The Journal of Immunology

Antigen Presentation to Celiac Lesion-Derived T Cells of a 33-Mer Gliadin Peptide Naturally Formed by Gastrointestinal Digestion

Shuo-Wang Qiao, Elin Bergseng, Øyvind Molberg, Jiang Xia, Burkhard Fleckenstein, Chaitan Khosla and Ludvig M. Sollid

J Immunol 2004; 173:1757-1762; ;
doi: 10.4049/jimmunol.173.3.1757
http://www.jimmunol.org/content/173/3/1757

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Antigen Presentation to Celiac Lesion-Derived T Cells of a 33-Mer Gliadin Peptide Naturally Formed by Gastrointestinal Digestion[1]

Shuo-Wang Qiao,[2]* Elin Bergseng,* Øyvind Molberg,* Jiang Xia,† Burkhard Fleckenstein,* Chaitan Khosla,† and Ludvig M. Sollid*

Celiac disease is an HLA-DQ2-associated disorder characterized by intestinal T cell responses to ingested wheat gluten proteins. A peptide fragment of 33 residues (α_2-gliadin 56–88) produced by normal gastrointestinal proteolysis contains six partly overlapping copies of three T cell epitopes and is a remarkably potent T cell stimulator after deamidation by tissue transglutaminase (TG2). This 33-mer is rich in proline residues and adopts the type II polyproline helical conformation in solution. In this study we report that after deamidation, the 33-mer bound with higher affinity to DQ2 compared with other monovalent peptides harboring gliadin epitopes. We found that the TG2-treated 33-mer was presented equally effectively by live and glutaraldehyde-fixed, EBV-transformed B cells. The TG2-treated 33-mer was also effectively presented by glutaraldehyde-fixed dendritic cells, albeit live dendritic cells were the most effective APCs. A strikingly increased T cell stimulatory potency of the 33-mer compared with a 12-mer peptide was also seen with fixed APCs. The 33-mer showed binding maximum to DQ2 at pH 6.3, higher than maxima found for other high affinity DQ2 binders. The 33-mer is thus a potent T cell stimulator that does not require further processing within APC for T cell presentation and that binds to DQ2 with a pH profile that promotes extracellular binding. ***The Journal of Immunology,*** **2004, 173: 1757–1762.**

Celiac disease is a chronic inflammatory disease of the small intestine caused by the ingestion of proline- and glutamine-rich wheat gluten (consisting of the gliadin and glutenin subcomponents) or related proteins from rye and barley. The lesion is characterized by villous atrophy, crypt hyperplasia, and increased number of infiltrating lymphocytes in both epithelium and lamina propia. Celiac disease has a strong HLA association; ~90% of the patients are HLA-DQ2, and the majority of the remaining patients are HLA-DQ8 (1). Gluten-reactive $CD4^+$ T cells can be readily isolated from the small intestine of celiac disease patients, but not of controls, and such gluten-reactive T cells are exclusively restricted by the disease-associated DQ2 or DQ8 HLA molecules. The activation of gluten-reactive T cells probably represents a key event in disease development. Gluten-reactive T cells from celiac lesions predominantly recognize gluten peptides in which glutamine residues at certain positions have been converted to glutamic acid by tissue transglutaminase (TG2)[3]-mediated deamidation (1). The negative charges introduced by TG2 generally increase the binding affinity of the gluten peptides to DQ2 (2, 3).

Several different celiac T cell epitopes derived from gliadin proteins have been identified during the last few years (2, 4–7), and these epitopes cluster in the proline-rich regions of the proteins (5). The epitopes identified in α-gliadins appear to be particularly important because they are recognized by intestinal T cells of the majority of adult celiac patients (2). By mimicking the enzymatic gastrointestinal digestion of a representative α-gliadin (α_2-gliadin), Shan and coworkers (8) found a 33-mer (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF; α_2-gliadin 56–88) to be particularly interesting. This fragment was resistant to further breakdown by luminal proteases and intestinal brush-border enzymes due to its high proline content (13 of 33 residues are proline). Moreover, it showed strong type II polyproline helical conformation in solution; the same conformation that is adopted by peptides bound to MHC class II molecules (9). The 33-mer contains six partly overlapping copies of three DQ2-restricted T cell α epitopes (Fig. 1), and the deamidated 33-mer was found to be an extremely potent T cell stimulator, several-fold more potent than any other known gluten peptide (8). Given the fact that this highly immunostimulatory peptide is an end product of the gastrointestinal proteolytic digestion and given its central role in the pathogenesis of celiac disease, we aimed to characterize its binding to the disease-associated DQ2 molecule and the mechanism of its presentation to T cells.

Materials and Methods

Peptide synthesis, gliadin protein Ag, and GST-TG2 production

The γI peptides YQQLPQP**E**QPQQSFP**E**Q**E**RPF[4] (γ-gliadin(Y134–153)E140E148E150, SWISSPROT GDB2_WHEAT) and qP**E**QPQQSF

*Institute of Immunology, Rikshospitalet University Hospital, University of Oslo, Oslo, Norway; and †Department of Chemistry, Stanford University, Stanford, CA 94305

Received for publication March 9, 2004. Accepted for publication May 19, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from the Research Council of Norway, the European Commission (QLK1–2000-00657), Rikshospitalet University Hospital, the Deutsche Forschungsgemeinschaft (SFB 510, Project D4), and the National Institutes of Health (DK63158). B.F. and S.-W.Q. hold fellowships from University of Oslo.

[2] Address correspondence and reprint requests to Dr. Shuo-Wang Qiao, Institute of Immunology, University of Oslo, Rikshospitalet University Hospital, N-0027 Oslo, Norway. E-mail address: s.w.qiao@medisin.uio.no

[3] Abbreviations used in this paper: TG2, tissue transglutaminase; B-LCL, EBV-transformed B lymphoblastoid cell line; DC, dendritic cell; HMWF-PT-gliadin, high m.w. fraction of PT-gliadin; Hsp, heat shock protein; MB, *Mycobacterium bovis*; PT-gliadin, pepsin trypsin-treated gliadin; TCC, T cell clone.

