

Diabetogenic T-Cell Clones Recognize an Altered Peptide of Chromogranin A

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Chromogranin A (ChgA) has been identified as the antigen target for three NOD-derived, diabetogenic CD4 T-cell clones, including the well-known BDC-2.5. These T-cell clones respond weakly to the peptide WE14, a naturally occurring proteolytic cleavage product from ChgA. We show here that WE14 can be converted into a highly antigenic T-cell epitope through treatment with the enzyme transglutaminase (TGase). The WE14 responses of three NOD-derived CD4 T-cell clones, each with different T-cell receptors (TCRs), and of T cells from BDC-2.5 TCR transgenic mice are increased after TGase conversion of the peptide. Primary CD4 T cells isolated from NOD mice also respond to high concentrations of WE14 and significantly lower concentrations of TGase-treated WE14. We hypothesize that posttranslational modification plays a critical role in the generation of T-cell epitopes in type 1 diabetes. *Diabetes* 61:3239–3246, 2012

Pathogenesis of type 1 diabetes (T1D) is mediated by autoreactive T cells directed toward β -cell antigens. The role of CD4 T cells in the development of autoimmune diabetes in the NOD mouse is well established, and islet-reactive CD4 T-cell clones have been valuable tools in the investigation of both disease progression and immunoregulation (1). The diabetogenic T-cell clone BDC-2.5 has been widely used in the study of autoimmune diabetes, and we recently identified the secretory granule protein chromogranin A (ChgA) as the target antigen for this and two other diabetogenic clones (2). ChgA was demonstrated to be the antigen for these clones through 1) the purification and mass spectrometric analysis of antigenic fractions from β -cell tumors, 2) the presence in ChgA of an amino acid motif sequence, WXR(M/D/E), that defined mimotope peptides for BDC-2.5 and other NOD T-cell clones, 3) the absence of antigenic activity in ChgA-deficient islets, and 4) the ability of WE14, a naturally processed 14-amino acid ChgA peptide (3) containing the WXR(M) motif, to stimulate the T-cell clones. Although the presence of ChgA in antigenic fractions was confirmed by mass spectrometric analysis, the peptide motif could not be detected, indicating the existence of a yet-unknown modification of ChgA in β -cells.

Despite the presence of the motif sequence in WE14, a very high concentration ($>100 \mu\text{g/mL}$ or $60 \mu\text{mol/L}$) of the peptide is required to activate BDC-2.5 and the other

clones, further indicating that the naturally occurring form of the ligand in β -cells may be altered. Due to the role of the enzyme transglutaminase (TGase) in the posttranslational modification (PTM) of proteins in various inflammatory, autoimmune, and degenerative conditions (4), we investigated whether treatment with TGase could convert WE14 to a more antigenic species. Our data indicate that enzymatic conversion of WE14 in vitro leads to the formation of a highly antigenic epitope for BDC-2.5 and other ChgA-reactive T cells from our panel. A recent report suggested that the unrelated ChgA_{29–42} peptide, rather than WE14, was the actual BDC-2.5 epitope (5). However, this peptide was not antigenic in our assays either before or after TGase treatment.

RESEARCH DESIGN AND METHODS

Mice. NOD, NOD.*scid*, BDC-2.5 TCR-Tg.NOD (6), BDC-6.9 TCR-Tg.NOD (7), and BALB/c mice were bred and maintained in the Biological Resource Center at National Jewish Health. All experimental procedures were in accordance with guidelines of the Institutional Animal Care and Use Committee and approved by the Animal Care and Use Committee of National Jewish Health.

Culture of T-cell clones. T-cell clones were maintained as previously described (8) by restimulation every 2 weeks with a β -cell granule membrane fraction (β -Mem) obtained from NOD β -cell tumors as a source of antigen, irradiated NOD spleen cells as antigen-presenting cells (APCs), and supernatant from the EL-4 cell line as a source of interleukin-2 (IL-2) in complete medium (CM). CM is Dulbecco's modified Eagle's medium supplemented with 44 mmol/L sodium bicarbonate, 0.55 mmol/L L-arginine, 0.27 mmol/L L-asparagine, 1.5 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 50 mg/L gentamicin sulfate, 50 mmol/L 2-mercaptoethanol, 10 mmol/L HEPES, and 10% FCS.

Assays for antigen. For T-cell clones, antigenicity of samples was assessed through interferon- γ (IFN- γ) responses measured by enzyme-linked immunosorbent assay (ELISA). Assay cultures in 96-well microtiter plates contained 2×10^4 responder T cells, 2.5×10^4 NOD peritoneal exudate cells as APCs, and β -cell or peptide antigen. The β -Mem described above was used as the antigen-positive control. For primary CD4 T-cell assays, single-cell suspensions were prepared by homogenizing in CM spleens of mice (6–8 weeks old). Spleen cells (1×10^6 cells/well) were cultured in 96-well plates with antigen at 37°C for 4–5 days, and IFN- γ production was determined by ELISA.

T-cell proliferation assay. Single-cell suspensions of spleens from BDC-2.5 TCR-Tg.NOD or BDC-6.9 TCR-Tg.NOD mice (2–5 months old) were labeled with 2.5 $\mu\text{mol/L}$ carboxyfluorescein succinimidyl ester (CFSE) and cultured in a 96-well plate (1×10^6 cells/well) in the presence of antigen or anti-CD3 ϵ (145-2C11; BD Biosciences). After 4 days of culture, cells were harvested, stained with anti-mouse CD4 APC (GK1.5; eBioscience), and analyzed on a FACSCalibur flow cytometer (BD Biosciences). Proliferation was assessed using the FlowJo software (Tree Star, Inc., Ashland, OR).

