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An Insertion Mutant in DQA1*0501 Restores Susceptibility to HLA-DM: Implications for Disease Associations

Tieying Hou,* Henriette Macmillan,* Zhenjun Chen,† Catherine L. Keech,† Xi Jin,‡ John Sidney,§ Michael Strohman,* Taejin Yoon,* and Elizabeth D. Mellins*

HLA-DM (DM) catalyzes CLIP release, stabilizes MHC class II molecules, and edits the peptide repertoire presented by class II. Impaired DM function may have profound effects on Ag presentation events in the thymus and periphery that are critical for maintenance of self-tolerance. The associations of the HLA-DQ2 (DQ2) allele with celiac disease and type 1 diabetes mellitus have been appreciated for a long time. The explanation for these associations, however, remains unknown. We previously found that DQ2 is a poor substrate for DM. In this study, to further characterize DQ2–DM interaction, we introduced point mutations into DQ2 on the proposed DQ2–DM interface to restore the sensitivity of DQ2 to DM. The effects of mutations were investigated by measuring the peptide dissociation and exchange rate in vitro, CLIP and DQ2 expression on the cell surface, and the presentation of α-II-gliadin epitope (residues 62–70) to murine, DQ2-restricted T cell hybridomas. We found that the three α-chain mutations (α+53G, α+53R, or αY22F) decreased the intrinsic stability of peptide-class II complex. More interestingly, the α+53G mutant restored DQ2 sensitivity to DM, likely due to improved interaction with DM. Our data also suggest that α-II-gliadin suppressed epitope. The DQ2 resistance to DM changes the fate of this peptide from a cryptic to an immunodominant epitope. Our findings elucidate the structural basis for reduced DQ2–DM interaction and have implications for mechanisms underlying disease associations of DQ2. The Journal of Immunology, 2011, 187: 2442–2452.

Epidemiological studies have revealed the now well-established association between the HLA-DQ2 allele (DQ2; A1*0501/B1*0201) and multiple autoimmune diseases, including celiac disease (CD) (1) and type 1 insulin-dependent diabetes (2). CD is induced by the ingestion of gluten, a component derived from wheat, barley, and rye. It is characterized by T and B cell-mediated autoimmunity that results in inflammation of the small intestine (3). About 90–95% of patients with CD express the DQ2 allele, and most of the remaining patients carry the HLA-DQ8 allele (4). Although the strong associations between DQ2 and CD have been appreciated for a long time, the explanations for these genetic links remain unclear.

The Journal of Immunology
interaction and provides additional insight into understanding the association of DQ2 with autoimmunity.

Materials and Methods

Cell lines

The T × B hybrid cell lines T2 (MHC II-DM) and T2DM (MHC II-DM) were obtained from the laboratory of Dr. Lisa Denzin (Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, New York, NY). They were established as described (20, 21). The T cell hybridoma cells, HH8 and DB4, were generous gifts from Prof. James McCluskey (University of Melbourne, Parkville, Australia) (22). Soluble (s)DQ2 was expressed in S2 Drosophila melanogaster insect cells using a baculovirus expression vector system (23).

Peptides

Biotin-labeled peptides MHC Ia 49–63 (APWIEQEGEYWDQ) and 65-kDa heat-shock protein of Mycobacterium bovis (MB 65KD Hsp 243–255, KPLLIIAEDVEGEY) were synthesized by AnaSpec (San Jose, CA). Unlabeled MHC Ia 49–63 was a kind gift from Dr. Alessandro Sette at La Jolla Institute for Allergy & Immunology (La Jolla, CA). The peptide α-gliadin 57–73 Q65E (QLQFPFPQELPPQPS) was a generous gift from Prof. James McCluskey.

cDNA constructs and transfection

The plasmids expressing wild-type (WT) DQ2α and β-prmHa3-DQA1*0501 and prmHa3-DQB1*0201 were obtained from the laboratory of Dr. Ludvig Oldfield (University of Oslo, Oslo, Norway). Site-directed mutagenesis was done by the GeneTailor Site-Directed Mutagenesis System from Invitrogen. The primer sets used to make mutants are summarized in Table I. The plasmids prmHa3-DQA1*0501 and prmHa3-DQB1*0201 were cotransfected with the neomycin-resistance plasmid pUC18neo into S2 cells using calcium phosphate. The positively transfected cells were selected with neomycin (1.5 mg/ml) for 4 wk.