[4] **E** (in bold) in the peptide sequences in this paper denotes Glu residues that are posttranslationally converted from Gln residues.

0022-1767/04/$02.00

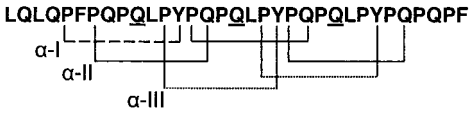

FIGURE 1. The 33-mer and its epitopes. The 33-mer (α_2-gliadin 56–88) contains, in total, six overlapping copies of three DQ2-restricted T cell epitopes. Three glutamine residues (underlined), which we predict to be primarily selected for deamidation by TG2 (10, 11), were converted to glutamic acid in the synthesis of the deamidated 33-mer.

PEQERP (γ-gliadin(Y139–152)E140E148E150; γI core region underlined) were purchased from Research Genetics (Huntsville, AL). The αI peptide QLQPFPQPELPY (corresponds to α_2-gliadin(57–68)E65 and α_9-gliadin(57–68)E65); αI core region underlined) and the αII/III peptide PQPELPYPQPELPY (α2-gliadin (62–75)E65E72; αIII core region underlined) were synthesized at University of Tubingen using F-moc/OtBu chemistry and 2-chlorotriityl resin (Senn Chemicals, Dielsdorf, Switzerland). The 33-mer and its deamidated variant (LQLQPFPQPELPYPQPELPYPQPELPYPQPQPF), with the substitution of Gln to Glu at three positions according to the previously characterized TG2 specificity motif (10, 11), were synthesized at Stanford University using Boc/HBTU chemistry starting from *N*-α-t-Boc-L-phenylalanine-phenylacetamidomethyl resin. After the cleavage of peptidyl resin in trifluoromethanesulfonic acid/trifluoroacetic acid (1/10, v/v) with thioanisole as scavenger reagent, the crude peptides were precipitated in cold ether and dissolved in acetonitrile/water (3/2, v/v). The peptides were purified by reverse phase HPLC, and the identities of the peptides were confirmed by electrospray mass spectrometry. The peptides were resolved in water, aliquoted, lyophilized, and then stored at −70°C until used.

Human TG2 was expressed as a GST fusion protein in *Escherichia coli* using the vector construct as described previously (12). For the production of the TG2-treated 33-mer, the 33-mer with native sequence was treated with 200 μg/ml GST-TG2 in PBS with 2 mM $CaCl_2$, for 2 h at 37°C. The peptide fraction was separated from GST-TG2, and TG2-peptide complexes were separated by size exclusion fast protein liquid chromatography on a Superdex 200 (Pharmacia Biotech, Uppsala, Sweden) column. The concentration of TG2-treated 33-mer was determined by OD measurement at 280 nm, using the synthetic deamidated 33-mer as standard. The identity of the TG2-treated 33-mer was assessed by matrix-assisted laser desorption-ionization mass spectrometry. A mass change of +3 was observed compared with the native sequence, suggesting that the three predicted Gln residues were deamidated to Glu by TG2.

Gliadin protein (Sigma-Aldrich, St. Louis, MO) was treated with pepsin and trypsin (PT-gliadin) as described previously (13) and dialyzed overnight against water in membrane with a molecular mass cut-off of 12–14 kDa (Spectrum Laboratories, Rancho Dominguez, CA). The resultant high m.w. PT-gliadin (HMWF-PT-gliadin) was then lyophilized and stored at −20°C until used. Alternatively, PT-gliadin was subjected to gel filtration fractionation in fast protein liquid chromatography on a Superdex Peptide 10/30 column (Pharmacia Biotech) with an exclusion size of 20 kDa, with 10 mM NH_4HCO_3 as the running buffer. High molecular mass fractions were collected and lyophilized.

HLA-DQ2 molecules and peptide binding assays

Detergent-solubilized DQ2 molecules were purified from DQ2 homozygous, EBV-transformed, B lymphoblastoid cell lysates as described previously (14). The *Mycobacterium bovis* (MB) 65-kDa heat shock protein (Hsp)-derived peptide (KPLLIIAEDVEGEY; MB 65 kDa Hsp 243–255Y), the synthetic deamidated 33-mer, and the 33-mer deamidated by TG2 were ^{125}I-labeled by the chloramine-T method (15). Labeled peptides (30,000 cpm; 1–5 nM) were incubated with DQ2 (1 μM for both deamidated 33-mer variants or 300 nM for MB 65 kDa Hsp 243–255Y) overnight at 37°C in the presence of a mixture of protease inhibitors (14). In the competitive inhibition assays, various concentrations of unlabeled peptides were added to inhibit binding of the isotope-labeled MB 65 kDa Hsp 243–255Y peptide. DQ2 molecules and peptides were separated on Sephadex G-50 Superfine (Pharmacia Biotech) mini spin columns as described previously (16). The radioactivity was counted, and the concentrations of the competing peptides required to give 50% inhibition of binding of the indicator peptide (IC_{50}) were calculated.

APCs

An EBV-transformed B lymphoblastoid cell line (B-LCL) and monocyte-derived dendritic cells (DC) were used as APC. The B-LCL of an HLA-DR3/DQ2 homozygous individual (CD114) was cultured in RPMI 1640 with 10% FCS (both from Invitrogen Life Technologies, Carlsbad, CA). The DC were prepared from PBMC of DR3/DQ2-positive blood donors. Monocytes were positively selected from the PBMC with anti-CD14 mAb-coated MicroBeads (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) and then cultured in RPMI 1640 with 10% FCS containing 1000 U/ml GM-CSF and 500 U/ml IL-4 (both from R&D Systems, Abingdon, U.K.). On day 6, some DC were matured with 150 ng/ml LPS (Sigma-Aldrich) for 48 h, and the rest were kept immature in the GM-CSF/IL-4 medium. Both immature and mature DC were used as APC on day 8, and both cell sets were >95% $CD11c^+$, as ascertained by flow cytometry. The mature DC expressed CD83 and showed up-regulated expression of CD86 and HLA class II molecules compared with the immature DC.