Adoptive transfer of transgenic T cells. CD4 T cells were isolated from BDC-2.5 TCR-Tg mice using magnetic beads (Miltenyi Biotec) and stimulated with irradiated spleen cells pulsed with WE14 or TGase-treated WE14 (20 $\mu\text{g/mL}$ or 12 $\mu\text{mol/L}$) and cultured in the presence of recombinant human (rh) IL-2 (50 units/mL). After 3 days of culture, cells were harvested and expanded for another 3–4 days in CM + rhIL-2 (rhIL-2) (50 units/mL) before injecting 5×10^6 cells intraperitoneally into adult NOD.*scid* mice. Mice were monitored daily for development of disease by urine glucose (Diasix; Bayer), and hyperglycemia was confirmed by OneTouch Ultra glucometer (LifeScan). Mice were considered diabetic when blood glucose levels were $>18 \text{ mmol/L}$. rhIL-2 was obtained from the National Cancer Institute.

Flow cytometry. Two weeks after restimulation, T-cell clones (1×10^5) were challenged with peritoneal exudate cells (1×10^5) and antigen in 96-well microtiter plates. After overnight incubation, cells were harvested and surface

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stained with the appropriate antibody combination, including anti-CD4 APC (GK1.5; eBioscience), anti-V β 8 PE (F23.1; BD Biosciences), and anti-CD4 FITC (GK1.5; BD Biosciences), in the presence of FcBlock (2.4G2; BD Biosciences). For intracellular staining, cells were fixed in 2% paraformaldehyde for 10 min in the dark. Cells were washed once more before resuspending in permeabilization buffer (staining buffer, 0.5% saponin), containing an isotype control or anti-IFN- γ allophycocyanin (XMG1.2; BD Biosciences). After 30 min of incubation, cells were washed three times in permeabilization buffer and resuspended in staining buffer. The "lymphocyte" gate was defined by sequential gates first set around intermediate forward scatter (FSC)/low side scatter (SSC) events; these events were then applied to a CD4/FSC plot to set a region consistent with the low SSC, intermediate FSC, CD4-high characteristics of live CD4 T cells.

Peptides. The following peptides (listed also in Table 2) were used for this study: WE14 (WSRMDQLAKELTAE), WE14-Q6E (WSRMDDELAKELTAE), ChgA₂₉₋₄₂ (DTKVMKCVLEVISD), B₉₋₂₃ (SHLVEALYLVCGERG), insulin B-chain (FVKQLHLCGSHLVEALYLVCGERGFFFTPMS), islet amyloid polypeptide (IAPP) KS20 (KCNTATCATQRLANFLVRSS) (9), and the HRPI (abbreviated form of HRPIWARMMD) peptide mimotope (EKAHRPIWARMMDAKK) for BDC-2.5 (10). B₉₋₂₃ was solubilized in 0.1 mol/L NaOH and then neutralized with 0.1 mol/L HCl. WE14 and WE14-Q6E were solubilized directly in sterile water. B-chain FS30 and ChgA₂₉₋₄₂ were only poorly soluble in water, and therefore peptide suspensions were used in assays. With the exception of HRPI, all peptides were obtained from CHI Scientific at a purity (high-performance liquid chromatography) >95%. **TGase treatment.** A reaction mixture containing 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 10 mmol/L CaCl₂, 50 mmol/L Tris (pH 8.0), 0.1 units/mL guinea pig transglutaminase (Sigma-Aldrich), and peptide (250–500 μ g/mL or 150–300 μ mol/L) was incubated for 4 h at 37°C. Samples were incubated in the presence (15.6, 7.8, 3.9, 1.95, 0.98, 0.49, 0.24, 0.12, 0.06, or 0.00 mmol/L) of putrescine, a competitive inhibitor of TGase.

Size exclusion chromatography. The chromatographic purification method is described elsewhere (2). The TGase reaction was carried out using the protocol described above except for the concentration of WE14 in the reaction mix, which was increased to 2.5 mg/mL (1.5 mmol/L).

Gel electrophoresis. SDS-PAGE was carried out on a 16% precast Tricine-Tris gel (Bio-Rad) applying a current of 65 mA for 10 min followed by 15 mA for 800 min. Gels were silver stained using the silver stain kit SilverSNAP (Pierce).

Statistics. For disease transfer experiments, statistical significance was determined by using a Wilcoxon rank sum test. A *P* value ≤ 0.05 was considered significant.

RESULTS

TGase treatment renders the peptide WE14 highly antigenic for several diabetogenic T cells. As shown in Table 1, the BDC panel of islet-reactive CD4 T cells contains three cell lines that recognize the ChgA-derived peptide WE14. The T-cell receptor (TCR) V β 4V α 1 is used by the T-cell clone BDC-2.5, whereas BDC-10.1 expresses a distinct TCR V β 15V α 13. Recently, we discovered that another T-cell line from our panel, BDC-9.46, also responds to WE14. As shown in Supplementary Fig. 1, this cell line is not clonal and consists of at least two different TCR V β populations, one of which is V β 8; cells in both BDC-9.46 populations respond to WE14. The diabetogenicity of BDC-9.46 has not yet been determined. T-cell clones from our panel therefore

express at least three different TCRs with specificity for the ChgA peptide WE14. The peptides that were used in this study, including the identified antigenic epitopes for the clones, are shown in Table 2.

