20051251/DC1). The serine-substituted peptide was used to avoid spontaneous oxidation of cysteine during the HLA binding assay. Both peptides also bound equally well to HLA DRB1*0401 (unpublished data). Thus, the vicinal disulfide bond did not alter binding to HLA DR4. The binding register of the insulin A1–13 epitope was determined to be the core nonamer VEQCCTSIS, using the ProPred algorithm (23). The formation of a vicinal disulfide bond between cysteine residues at A6 and A7 impacts on the conformation and solvent accessibility of the central portion of the epitope (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20051251/DC1). The vicinal disulfide bond creates a structure that is unable to be mimicked by other naturally occurring amino acids; this explains the failure of serine substitution to stimulate, even in part, the T cell clones.

Analysis of T cell clones from an individual at risk for T1D and healthy donors
To determine whether T cells specific for the cysteine-modified epitope are present before the onset of clinical T1D, we isolated 11 proinsulin-specific CD4+ T cell clones from an asymptomatic HLA DRB1*0404+ 12-yr-old with autoantibodies to insulin. Three of the clones responded to proinsulin, insulin, KR-A1-13, S-6, S-7, or S-11 peptides (10 μM each), or without antigen (Fig. S1). Further more, the RP-HPLC fraction containing the A1-13 epitope with the vicinal disulfide bond stimulated these clones (Fig. 5 B). Furthermore, the RP-HPLC fraction containing the A1-13 epitope with the vicinal disulfide bond stimulated these clones (Fig. 5 B).
5 C). In contrast, none of the 13 proinsulin-specific CD4+ T cell clones that were isolated from two healthy HLA DR4+ donors (6 and 7 clones, respectively) proliferated in response to KR-A1–13 peptide (unpublished data). Hence, T cells specific for the A1–13 epitope were not detected in healthy subjects, but were present in the blood of an individual with insulin autoantibodies.

Kent et al. (15) recently reported that CD4+ T cells that are specific for the first 15 amino acids of the A-chain of insulin were overrepresented in the pancreatic lymph nodes from two HLA DR4+ diabetic subjects, but not in the pancreatic lymph nodes from one HLA DR4+ subject and two other control subjects. We isolated proinsulin-specific CD4+ T cell clones from blood, the only tissue that is available for routine analysis in humans. T cell clones that recognized the first 15 amino acids of the A-chain of insulin also responded to islet cell lysate, which demonstrated that the A-chain epitope is naturally processed and presented. The epitope requires a vicinal disulfide bond between cysteines A6 and A7, which is essential for T cell recognition, but not for binding to HLA DR4. T cell responses to A1–13 were not a result of injecting insulin, because CD4+ T cells that recognized the A1–13 epitope were isolated from a donor who had never injected insulin, but had evidence of insulin autoimmunity. Our findings extend to T1D the paradigm that autoimmune responses can target epitopes that are formed by posttranslational modification.

MATERIALS AND METHODS

Human subjects and peripheral blood mononuclear cell isolation. Blood was obtained by venepuncture with informed consent and ethics committee approval. The HLA typing and clinical status of each donor are shown in Table S1 (available at http://www.jem.org/cgi/content/full/jem.20051251/DC1). PBMCs were cultured in IMDM (GIBCO BRL) supplemented with 5% pooled human serum, 2 mM glutamine (Glutamax; GIBCO BRL), 5 × 10−5 M 2-mercaptoethanol (Sigma-Aldrich), penicillin (100 U/ml), streptomycin (100 μg/ml; GIBCO BRL), and 100 μM nonessential amino acids (GIBCO BRL). Islets and spleen samples were obtained from the Tom Mann del Ilet Transplant Program with the approval of the St Vincent’s Hospital Human Ethics Committee.