T cell assays

For use in T cell assays, HMWF-PT gliadin and the 33-mer were preincubated with 200 μg/ml GST-TG2 in PBS with 2 mM $CaCl_2$ for 2 h at 37°C. In glutaraldehyde fixation of the APC, B-LCL or DC were washed with serum-free RPMI 1640 and incubated with 0.05% glutaraldehyde in RPMI 1640 for 90 s at 22°C before 0.2 M glycine were added for an additional 60 s. The cells were then thoroughly washed. Live B-LCL were irradiated (75 Gy). Fifty thousand fixed or live B-LCL or 20,000 fixed or live DC were coincubated overnight at 37°C in U-bottom, 96-well plates with various concentrations of Ags in RPMI 1640/10% FCS. After the incubation, the APC were washed twice with cold RPMI 1640 to remove unbound Ags before 50,000 gliadin-specific T cells were added to each well. The final part of the T cell assay was performed in RPMI 1640 with 15% heat-inactivated, pooled human serum. The following T cell clones (TCC) were used: TCC387.E9, TCC430.1.142 and TCC423.1.3.8. TCC387.E9 and TCC430.1.142 both recognize the DQ2-αI-epitope (9mer core region PFPQPELPY), and the closely related α-III epitope (9-mer core region PYPQPELPY). TCC423.1.3.8 recognizes the DQ2-γI-epitope (9-mer core region PQQSFPEQE). All three TCC are derived from intestinal biopsies of celiac disease patients and are DQ2 restricted (17). T cell proliferation was measured by the uptake of [^{3}H]thymidine (1 μCi/well (0.037 MBq/well); Hartmann Analytic, Braunshweig, Germany), which was added 24 h before harvesting. Cells were harvested after 72 h onto glass-fiber filter paper with an automated harvester (Mach III; TomTec, Hamden, CT), and [^{3}H]thymidine incorporation was measured by liquid scintillation counting (Wallac MicroBeta TriLux 1450; PerkinElmer, Wellesley, MA). Each Ag concentration was studied in triplicate. At least two independent experiments were performed for each assay.

Results

The 33-mer has a relatively high binding affinity to DQ2

Binding of the 33-mer to DQ2 was assessed in a competitive inhibition assay at pH 4.9 using an ^{125}I-labeled MB 65 kDa Hsp

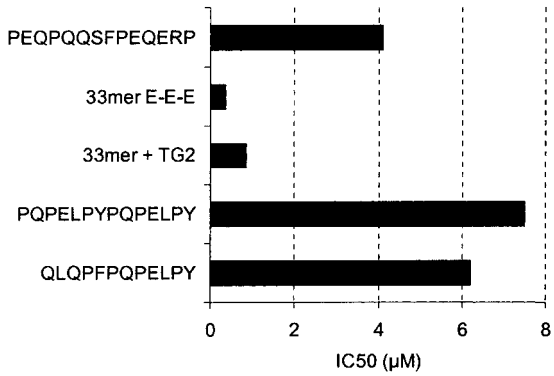

FIGURE 2. The 33-mer binds to DQ2 with relatively high affinity and with higher affinity than the shorter peptides containing one or two epitopes within the 33-mer. Peptides representing different deamidated gliadin epitopes and two variants of deamidated 33-mer were assessed for DQ2 binding in a cell-free, competitive binding assay at pH 4.9. The peptide PEQPQQSFPEQERP represents the DQ2-γI epitope. The peptides 33-mer E-E-E and 33-mer TG2 represent the synthetic deamidated and the TG2-treated 33-mer, respectively. The peptides PQPELPYPQPELPY and QLQPFPQPELPY represent DQ2-αII/III and -αI epitopes, respectively.

243–255Y peptide (KPLLIIAEDVEGEY) as the indicator peptide (14). Binding affinities, given as IC_{50} values, of some selected peptides representing gliadin-derived T cell epitopes are shown in Fig. 2. The two variants of the deamidated 33-mer had higher binding affinities to DQ2 molecules than other gliadin-derived peptides tested. The IC_{50} value for the 33-mer treated with TG2 was slightly higher than that of the synthetic deamidated 33-mer.

Processing dependence of the 33-mer and other gliadin T cell epitopes

To address whether presentation of the 33-mer requires further processing, we first used live and glutaraldehyde-fixed B-LCL as APC. To benchmark the assay, we used DQ2-γI epitope, which has previously been described to be processing dependent when part of partially proteolysed and dialyzed gliadin (18). By using pepsin- and trypsin-digested gliadin that was dialyzed against a membrane with an exclusion size of 12–14 kDa (HMWF-PT-gliadin) and the DQ2-γI-specific T cell clone TCC423.1.3.8, we reproduced this finding. A 21-mer deamidated peptide containing the DQ2-γI epitope (underlined; YQQLPQPEQPQQSFPEQERPF) was presented by both live and fixed B-LCL (Fig. 3*A*). In contrast, HMWF-PT-gliadin was presented to TCC423.1.3.8 by live $DQ2^+$ B-LCL, but not by same cells fixed by glutaraldehyde (Fig. 3*B*).

Using the same live and fixed B-LCL, a 12-mer peptide containing the DQ2-αI epitope (underlined; QLQPFPQPELPY) was efficiently recognized by the DQ2-αI/III-specific T cell clone TCC430.1.142 (Fig. 4*A*). The TG2-treated 33-mer, which contains three copies of DQ2-αI and -αIII epitopes, was also presented to the same T cell clone equally well by live and fixed B-LCL. The deamidated 33-mer was much more potent than the αI peptide, and this increased relative stimulatory potency was unchanged by using fixed B-LCL. Also, HMWF-PT-gliadin was presented by B-LCL (Fig. 4*B*), suggesting that even among the higher m.w. material retained by the dialysis membrane there were fragments that did not require further processing. Similar results were obtained with another DQ2-αI/III-specific T cell clone (TCC387.E9) as the readout (data not shown). These results indicate that the presentation of the 33-mer and larger fragments containing the DQ2-αI or DQ2-αIII epitopes does not require processing by B-LCL for effective T cell presentation.