The enzyme TGase is known to catalyze two major reactions that could be involved in PTMs occurring in autoimmune disease. In the first, deamidation leads to the conversion of the amino acid glutamine into glutamic acid, as exemplified by the modification of gliadin in celiac disease (11). The second major reaction catalyzed by TGase is the covalent cross-linking of glutamine (Q) to lysine (K) residues through the formation of an isopeptide bond (12,13). WE14 is an ideal substrate for TGase because it contains both amino acids, Q and K. To determine whether TGase could affect the antigenicity of WE14, we tested various concentrations of the peptide, with or without TGase treatment, with the ChgA-reactive T-cell clones BDC-2.5 and BDC-10.1, the T-cell line BDC-9.46, and the insulin-reactive T-cell clone PD-12.4.4 (14). As shown in the top panel of Fig. 1A, the ChgA-reactive T cells responded to high concentrations of WE14 (>100 μ g/mL or 60 μ mol/L), whereas PD-12.4.4 did not respond to this peptide at any concentration tested. The bottom panel of Fig. 1A shows that TGase treatment significantly increases the antigenicity of the peptide WE14 for the three ChgA-reactive T cells, with responses occurring at lower concentrations of peptide; PD-12.4.4 did not respond to the modified peptide. As shown in Table 3, the half-maximal effective concentrations (EC₅₀) of treated and untreated WE14 were determined using data from Fig. 1A, and the increase of peptide antigenicity was measured for the three ChgA-reactive T-cell clones. The increase of antigenicity for TGase-treated WE14 over untreated peptide varied between 2-fold for BDC-9.46 and 44-fold for BDC-2.5. Generally, the increase for BDC-2.5 was the highest and reached up to 100-fold in some cases. The increase for BDC-9.46 was the lowest but was at least twofold in all of the experiments performed. The data are representative for the three T-cell clones. We also observed that CD69, another marker of activation, was upregulated on the clone BDC-2.5 with much lower concentrations of TGase-treated WE14 than of unmodified peptide (data not shown).

As antigen controls, we tested two non-ChgA peptides before and after treatment with TGase for their ability to stimulate IFN- γ production by BDC-2.5 (Fig. 1C). These peptides, IAPP KS20 (9) and the insulin B-chain, also contain glutamines but remain inactive with BDC-2.5 after treatment with TGase. We also tested the vasostatin peptide ChgA₂₉₋₄₂ reported recently to be a more likely epitope for BDC-2.5 (5), despite the fact that this peptide sequence does not contain the TCR motif WXR. In two of

TABLE 1
Islet antigen-reactive CD4 T-cell clones from the NOD mouse

T cell	TCR	Antigen	Epitope
BDC-2.5	V β 4V α 1	ChgA	WE14
BDC-10.1	V β 15V α 13	ChgA	WE14
BDC-9.46	V β 8V α (ND)	ChgA	WE14
BDC-5.2.9	V β 6V α 12	IAPP	KS20
BDC-4.38*	V β 6 and V β 12	Insulin	B ₉₋₂₃
PD-12.4.4**	V β 1	Insulin	B ₉₋₂₃
BDC-6.9	V β 4V α 13.1	ND	ND
BDC-9.3	V β 4V α 13.1	ND	ND
BDC-6.3	V β 4V α 3.1	ND	ND

ND, not determined. *T-cell line contains two V β populations, both of which react to insulin B₉₋₂₃. **Daniel et al. (14).

TABLE 2
Peptides used in this study

Peptide	Amino acid sequence*
WE14	WSRMDQLAKELTAE
B ₉₋₂₃	SHLVEALYLVCGERG
KS20	KCNTATCATQRLANFLVRSS
WE14-Q6E	WSRMDDELAKELTAE
ChgA ₂₉₋₄₂	DTKVMKCVLEVISD
B-chain	FVKQLHLCGSHLVEALYLVCGERGFFFTPMS
HRPI	EKAHRPIWARMMDAKK

*Single letter code.