To stably express full-length DQ2 in T2/T2DM cells, cDNA of WT DQ2α was amplified by PCR using sense primer 5′-CCGCGATCCAT-GATCCTAAACAAAGCT-3′ and antisense primer 5′-ACGGTGTGAAGGCCGCCTTGGT-3′ to add a BamHI site to the 5′ end and a Sall site to the 3′ end. The digested DQ2α was cloned into the retrovirus vector, PBMM-ZIN-neo, which was cut with the same enzymes. Full-length cDNA of WT DQ2β was amplified with the primer set 5′-CCCAAGGGCCCTTGGTG-3′ and 3′-CCCAA-TGTCTTGGAAAAAGGCT-3′ to add a SalI site to the 3′ end and a HindIII site to the 5′ end. The PCR product was cut with HindIII and EcoRI and ligated into the retrovirus vector PBMM-LZRS-puro, which was digested with the same enzymes. Mutants were generated with the same primer sets and system mentioned above.

The α-chain of DQ2 was first transfected into Phoenix Retroviral System, and the supernatant was collected to infect T2 or T2DM cells. Forty-eight hours postinfection, the positively infected cells were treated with neomycin (1 mg/ml) for 1 wk. The DQ2β-chain was then introduced into cells expressing the α-chain by the same approach. Finally, cells expressing a DQ2 dimer on the surface were enriched by PE-conjugated anti-DQ2 Ab (SPVL3, BD Biosciences) and anti-PE MACS microbeads (Miltenyi Biotec).

In vitro peptide loading assay

sDQ2 was engineered to have a CLIP peptide tethered to the β-chain via a thrombin cleavable linker, and an Acid-Base Zipper replaced the transmembrane domains. sDQ2 protein was expressed in S2 cells and purified with anti-Flag affinity M2 column. sDQ2 was predigested with thrombin for 2 h at room temperature to free the tethered CLIP peptide. Digested sDQ2 was then incubated with biotin-labeled peptides, MHC Ia 49–63, or MB 65KD Hsp 243–255, with or without DM, at pH 4.7, 37°C for 2 h, to allow peptide loading into DQ2. The reaction buffer contained 150 mM NaCl, 150 mM NaCl, 1% BSA, 0.5% Nonidet P-40 (NP-40), and 0.1% NaN3. After 2 h of incubation, the reaction was stopped by neutralizing pH with a buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% BSA, 0.5% NP-40, and 0.1% NaN3 (pH 8.6). DQ2-peptide complexes (100 µl) were transferred to a 96-well plate and captured by anti-DQ2 Ab SPVL3 for 1 h at room temperature. The loaded biotin-peptide-DQ2 complexes were streptavidin-activated using NeutrAvidin to minimize nonspecific binding. The europium emission fluorescence was measured in a time-resolved fluorometer (EG&G Wallac, Gaithersburg, MD) after enhancer was added.

In vitro peptide dissociation assay

Thrombin-digested sDQ2 was loaded with the peptide biotin-MHC Ia 49–63 at 37°C overnight. MHC Ia-DQ complexes were separated from free peptide using Sephadex G50-Superfine spin columns (Pharmacia Biotech, #17-0043-01). Unlabeled MHC Ia 49–63 peptide was added to a concentration five times higher than the biotin-labeled peptide to prevent re-binding of dissociated biotinylated MHC Ia 49–63. The dissociation was then allowed to proceed at 37°C with or without sDM for various times. At each time point, reactions were stopped by adding two volumes of ice-cold neutralization buffer (50 mM Tris-HCl, 150 mM NaCl, 1% BSA, 0.5% NP-40, and 0.1% NaN3 [pH 8.6]). Neutralized reaction mixtures (100 µl) were transferred to SPVL3-coated, blocked flat-bottom 96-well microtiter plates. After 2 h of capture at room temperature, plates were washed in PBS containing 0.05% Tween-20. DQ-bound biotinylated peptide was detected by addition of streptavidin-europium. After 1 h of incubation and further washes, enhancement solution (100 µl) was added, and time-resolved fluorescence was detected using a fluorescence plate reader (EG&G Wallac). The signals of each time point were normalized to the values of time zero, which was considered as one, and single-exponential decay curves were fitted with Prism5 (GraphPad) to calculate t1/2.