We next repeated some of these experiments with the T cell clone TCC430.1.142 using live and fixed mature and immature DC as APC. The results are shown for the immature DC (Fig. 4, *C* and *D*), but the results were comparable with those for the mature DC. The 12-mer DQ2-αI peptide was equally efficiently presented by fixed and live DC. The TG2-treated 33-mer was also effectively presented by fixed DC, but not as efficiently as live DC (Fig. 4*C*). Fixed DC also presented the HMWF-PT-gliadin, but as with the 33-mer, the live cells were more effective than the fixed cells (Fig. 4*D*). The increased potency of the 33-mer compared with the 12-mer was also observed with DC as APC (Fig. 4*C*).

To further assess the molecular sizes of PT-gliadin fragments that are presented by fixed APC, PT-gliadin was subjected to fractionation by gel filtration. Fractions 7 and 8, containing larger molecules than the 33-mer (which elutes in fraction 10 under the same chromatographic conditions; Fig. 5*A*), were tested in T cell assays using both live and fixed B-LCL. Similar to results obtained with dialyzed PT-gliadin, the DQ2-αI/III-specific T cell clone TCC430.1.142, but not the DQ2-γI-specific T cell clone TCC423.1.3.8, recognized the high m.w. fractions 7 and 8 when presented by fixed B-LCL (Fig. 5*B*). Using live B-LCL, both T cell clones recognized the same fractions, showing that both the DQ2-αI/III and DQ2-γI epitopes were present in fractions 7 and 8 (Fig. 5*B*).

The 33-mer binds DQ2 efficiently at neutral pH in peptide binding assays and T cells assays with fixed B-LCL

We next analyzed the pH dependence of binding of the 33-mer to DQ2. The binding to DQ2 molecules purified from B-LCL was first tested, then in another set of experiments binding to fixed APC was tested using T cell proliferation as the readout. In the experiments with purified DQ2 molecules, the synthetic deamidated 33-mer, the 33-mer deamidated by TG2, and MB 65 kDa Hsp 243–255Y were labeled with ^{125}I and incubated overnight with detergent-solubilized DQ2 at different pH, and binding was assessed in a gel filtration assay. Peptide MB 65 kDa Hsp 243–255Y bound to DQ2 with a maximum of approximately pH 5.5, in accordance with earlier reports (14), whereas the binding of both 33-mer variants were maximal at pH 6.3 (Fig. 6).

In the T cell assay either TG2-treated 33-mer or synthetic 33-mer peptide with three glutamine residues substituted with glutamic acid were coincubated overnight with fixed B-LCL at three different pH values. The APC were then thoroughly washed to remove unbound peptides and pH-adjusting buffers. Thereafter, the DQ2-αI/III-reactive T cell clone TCC430.1.142 was added to cell culture medium at pH 7.3 and cultured in a standard 3-day T cell proliferation assay. The T cell responses to both deamidated 33-mer variants were higher when APC and 33-mer coincubation was performed at pH 6.3 compared with those at pH 4.9 and pH 7.2 (Fig. 7).

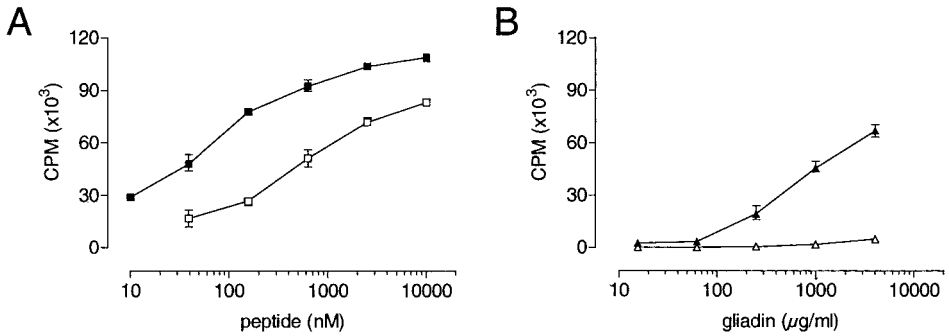

FIGURE 3. The DQ2-γI epitope is processing dependent when part of the HMWF of pepsin/trypsin-treated gliadin. The processing requirements of Ags containing the DQ2-γI epitope were assessed in a T cell assay with T cell clone TCC423.1.3.8 using either live APC (■ and ▲) or glutaraldehyde-fixed APC (□ and △). The DQ2-γI peptide YQQLPQPEQPQQSFPEQERPF (■ and □) can be presented by both live and fixed APC (*A*). HMWF-PT-gliadin (▲ and △) can only be presented by live APC (*B*). Error bars indicate the observed range within triplicate determinations.