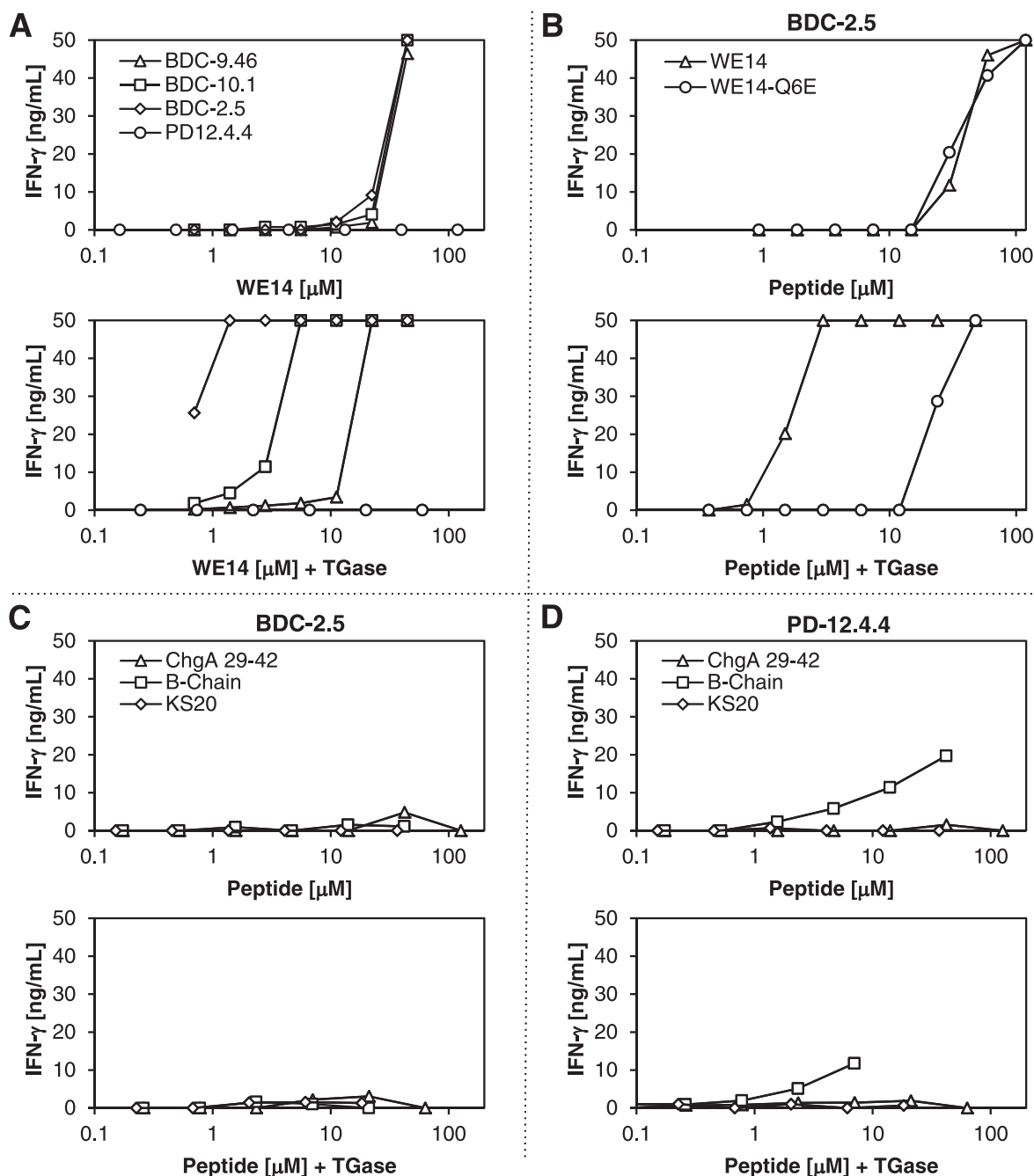


FIG. 1. Treatment of WE14 with TGase significantly increases peptide antigenicity for ChgA-reactive T-cell clones. **A:** T cells (BDC-2.5, BDC-10.1, BDC-9.46, and PD12.4.4) and APCs were incubated with increasing concentrations of the TGase-treated (*bottom*) or untreated (*top*) WE14. T-cell responses were assessed through IFN- γ production measured by ELISA. Data are representative of at least three separate experiments. **B:** BDC-2.5 was incubated with APCs and increasing concentrations of TGase-treated (*bottom*) or untreated (*top*) peptides WE14 and deamidated WE14-Q6E. Data are representative of at least three separate experiments. **C:** BDC-2.5 (*C*) or PD-12.4.4 (*D*) were incubated with APCs and increasing concentrations of the TGase-treated (*bottom*) or untreated (*top*) peptides ChgA₂₉₋₄₂, IAPP KS20, and the insulin B-chain. Data are representative of at least three separate experiments.

three assays with BDC-2.5, ChgA₂₉₋₄₂ triggered a very weak T-cell response at 66 $\mu\text{g}/\text{mL}$ (42 $\mu\text{mol}/\text{L}$) but was not antigenic at higher or lower peptide concentrations; in the third assay, no response at any concentration could be detected. Furthermore, treatment of ChgA₂₉₋₄₂ with TGase did not lead to an increase of antigenicity, as would be predicted since this sequence does not contain glutamine. The ChgA₂₉₋₄₂ peptide does not qualify as an antigen in our system due to the inconsistent response from BDC-2.5, the

lack of reactivity with other ChgA-reactive T-cell clones, and the fact that the peptide could not be titrated.

The response of the insulin-reactive T-cell clone PD-12.4.4 to these peptides was also tested (Fig. 1D). As expected, this clone did not respond to KS20, with or without TGase treatment. In this assay, there was a very small PD-12.4.4 response to ChgA₂₉₋₄₂ at 66 $\mu\text{g}/\text{mL}$ (42 $\mu\text{mol}/\text{L}$) in the absence of TGase, but as was observed with BDC-2.5, the peptide was not antigenic at higher or lower

TABLE 3
EC₅₀ values for T-cell stimulation

T-cell clone	EC ₅₀ values for T-cell stimulation		Fold increase*
	WE14 (μmol/L)	WE14 + TGase (μmol/L)	
BDC-2.5	31.0	0.7	44.3
BDC-10.1	32.5	3.8	8.6
BDC-9.46	34.0	16.4	2.1

*EC₅₀ values were determined from Fig. 1A.

peptide concentrations. PD-12.4.4 responded to the untreated insulin B-chain peptide, and this response was not increased significantly by TGase treatment.

Glutamine is required for TGase conversion of WE14.

To determine whether deamidation of the peptide WE14 could explain the increased antigenicity of TGase-treated WE14, we tested the deamidated peptide WE14-Q6E (Fig. 1B). This peptide is a potential reaction product of WE14 and TGase because it contains a glutamic acid (E) instead of glutamine (Q) residue at position 6. WE14-Q6E was no better than unmodified WE14 in activating BDC-2.5, indicating that deamidation of WE14 is not the cause of increased antigenicity after TGase treatment. Upon reaction with TGase, antigenic activity of WE14-Q6E, used at the same concentrations as WE14, was not affected, because

the Q6E peptide lacks the glutamine required for enzyme activity. We conclude that TGase is a potent activator of WE14 via Q6 but does not simply convert the Q to E.

BDC-2.5 TCR-Tg CD4 T cells proliferate and secrete IFN-γ in response to WE14. To assess activation of BDC-2.5 TCR-Tg CD4 T cells in response to various peptides or anti-CD3 (as a positive control), we measured T-cell proliferation of spleen cells labeled with CFSE. T cells from the BDC-6.9 TCR-Tg mouse (6) were used as a negative control because the antigen for the BDC-6.9 T-cell clone is not ChgA; a peptide KS20 from IAPP was used as a negative antigen control for both types of TCR-Tg T cells. The vasostatin peptide ChgA₂₉₋₄₂ and the HRPI peptide mimotope for BDC-2.5 were also tested in this assay. As shown in Fig. 2A, both BDC-2.5 and BDC-6.9 TCR-Tg CD4 cells responded very well to anti-CD3 stimulation. CD4 T cells from BDC-2.5 TCR-Tg (but not BDC-6.9 TCR-Tg) mice responded to the peptide WE14 at a concentration of 200 μg/mL (120 μmol/L) as well as to the BDC-2.5 peptide mimotope HRPI. There was no proliferative response by transgenic T cells to either the ChgA₂₉₋₄₂ or the IAPP KS20 peptide. As indicated in Fig. 2B, the proliferative response of BDC-2.5 transgenic T cells to various concentrations of TGase-treated WE14 was greater than to untreated WE14. Treatment of the irrelevant peptide KS20 with TGase did not alter the T-cell proliferation responses at any peptide concentration tested, indicating that the enzyme itself did not affect reactivity (data not shown). Supplementary Fig. 2 shows that the proliferative responses of BDC-2.5