Antigens. Synthetic peptides were purchased from Mimotopes, and reconstituted to 5 mM in 0.5% acetic acid, 40% acetonitrile in water. A library of 15-mer peptides overlapping by 12 amino acids, comprising the entire sequence of human proinsulin, was used for initial epitope-mapping experiments. All single peptides were ≥80% pure, and are summarized in Table S2. The vicinal disulfide bond (represented by C–C) in peptide S–11 was generated by incubating peptide in serum-free IMDM for 1 h. Modified peptide was purified by RP-HPLC, and analyzed as described below. Recombinant human proinsulin was produced in-house using a published protocol (24), and was shown to be folded correctly and free of posttranslational modification of modified and unmodified insulin A1–13 peptide complexed with HLA DR*0404. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20051251/DC1.

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1H-thymidine incorporation assays. Assays were performed in 96-well round-bottom plates in 5% pooled human serum/IMDM. APCs were (a) irradiated (20 Gy) autologous or HLA-matched PBMCs (fresh or thawed); (b) HLA-typed EBV-transformed B cell lines from the 9th International HLA Typing Workshop; or (c) EBV-transformed B cells, from a donor who had bare lymphocyte syndrome (BLS), that were transfected with different HLA genes. EBV lines were irradiated at 50 Gy. In some experiments, APCs were fixed with 1% paraformaldehyde for 20 min at room temperature, and washed twice in PBS and once in culture medium before use in proliferation assays. In other experiments, freshly prepared TCEP (Tris (2-carboxyethyl) phosphine hydrochloride (Pierce Chemical Co.) was added to the antigen solution, before dilution by medium and addition of APCs and T cells to the final concentration shown in the figures. For all experiments, T cells and APCs were cultured in triplicate in 96-well round-bottom plates. After 2 d, 1H-thymidine (0.5 μCi/well) was added for 18 h, after which the cells were harvested and incorporated radioactivity was measured by β-scintillation counting. Results are expressed as the mean ± SEM of triplicate wells. All experiments were done at least twice with at least two clones.

HPLC fractionation and mass spectrometry. Modifications of KR-A1–13 peptide in culture media were investigated after incubating 1.8 mg of peptide with 1.8 ml of culture media for 1 h at 37°C. The incubate was fractionated by RP-HPLC using an AKTA Basic HPLC equipped with a multi-wavelength tunable UV detector and a Frac 950 fraction collector (GE Healthcare). Proteins were separated on a Vyda C18 column (4.6 i.d. × 250 mm, 300 Å pore size, 5-μm nominal particle size), using a linear gradient of buffer A (0.1% TFA) to 60% B (acetonitrile/0.09% TFA; 0.86%/min), at a flow rate of 1 ml/min. Fractions (500 μl) were collected and 1-μl aliquots were mixed with 1 μl of 2,5-dihydroxybenzoic acid (Agilent Technologies) and dried onto a sample stage for analysis by MALDI-QuTOF MS (QSTAR pulsar + Applied Biosystems). Selected ions were subjected to further analysis using MS/MS analysis. Fragment ions that were generated in this way were assigned manually, based on the known sequence of the parent peptide and modified amino acid residues identified within the sequence, as described (25).

Online supplemental material. Table S1 shows subjects defined by their clinical status and HLA type. Table S2 shows that insulin A-chain peptides used in this study. Table S3 shows the assignment of the b-series ions and their clinical status and HLA type. Table S1 shows subjects defined by Table S2 shows the assignment of the b-series ions and their clinical status and HLA type. Table S3 shows the assignment of the b-series ions and their clinical status and HLA type. Table S1 shows subjects defined by Table S2 shows the assignment of the b-series ions and their clinical status and HLA type. Table S3 shows the assignment of the b-series ions and their clinical status and HLA type. Table S1 shows subjects defined by Table S2 shows the assignment of the b-series ions and their clinical status and HLA type. Table S3 shows the assignment of the b-series ions and their clinical status and HLA type. Table S1 shows subjects defined by Table S2 shows the assignment of the b-series ions and their clinical status and HLA type. Table S3 shows the assignment of the b-series ions and their clinical status and HLA type. Table S1 shows subjects defined by
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