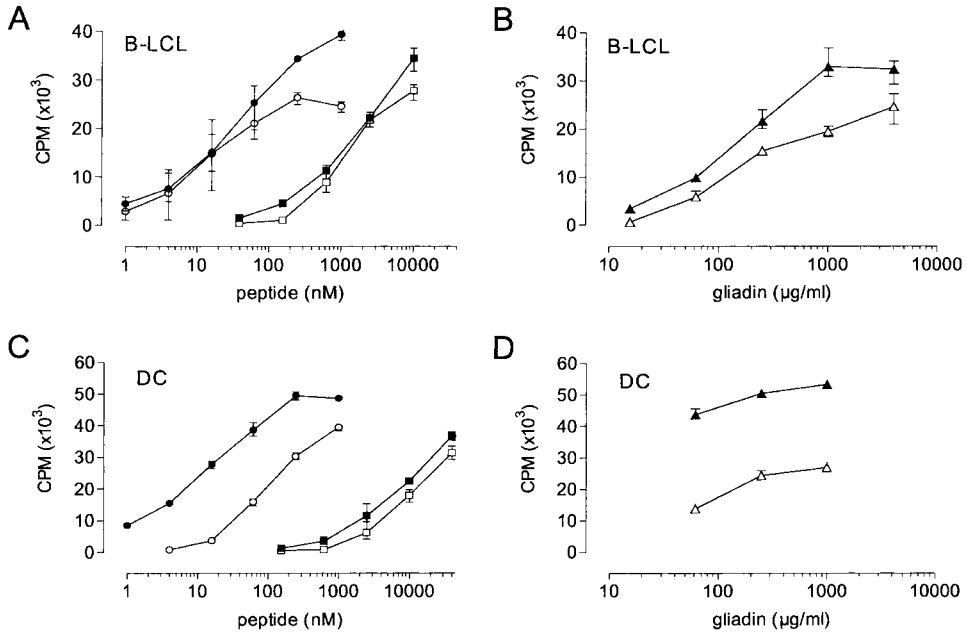

FIGURE 4. Processing requirements of Ags containing the DQ2-αI epitope. Two types of APC sources, either EBV-transformed B cells (B-LCL; *A* and *B*), or immature monocyte-derived DC (*C* and *D*) were tested as live (■ and ▲) or glutaraldehyde fixed cells (□ and △) in T cell assays using the T cell clone TCC430.1.142. The 12-mer DQ2-αI peptide (■ and □), the TG2-treated 33-mer (● and ○) (*A* and *C*) and TG2-treated HMWF-PT-gliadin (▲ and △; *B* and *D*) were presented by both live and fixed APC of both types. The 33-mer was more potent for T cell stimulation than the DQ2-αI peptide with all types of APC. Error bars indicate the observed range within triplicate determinations.

Discussion

The 33-mer (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF, α_2-gliadin 56–88) is produced by normal gastrointestinal digestion of recombinant α_2-gliadin. It is extremely resistant to proteolytic degradation, contains six partly overlapping copies of three different DQ2-restricted T cell epitopes, and is superstimulatory for T cells (8). Understanding how this peptide binds to DQ2 and how it is handled by APC for presentation to T cells is thus particularly instructive for the learning about the disease mechanisms of celiac disease. In this study we confirm the initial observations on the stimulatory potency of the 33-mer with live APC and show that the 33-mer is presented efficiently by fixed APC. Notably, this increased stimulatory potency is also retained after fixation of the APC.

Extracellular Ags can be presented by APC in either of two ways. The classical Ag processing and presentation pathway includes Ag uptake into the endosomal compartment, proteolytic fragmentation in the lysosomal compartments, and binding of the resultant peptide fragments to MHC class II molecules. Most proteins are processing dependent and presented via this pathway. The alternative pathway for Ag binding is the direct binding of

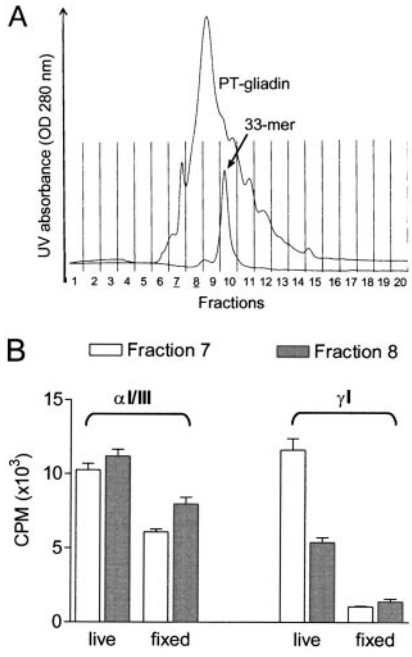

FIGURE 5. Fragments of peptic/tryptic digest of gliadin even longer than the 33-mer do not require processing by APC for T cell recognition. Pepsin/trypsin-treated gliadin (500 μg) and 33-mer (60 μg) were subjected to FPLC gel filtration (Superdex Peptide 10/30 column; exclusion size, 20 kDa) and monitored by UV absorbance at OD 280 (*A*). The 33-mer was almost exclusively eluted in fraction 10. Materials from fractions 7 and 8 were tested for T cell recognition with either live or fixed APC (*B*). Both fraction 7 (□) and fraction 8 (■) were recognized by the T cell clones specific for DQ2-αI/III (TCC430.1.142) and DQ2-γI (TCC423.1.3.8) when presented by live B-LCL. Using glutaraldehyde-fixed B-LCL, both fractions were only recognized by the DQ2-αI/III-specific T cell clone, not the DQ2-γI-specific T cell clone. Error bars indicate the observed range within triplicate determinations.

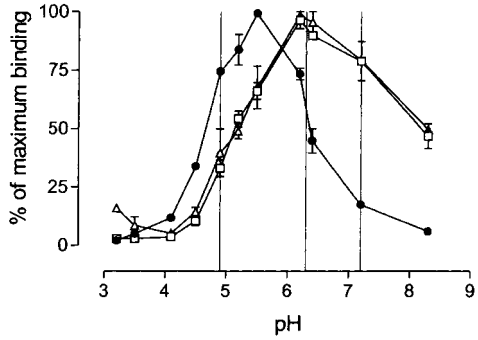

FIGURE 6. The 33-mer binds HLA-DQ2 most efficiently at approximately pH 6.3 in cell-free peptide binding assay. The binding efficiency of ^{125}I-labeled synthetic deamidated 33-mer (□), TG2-treated 33-mer (△), and the high affinity binder MB 65 kDa Hsp 243–255Y (●) to purified detergent-solubilized DQ2 molecules were assessed in a direct peptide binding assay at various pH. For each peptide, the binding is presented as a percentage of the maximum binding of this peptide. Maximum binding was 14.3% for the synthetic deamidated 33-mer, 7.2% for the TG2-treated 33-mer, and 10.7% for MB 65 kDa Hsp 243–255Y. Error bars indicate the observed range. Three vertical lines were drawn at pH 4.9, 6.3, and 7.2.