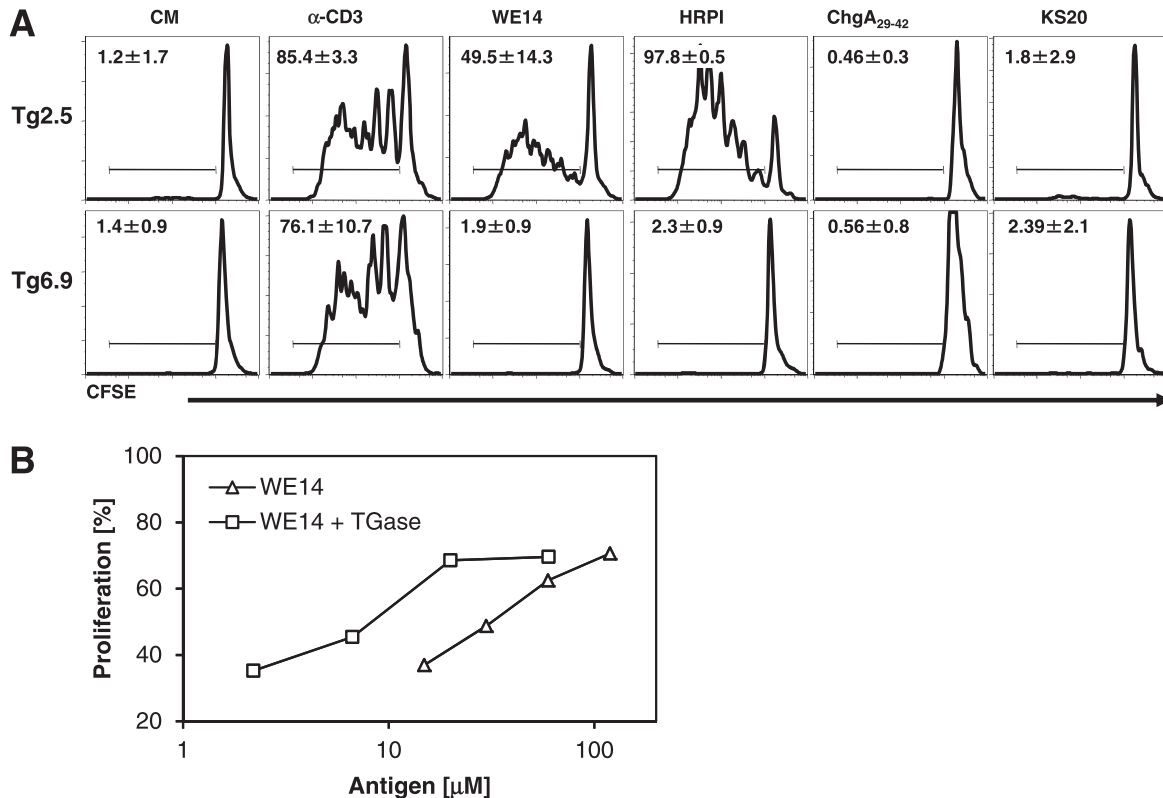


FIG. 2. CD4 T cells from BDC-2.5 TCR-Tg mice proliferate upon stimulation with WE14. Spleen cells were isolated from BDC-2.5 TCR-Tg ($n = 6$) or BDC-6.9 TCR-Tg ($n = 2$) mice and were labeled with CFSE. CFSE dilution was analyzed by flow cytometry, gates were set on the CD4/lymphocyte gate, and average percentages of divided cells and SDs from two independent experiments are indicated. *A*: Single-cell suspensions (1×10^6 /well) of transgenic T cells were cultured in the presence of CM, α-CD3 (0.1 μg/mL), WE14 (200 μg/mL or 120 μmol/L), HRPI (0.4 ng/mL or 0.21 nmol/L), ChgA₂₉₋₄₂ (200 μg/mL or 127 μmol/L), or KS20 (1 μg/mL or 0.45 nmol/L). Histograms are from one representative mouse, and average percentages of proliferation and SD are from six BDC-2.5 TCR-Tg and two BDC-6.9 TCR-Tg mice. *B*: Single-cell suspensions (1×10^6 /well) were labeled with CFSE and were cultured in IL-2 containing media in the presence of WE14 or TGase-treated WE14. Data shown are average percentages of proliferation from three individual BDC-2.5 TCR-Tg mice and are representative of three independent experiments.

TCR-Tg T cells increased with increasing concentrations of the HRPI mimotope or the β -Mem control antigen, whereas the transgenic T cells did not respond to any concentration of ChgA₂₉₋₄₂.

Figure 3 illustrates the IFN- γ response of CD4 T cells from BDC-2.5 TCR-Tg mice (six individual animals) to WE14 and TGase-treated WE14. T cells from BDC-6.9 TCR-Tg (two individual mice) were again used as negative controls. TCR-Tg spleen cell suspensions were incubated with various antigen concentrations, and supernatants were assayed for the presence of IFN- γ . As shown in Fig. 3, cells obtained from four out of six of the BDC-2.5 TCR-Tg mice responded weakly to high concentrations (200 μ g/mL or 120 μ mol/L) of the unmodified peptide WE14. The TGase-treated peptide, used at significantly lower concentrations (<10 μ g/mL or 6 μ mol/L), elicited much greater T-cell responses from five out of six BDC-2.5 TCR-Tg mice. This experiment illustrates the considerable mouse-to-mouse variability of TCR-Tg T-cell responses to antigen, with T cells from some mice responding strongly and from other mice only weakly or not at all. Cells obtained from 6.9 TCR-Tg mice did not respond to WE14 or TGase-treated WE14.