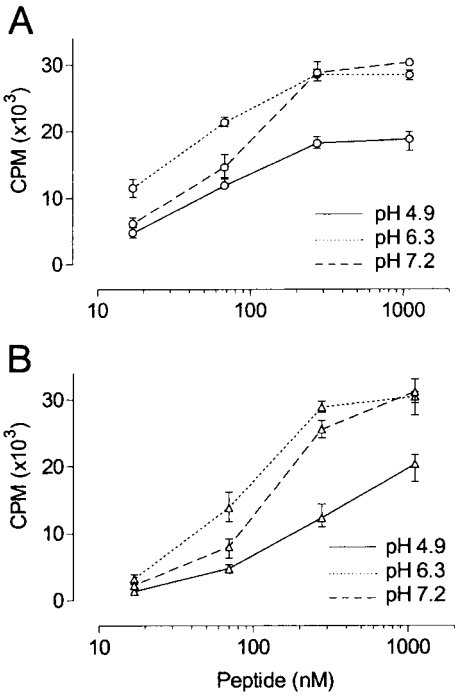

FIGURE 7. The 33-mer is most efficiently presented to T cells when incubated with fixed APC at pH 6.3 compared with pH 4.9 or 7.2. T cell proliferation of the T cell clone TCC430.1.142 was measured after stimulation with fixed and washed APC that had been incubated with either the deamidated 33-mer (*A*) or the TG2-treated 33-mer (*B*) at three different pH (solid line, 4.9; dotted line, 6.3; broken line, 7.2). Error bars indicate the observed range within triplicate determinations.

polypeptides to surface-expressed MHC class II molecules, without being internalized and processed. Shorter peptides are usually able to use this presentation pathway, but even some longer peptides of 30–39 aa (19, 20), intact proteins (i.e., fibrinogen) (21), and denatured proteins (i.e., bovine albumin, hen egg lysozyme, OVA, and human transferrin) (22) have been shown to bind to the appropriate MHC class II molecules in the absence of processing. Our observations that the 33-mer α-gliadin peptide can bind directly to DQ2 and that both 33-mer and pepsin, trypsin-generated fragments >10 kDa, can be presented by fixed APC, suggest that the 33-mer and larger fragments containing this peptide are not dependent on further processing by APC for T cell presentation. The observation that live DC present the TG2-treated 33-mer better than fixed DC is interesting, particularly because no such difference is observed for the shorter TG2-treated 12-mer T cell epitope. DC may thus be particularly suited to present the 33-mer. The lack of difference in presentation of the TG2-treated 33-mer between live and fixed B-LCL and the data showing that the deamidated 33-mer is a good DQ2 binder by itself suggest that it is impaired Ag uptake or HLA loading, rather than processing, that is affected by fixation of the DC. In this respect it is interesting that TG2 associated with surface integrins may be involved in the uptake of gluten peptides in DC and monocytes (1).

Our data provide some insight into why the 33-mer is a more potent T cell stimulator than the shorter peptides containing single epitopes within the 33-mer. The relative stimulatory potency of the 33-mer cannot be solely explained by the increased number of epitope copies contained, because the 33-mer has only three copies of the DQ2-αI and DQ2-αIII epitopes. The 33-mer has increased binding affinity to detergent-solubilized DQ, and the increased potency is seen also with fixed APC that are metabolically inactive. This suggests that at least part of the effect is related directly to HLA binding and does not involve signal transduction in the APC, which has been shown to be important for the superstimulatory activity of artificially multimerized T cell epitopes (23). Increasing antigenicity as a function of peptide length has also been reported for a cytochrome *c* epitope (24). T cell recognition of this epitope was independent of processing, and the antigenicity was continuously improved by extension of the core peptide region. A 23-mer peptide was >30 times more potent than the 12-mer core peptide. Moreover, a hybrid peptide of 51 residues containing the cytochrome *c* epitope and a T cell epitope derived from tetanus toxoid not only required no processing, but was 1 log more efficient than the cytochrome *c* of less than half its size. This length-dependent antigenicity of the cytochrome *c* peptides was also observed using lipid membrane-associated I-E^k, suggesting that this phenomenon is related to MHC binding per se. Our results with the 33-mer, including the binding data, are analogous to these observations. They raise the possibility that long gluten protein fragments surviving gastrointestinal digestion have the potential to be particularly immunogenic as a function of their length or because they can act multivalently for binding to DQ2. The identification of additional gluten T cell epitopes in celiac disease should take this into account.

Among the peptides eluted from DQ2 molecules of B-LCL, CLIP peptides are remarkably abundant (25–28). This may relate to binding of an unusual register of CLIP peptides in addition to the conventional register (25–26) as well as a potentially low efficacy of HLA-DM to unload CLIP peptides from DQ2. At any rate, cell surface DQ2 with abundant CLIP peptides available for exchange reactions should be particularly fit for cell surface binding of peptides. This may be one of the reasons why DQ2 predisposes to celiac disease. Not only can DQ2 bind the register-constraining, proline-rich gluten peptides with high affinities by accommodating glutamate residues in the P4, P6, or P7 pocket (29), but it can also directly bind gluten peptides that are produced by the gastrointestinal enzymatic digestion. The 33-mer is particularly proline rich and adopts type II polyproline helical conformation in solution. The fact that the free 33-mer has already adopted the required conformation for MHC class II binding may promote its binding to cell surface DQ2 by exchange with CLIP or other prebound peptides without the need for HLA-DM.

A limited set of peptides with high affinity binding to DQ2 has been found to exhibit a pH binding optimum at ~5 (14). Interestingly, the 33-mer has a pH optimum closer to what is found in the extracellular milieu, which is relevant for binding to DQ2 at the cell surface. At this point, we do not know the factors involved in this peptide-dependent pH effect on binding. It could relate to variable protonation of the peptides. The fact that the 33-mer adopts a type II polyproline helical conformation in solution may also be relevant.

The requirement for processing of food Ags by APC in the gut may be a special case, because these Ags are preprocessed by gastrointestinal digestion. Although most dietary proteins are broken down to amino acids or di- or tripeptides by the consecutive proteolytic actions of gastric, pancreatic, and small intestine brush-border membrane enzymes (30), significant amounts of the 33-mer remain intact after the digestion of α_2-gliadin by all these enzymes (8). Interestingly, a corresponding 12-mer peptide, QLQPFPQ PQLPY (α_9-gliadin 57–68), containing the native sequence of the αI epitope, is produced by similar proteolytic digestion of α_9-gliadin (2). T cells that become activated in intestinal biopsies by gluten stimulation in vitro are mainly CD4$^+$ T cells located in the lamina propria (1, 13). The gluten peptides will thus have to access DQ2-positive APCs in this subepithelial compartment. Although

enterocytes have been suggested to play a role in gliadin uptake and presentation in celiac disease (31), these cells generally lack expression of HLA-DQ molecules (32), and it therefore seems unlikely that DQ2-binding gliadin peptides should associate with HLA molecules of epithelial cells. Recently, intestinal transport and degradation of the 33-mer and other gliadin peptides were studied by mounting duodenal biopsies in Ussing chambers (33). After intestinal transport, gliadin peptides were found to be fully digested by enterocytes in controls and celiac patients in remission and to a somewhat slighter degree in patients with active celiac disease. Of note, however, selective uptake of luminal peptides by DC would not be measured in this system. Interestingly, DC express tight junction proteins and penetrate gut epithelial monolayers; they can thereby sample Ags directly from the intestinal lumen (34). DC also express vast amounts of TG2 (35). Our observation speaks to the possibility that luminal 33-mer and other gluten peptides produced by the gastrointestinal digestion can be deamidated by TG2 expressed in the enterocyte brush border or by DC and then be picked up by DC, partly by direct binding to DQ2, for later presentation to T cells in the lamina propria.

In summary, we demonstrate that a 33-mer of α-gliadin that is the end product of normal gastrointestinal proteolysis can be presented to T cells without further processing by APCs. The binding of the 33-mer does not show the pH 5 optimum found for other DQ2 ligands (14), but, rather, has an optimum around pH 6, closer to the pH at the cell surface. Direct binding of this peptide to cell surface DQ2 molecules may be an important route for Ag presentation in vivo. These results are interesting in relation to blocking of peptide presentation by competing compounds, a possible therapeutic approach for treatment of celiac disease.

Acknowledgments

We thank Günther Jung and Nicole Sessler (University of Tubingen) for providing synthetic peptides.