NOD-derived splenic cells are activated by low concentrations of TGase-treated WE14. In addition to testing CD4 T-cell responses from the BDC-2.5 transgenic mice, we analyzed the response of primary CD4 T cells from four nontransgenic NOD mice to the peptide WE14, before and after TGase treatment. As shown in Fig. 4, splenic T cells from all but one of four mice responded to

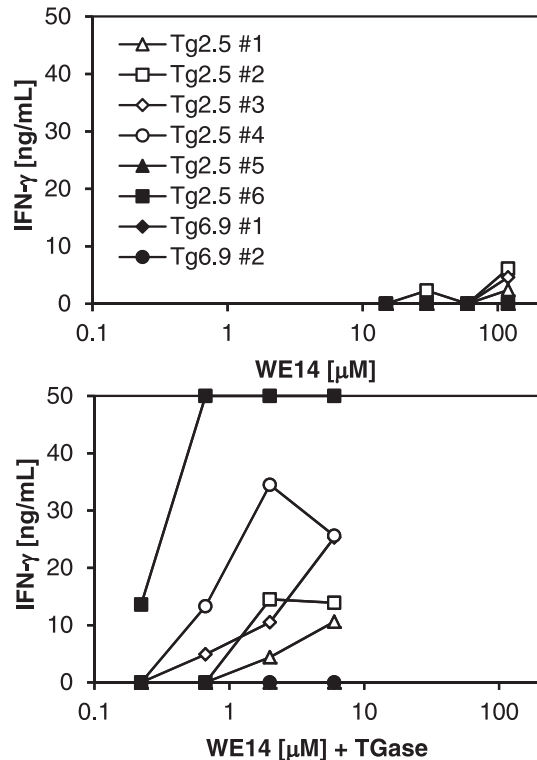


FIG. 3. IFN- γ responses of CD4 T cells from BDC-2.5 TCR-Tg mice to WE14 are increased after conversion of the peptide by TGase. Spleen cells from BDC-2.5 TCR-Tg and BDC-6.9 TCR-Tg mice were incubated with WE14 or TGase-treated WE14 in 96-well plates. Supernatants were harvested after 4 days and assayed for the presence of IFN- γ by ELISA. Data are from two separate experiments with a total of six BDC-2.5 TCR-Tg and two BDC-6.9 TCR-Tg mice.

the peptide WE14, but only at a very high peptide concentration (500 μ g/mL or 300 μ mol/L). NOD T cells demonstrated a much stronger response to TGase-treated WE14 peptide used at a significantly lower concentration (22 μ g/mL or 13 μ mol/L). Spleen cells from BALB/c mice did not produce IFN- γ in response to WE14 or converted WE14. As would be expected with NOD mice, a large degree of mouse-to-mouse variability in T-cell responses to the peptide WE14 was observed, with some animals responding well to WE14 or TGase-treated WE14 and other mice not responding.

Transfer of disease with BDC-2.5 TCR-Tg T cells is accelerated after activation with modified WE14. We also compared the antigenicity of TGase-treated peptide versus unmodified WE14 in the activation of transgenic T cells used in an adoptive transfer experiment. As illustrated in Fig. 5, all four mice receiving BDC-2.5 TCR-Tg T cells activated in vitro with TGase-treated WE14 became diabetic 9 days after injection. The kinetics of disease transfer with T cells activated with the same concentrations of unmodified WE14 were significantly slower.

TGase treatment of WE14 leads to the generation of peptide oligomers. In addition to deamidation, the other principal reaction catalyzed by the enzyme TGase is the formation of isopeptide bonds between glutamine and lysine residues. To determine whether the WE14 peptide could be cross-linked to itself by TGase to form multimers, we analyzed the reaction products of TGase conversion by electrophoresis on a Tricine-Tris peptide gel. As shown in Fig. 6A, treatment of WE14 with TGase leads to the formation of peptide-peptide aggregates (lane 1, bottom), which are not present in untreated peptide preparations (lane 11, bottom). Moreover, formation of WE14 multimers corresponds to the increased antigenicity of TGase-treated WE14 for BDC-2.5 in comparison with untreated peptide at the same concentration (lane 1 vs. lane 11, top). Competitive TGase inhibitors, such as putrescine, can be added to TGase reaction mixtures to compete with lysine residues over the binding to glutamine, leading to the alternate cross-linking between putrescine and glutamine. As shown in the figure, the addition of increasing concentrations of putrescine to reaction mixtures led to decreasing peptide

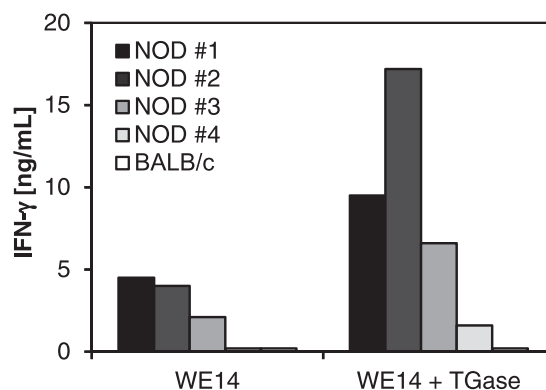


FIG. 4. T cells from NOD mice are activated by WE14, and responses are increased after TGase treatment of the peptide. Spleen cells from 6–8-week-old NOD or BALB/c mice were incubated with WE14 (500 μ g/mL or 300 μ mol/L) or TGase-treated WE14 (22 μ g/mL or 13 μ mol/L) in 96-well plates. Supernatants were harvested after 5 days and assayed for the presence of IFN- γ by ELISA. Data are representative of two separate experiments, with four NOD mice and one BALB/c mouse in each experiment.

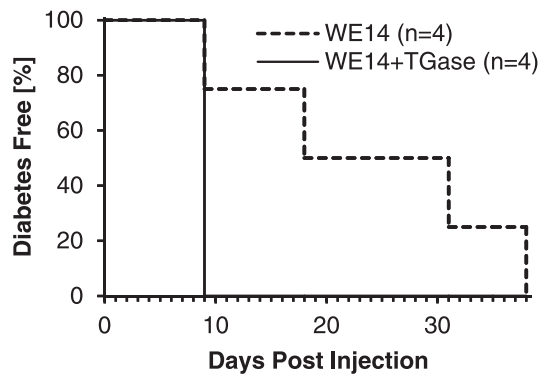


FIG. 5. T cells from BDC-2.5 TCR-Tg mice transfer disease more rapidly when activated with TGase-treated WE14. BDC-2.5 TCR-Tg cells were activated with TGase-treated WE14 (solid line) or WE14 (dashed line), and 5×10^6 cells were transferred intraperitoneally into adult NOD.*scid* mice. Urine glucose was monitored daily, and mice were considered diabetic when two consecutive measurements were >18 mmol/L. Data are from one experiment, and the number of recipient mice per group is indicated. Disease transfer was significantly slower when T cells were activated with WE14 compared with TGase-treated WE14 ($P = 0.04$).

oligomerization (lanes 2–10, bottom) and, at the same time, to diminished T-cell responses to converted peptide (lanes 2–10, top). The presence of putrescine did not affect the antigenicity of the β -Mem-positive control antigen in the assay (data not shown).

TGase treatment leads to the generation of low- and high-molecular-weight aggregates. As shown in Fig. 6B,

the purification of the untreated peptide WE14 by size exclusion chromatography leads to a single protein peak of low molecular weight (MW) (~ 2 kDa). The T-cell clone BDC-2.5 recognizes the peptide present in those fractions. When we purified TGase-treated WE14, the peptide peak broadened and, in addition, a protein absorbance above background could be detected between 1 kDa and very large MWs (see enlarged protein absorbance graph in the bottom of Fig. 6B). A small protein peak at an elution volume of 40 mL could also be detected; high-MW molecules not retained by the column appear at this elution volume. BDC-2.5 recognized antigen present in two separate regions of the chromatogram. The first region of antigenicity (<44 kDa) contains molecules of a molecular size between a single peptide and oligomers up to 26 peptides. The area containing antigenic activity at high MWs (>158 kDa) is likely to contain aggregates of TGase and WE14. As indicated in the graph, TGase elutes between 44 and 158 kDa, a range that does not contain antigenic activity.

DISCUSSION

It is well established that PTMs can affect antigenicity and presentation of proteins in autoimmune diseases. Citrullination in rheumatoid arthritis and multiple sclerosis, phosphorylation in systemic lupus erythematosus, and the TGase-catalyzed deamidation of gliadin in celiac disease are but a few examples of PTMs in autoimmunity (15,16). However, with the exception of vicinal disulfide