References

1. Sollid, L. M. 2002. Coeliac disease: dissecting a complex inflammatory disorder. *Nat. Rev. Immunol. 2:647.*
2. Arentz-Hansen, H., R. Korner, Ø. Molberg, H. Quarsten, W. Vader, Y. M. C. Kooy, K. E. A. Lundin, F. Koning, P. Roepstorff, L. M. Sollid, et al. 2000. The intestinal T cell response to α-gliadin in adult celiac disease is focused on a single deamidated glutamine targeted by tissue transglutaminase. *J. Exp. Med. 191:603.*
3. Molberg, O., S. N. McAdam, R. Korner, H. Quarsten, C. Kristiansen, L. Madsen, L. Fugger, H. Scott, O. Noren, P. Roepstorff, et al. 1998. Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. *Nat. Med. 4:713.*
4. Sjöström, H., K. E. A. Lundin, Ø. Molberg, R. Korner, S. N. McAdam, D. Anthonsen, H. Quarsten, O. Noren, P. Roepstorff, E. Thorsby, et al. 1998. Identification of a gliadin T-cell epitope in coeliac disease: general importance of gliadin deamidation for intestinal T-cell recognition. *Scand. J. Immunol. 48:111.*
5. Arentz-Hansen, H., S. N. McAdam, Ø. Molberg, B. Fleckenstein, K. E. Lundin, T. J. Jorgensen, G. Jung, P. Roepstorff, and L. M. Sollid. 2002. Celiac lesion T cells recognize epitopes that cluster in regions of gliadins rich in proline residues. *Gastroenterology 123:803.*
6. Vader, W., Y. Kooy, P. van Veelen, A. de Ru, D. Harris, W. Benckhuijsen, S. Pena, L. Mearin, J. W. Drijfhout, and F. Koning. 2002. The gluten response in children with celiac disease is directed toward multiple gliadin and glutenin peptides. *Gastroenterology 122:1729.*
7. van de Wal, Y., Y. Kooy, P. van Veelen, S. Pena, L. Mearin, G. Papadopoulos, and F. Koning. 1998. Cutting edge: selective deamidation by tissue transglutaminase strongly enhances gliadin-specific T cell reactivity. *J. Immunol. 161:1585.*
8. Shan, L., Ø. Molberg, I. Parrot, F. Hausch, F. Filiz, G. M. Gray, L. M. Sollid, and C. Khosla. 2002. Structural basis for gluten intolerance in celiac sprue. *Science 297:2275.*
9. Jardetzky, T. S., J. H. Brown, J. C. Gorga, L. J. Stern, R. G. Urban, J. L. Strominger, and D. C. Wiley. 1996. Crystallographic analysis of endogenous peptides associated with HLA-DR1 suggests a common, polyproline II-like conformation for bound peptides. *Proc. Natl. Acad. Sci. USA 93:734.*
10. Vader, L. W., A. de Ru, W. Y. van der, Y. M. Kooy, W. Benckhuijsen, M. L. Mearin, J. W. Drijfhout, P. van Veelen, and F. Koning. 2002. Specificity of tissue transglutaminase explains cereal toxicity in celiac disease. *J. Exp. Med. 195:643.*
11. Fleckenstein, B., Ø. Molberg, S. W. Qiao, D. G. Schmid, F. der Mulbe, K. Elgstoen, G. Jung, and L. M. Sollid. 2002. Gliadin T cell epitope selection by tissue transglutaminase in celiac disease. *J. Biol. Chem. 277:34109.*
12. Lai, T. S., T. F. Slaughter, C. M. Koropchak, Z. A. Haroon, and C. S. Greenberg. 1996. C-terminal deletion of human tissue transglutaminase enhances magnesium-dependent GTP/ATPase activity. *J. Biol. Chem. 271:31191.*
13. Lundin, K. E., H. Scott, T. Hansen, G. Paulsen, T. S. Halstensen, O. Fausa, E. Thorsby, and L. M. Sollid. 1993. Gliadin-specific, HLA-DQ(α1*0501, β1*0201) restricted T cells isolated from the small intestinal mucosa of celiac disease patients. *J. Exp. Med. 178:187.*
14. Johansen, B. H., S. Buus, F. Vartdal, H. Viken, J. A. Eriksen, E. Thorsby, and L. M. Sollid. 1994. Binding of peptides to HLA-DQ molecules: peptide binding properties of the disease-associated HLA-DQ(α1*0501, β1*0201) molecule. *Int. Immunol. 6:453.*
15. Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. *Biochem. J. 89:114.*
16. Buus, S., A. Stryhn, K. Winther, N. Kirkby, and L. O. Pedersen. 1995. Receptor-ligand interactions measured by an improved spun column chromatography technique: a high efficiency and high throughput size separation method. *Biochim. Biophys. Acta 1243:453.*
17. Molberg, Ø., S. N. McAdam, K. E. A. Lundin, and L. M. Sollid. 2000. Studies of gliadin-specific T cells in celiac disease. In *Celiac Disease: Methods and Protocols.* M. N. Marsh, ed. Humana Press, Totowa, p. *105.*
18. Quarsten, H., Ø. Molberg, L. Fugger, S. N. McAdam, and L. M. Sollid. 1999. HLA binding and T cell recognition of a tissue transglutaminase-modified gliadin epitope. *Eur. J. Immunol. 29:2506.*
19. Kovac, Z., and R. H. Schwartz. 1985. The molecular basis of the requirement for antigen processing of pigeon cytochrome *c* prior to T cell activation. *J. Immunol. 134:3233.*
20. Ria, F., B. M. C. Chan, M. T. Scherer, J. A. Smith, and M. L. Gefter. 1990. Immunological activity of covalently linked T-cell epitopes. *Nature 343:381.*
21. Lee, P., G. R. Matsueda, and P. M. Allen. 1988. T-cell recognition of fibrinogen: a determinant on the A_α-chain does not require processing. *J. Immunol. 140:1063.*
22. Sette, A., L. Adorini, S. M. Colon, S. Buus, and H. M. Grey. 1989. Capacity of intact proteins to bind to MHC class-II molecules. *J. Immunol. 143:1265.*
23. Rotzschke, O., K. Falk, and J. L. Strominger. 1997. Superactivation of an immune response triggered by oligomerized T cell epitopes. *Proc. Natl. Acad. Sci. USA 94:14642.*
24. Srinivasan, M., S. Z. Domanico, P. T. P. Kaumaya, and S. K. Pierce. 1993. Peptides of 23 residues or greater are required to stimulate a high-affinity class II-restricted T-cell response. *Eur. J. Immunol. 23:1011.*
25. Vartdal, F., B. H. Johansen, T. Friede, C. J. Thorpe, S. Stevanovic, J. E. Eriksen, K. Sletten, E. Thorsby, H. G. Rammensee, and L. M. Sollid. 1996. The peptide binding motif of the disease associated HLA-DQ(α1*0501, β1*0201) molecule. *Eur. J. Immunol. 26:2764.*
26. van de Wal, Y., Y. M. C. Kooy, J. W. Drijfhout, R. Amons, and F. Koning. 1996. Peptide binding characteristics of the coeliac disease-associated DQ(α1*0501, β1*0201) molecule. *Immunogenetics 44:246.*
27. Chicz, R. M., R. G. Urban, J. C. Gorga, D. A. Vignali, W. S. Lane, and J. L. Strominger. 1993. Specificity and promiscuity among naturally processed peptides bound to HLA-DR alleles. *J. Exp. Med. 178:27.*
28. Chicz, R. M., W. S. Lane, R. A. Robinson, M. Trucco, J. L. Strominger, and J. C. Gorga. 1994. Self-peptides bound to the type-I diabetes-associated class-II MHC molecules HLA-DQ1 and HLA-DQ8. *Int. Immunol. 6:1639.*
29. Kim, C. Y., H. Quarsten, E. Bergseng, C. Khosla, and L. Sollid. Structural basis for HLA-DQ2 mediated presentation of gluten epitopes in celiac disease. 2004. *Proc. Natl. Acad. Sci. USA 101:4175.*
30. Erickson, R. H., and Y. S. Kim. 1990. Digestion and absorption of dietary protein. *Annu. Rev. Med. 41:133.*
31. Zimmer, K. P., C. Poremba, P. Weber, P. J. Ciclitira, and E. Harms. 1995. Translocation of gliadin into HLA-DR antigen containing lysosomes in coeliac disease enterocytes. *Gut 36:703.*
32. Scott, H., L. M. Sollid, O. Fausa, P. Brandtzaeg, and E. Thorsby. 1987. Expression of major histocompatibility complex class-II subregion products by jejunal epithelium in patients with celiac disease. *Scand. J. Immunol. 26:563.*
33. Matysiak-Budnik, T., C. Candalh, C. Dugave, A. Namane, C. Cellier, N. Cerf-Bensussan, and M. Heyman. 2003. Alterations of the intestinal transport and processing of gliadin peptides in celiac disease. *Gastroenterology 125:696.*
34. Rescigno, M., M. Urbano, B. Valzasina, M. Francolini, G. Rotta, R. Bonasio, F. Granucci, J. P. Kraehenbuhl, and P. Ricciardi-Castagnoli. 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat. Immunol. 2:361.*
35. Le Naour, F., L. Hohenkirk, A. Grolleau, D. E. Misek, P. Lescure, J. D. Geiger, S. Hanash, and L. Beretta. 2001. Profiling changes in gene expression during differentiation and maturation of monocyte-derived dendritic cells using both oligonucleotide microarrays and proteomics. *J. Biol. Chem. 276:17920.*