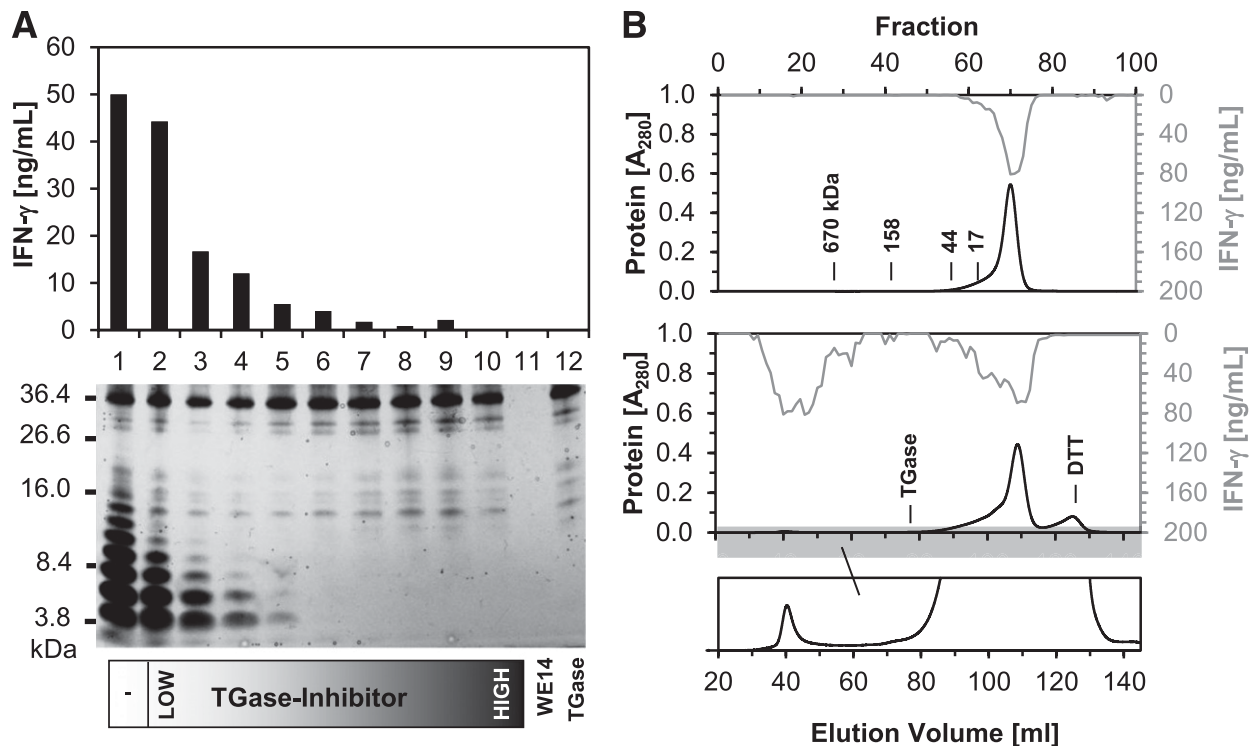


FIG. 6. The formation of high-MW as well as small-MW aggregates plays a critical role in the increased antigenicity of TGase-treated WE14. **A:** Silver-stained, Tricine-Tris peptide gel of untreated WE14 and TGase (lanes 11 and 12) and TGase-treated WE14 in the presence of low to high concentrations of the TGase inhibitor putrescine (lanes 2–10) and in the absence of inhibitor (lane 1). In the absence of inhibitor, the peptide forms multimers upon reaction with TGase (bottom, lane 1). The graph in the top panel shows the response of BDC-2.5 to fractions corresponding to 1–12 shown in the gel, with the highest response in the first column, corresponding to lane 1. BDC-2.5 does not respond to untreated WE14 (lane 11) at the low concentration used in this assay. Data are representative of at least three separate experiments. **B:** Size exclusion chromatography of 5 mg WE14 (top) and 5 mg TGase-treated WE14 (bottom). Protein absorbances were measured at 280 nm. Aliquots of fractions were tested for the presence of antigen using BDC-2.5. Data are representative of two separate experiments.

bond formation in the insulin A-chain epitope for human T cells (17), there has been little evidence for PTM contributing to T1D. Here we report our observations that treatment of the ChgA peptide WE14 with the enzyme TGase converts the peptide from a weakly stimulating antigen into a potent agonist for ChgA-reactive, I-A^{G7}-restricted CD4 T cells. Low concentrations of the converted WE14 peptide elicit strong responses not only from the diabetogenic CD4 BDC T-cell clones and BDC-2.5 TCR-Tg T cells but also from T cells obtained from nontransgenic NOD mice. The presence of primary CD4 T cells in polyclonal NOD populations that respond to the altered WE14 peptide suggests that it could be an important autoantigenic epitope in T1D.

The NH₂ terminus of WE14 begins with the WXR amino acid sequence, and alignment of WE14 with peptide mimotopes for BDC-2.5 suggests that the peptide only fills half of the major histocompatibility complex (MHC) class II binding groove, leaving the P1–P4 anchor positions unoccupied. Yet extension of the peptide WE14 at the NH₂ terminus with the naturally preceding ChgA amino acids, a sequence that would fill the I-A^{G7} groove, results in loss of antigenic recognition by the autoreactive T-cell clones (2). In the absence of contact with the P1–P4 positions, the WE14 peptide would be predicted to be a poor binder of I-A^{G7}, and this could explain its weak antigenicity for the BDC-2.5 clone. The majority of amino acids in WE14 extend beyond the P9 position of the class II binding groove, and our previous experiments indicate that COOH-terminal truncations of the flanking residues lead to decreased antigenicity, suggesting that these amino acids may be involved in peptide-MHC interactions outside the binding groove. A PTM in WE14 could alter peptide binding and increase affinity to class II.

Modification of WE14 through deamidation does not explain the increased antigenicity of TGase-converted WE14 because, as demonstrated in Fig. 1B, when glutamic acid is substituted for the glutamine (WE14-Q6E), there is no increase in antigenic activity. On the other hand, cross-linking of WE14 (Fig. 6) leads to the formation of high- and low-MW aggregates, which could result in preferential uptake by APCs and thereby contribute to the heightened antigenicity of the altered peptide. Another possible explanation for the strong T-cell response to converted WE14 reaction mixtures (which still contain the active enzyme TGase) is that during processing by the APC, the peptide binds to another protein, such as the MHC class II. If TGase treatment resulted in a covalent bond between WE14 and I-A^{G7}, the peptide could thus be bound to class II, leading to increased antigenicity. Peptide affinity studies between TGase-treated WE14 and soluble I-A^{G7} may address this question but have to wait for a procedure that can successfully purify the altered peptide and still retain antigenic activity.

To date, a total of eight different isoforms of TGase have been characterized in humans (18). It has been shown that the gene expression of tissue TGase (TGase2), which was used for this study, is enriched in pancreatic islets (19). Furthermore, it has been suggested that the inhibition of glucose-stimulated insulin release by TGase inhibitors is likely to be attributed to its interference with islet TGase activity (20). This observation is supported by a report demonstrating that islets from TGase2-deficient mice have an impaired insulin secretion (21) and that naturally occurring TGase2 mutations associated with familial, early-onset type 2 diabetes show a defective transamidating activity (22,23). Furthermore, recent electron microscopy

studies revealed that TGase2 is localized close to the secretory granules in human β -cells, and it was suggested that its role in insulin secretion may involve cytoplasmic actin remodeling and regulatory action on other proteins during granule movement (24). Among several TGase2 transamidating substrates in a β -cell line, mass spectrometry identified various molecules that participate in secretory granule structure, glucose metabolism, or Ca²⁺ sensing (24). The direct interaction between TGase and ChgA in β -cells has yet to be demonstrated.

Because WE14 contains the WXR motif required for BDC-2.5 TCR recognition, and is a natural proteolytic cleavage product of ChgA, it is a strong candidate ligand for BDC-2.5 and other ChgA-reactive T-cell clones from our panel. The increased antigenicity of WE14 upon treatment with TGase strongly supports this hypothesis. Preliminary results from a pilot study in humans indicate that a similarly enhanced reactivity to TGase-treated WE14 can be observed in T cells from human T1D patients (P. Gottlieb, T.D., L. Fitzgerald-Miller, K.H., unpublished data). The molecular basis of the modified WE14 is yet to be resolved, but the demonstration that this weakly antigenic peptide can be converted to a strongly stimulating ligand through enzymatic modification provides a compelling reason to further investigate the role of PTMs in the generation of autoantigens in T1D.

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T.D. and R.L.B. designed and performed the experimental work and wrote the manuscript. J.H. designed and performed the experimental work. G.B. provided T-cell clones and assisted with T-cell assays. B.B. provided mice, assisted with in vivo experiments, and helped with figures. K.H. assisted in design, interpretation, and oversight of all experimental work and wrote the manuscript. K.H. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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