Flow cytometry

Surface DQ2 was detected by anti-DQ2 Ab, SPVL3, Ia3, or 2E11.12 and the secondary Ab, PE-conjugated goat anti-mouse IgG (BD Biosciences). In the double staining of DQ2 and CLIP, surface DQ2 was detected with PE-conjugated Ia3, and CLIP was stained by FITC-conjugated anti-CLIP Ab (Kingston CLIP, BD Biosciences, Flemington, NJ). For intracellular staining of DQ2, T2DM cells were first fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences) and then stained with PE-conjugated anti-DM Ab (MAbDM1; BD Biosciences). Cells were analyzed using a FACScan flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

Processing of gluten

Gluten (750 mg, G5004; Sigma-Aldrich) was added into 50 ml 0.01 M HCl (pH 2) and digested with 30 mg pepsin (P6887; Sigma-Aldrich) for 1 h at 37°C. Na2HPO4 (175 mg) was added after pepsin digestion, and pH was adjusted to 6. For 1 ml gluten suspension, 7.8 µl trypsin (50 mg/ml, T8802; Sigma-Aldrich) and 7.8 µl chymotrypsin (50 mg/ml, C4129; Sigma-Aldrich) were added for further digestion at 37°C for 2 h. Both trypsin and chymotrypsin were prepared in 25 mM Na2HPO4 (pH 6). Enzymes were inactivated by heating for 5 min at 95°C. After inactivation, samples were spun down quickly to pellet the undigested gluten. The supernatant was collected and dialyzed against water with a membrane of a molecular mass cutoff of 12–14 kDa (Spectrum Laboratories) at 4°C overnight. The resultant high-m.w. fraction of pepsin- and trypsin/chymotrypsin-digested gluten (PT-gluten) was concentrated with Amicon Ultra Centrifugal Filters (Millipore Ireland, #UFC901024) with a cutoff of 10 kDa. Finally, PT-gluten was incubated with 100 µg/ml guinea pig transglutaminase (stock 10 mg/ml, T-5398; Sigma-Aldrich) at 37°C for 2 h with 1 mM CaCl2 for deamidation.

T cell proliferation assay

Irradiated (8000 rad) T2 or T2DM cells expressing WT or mutant (100,000) were incubated with the titrated α-gliadin 57–73 Q65E or PT-gluten and T cell hybridoma DB4 or HH8 (100,000) for 16–24 h. After incubation, medium was collected, and the IL-2 in the supernatant was measured by a mouse IL-2 ELISA kit (BD Biosciences).

Results

Rationale for choice of DQA*0501 and DQB*0201 mutations

We have previously mapped the interface on DR3 that interacts with DM (24) as well as the interface on DM that interacts with DR3 (25). Based on these studies and available crystal structures, we know that the solvent-accessible F51 in DRA1*0101 is critical for DM interaction. As indicated in Fig. 1A ( magnified view in bottom right panel), F51 in DR3 α-chain protrudes out and provides a putative interaction site for DM. In contrast, its structural homolog in DQ2 (DQA*0501, DQB*0201) is protected by a steric block (Fig. 1A, magnified view in bottom left panel). To test whether this is the basis of reduced DM–DQ2 binding, we mutated the αa Q50 in DQA1*0501 to F. In addition, DQA1*0501 contains a deletion of residue 53, which is not observed in DQA1 (DQA1*0101, DQB1*0501) or DQ8 (DQA1*0301, DQB1*0302) (Fig. 1B, middle panel), both of which are susceptible to DM function. To study the role of α53 in DM interaction, the deletion

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was repaired with the homologous residues from DQA1*0101 (G) or DQA1*0301 (R). Either of these two mutations is expected to reorient the DM-contact region in DQ2 and improve DM–DQ2 interaction. Our previous studies of DR–DM interaction also show that DR1 and DR4 are both better substrates for DM than DR3, because their β-chains contain polymorphisms that favor DM binding, with K (DRb*0101) or E (DRb*0401) at position 98 (Fig. 1B, bottom panel, and R.C. Doebele and E.D. Mellins, unpublished observations). Based on these findings, point mutation T98K or T98E was introduced into the β-chain of DQ2, representing the polymorphism in DR1 or DR4. DQ2.2 (DQA1*0201, DQB1*0201) is another HLA-DQ2 molecule that has high homology to DQ2.5 (DQA1*0501, DQB1*0201) but does not confer risk for CD. A recent study has shown that DQα22 polymorphism controls the kinetic stability of DQ2–peptide complexes (26).

Replacement of αY22 in DQ2.5 with the homologous residue of DQ2.2 (F) decreases the overall peptide binding stability and inhibits T cell proliferation in response to gliadin peptide (26). This mutant (αY22F) is also included in our study to investigate its influence on DM–DQ2 interaction. The mutations that were chosen are summarized in Fig. 1C. The primer sets used to introduce point mutations are listed in Table I.

Both DQ2α and β-chain of WT and mutants were sequentially transfected into T2DM− (T2) and T2DM+ (T2DM) cells using retroviral transduction (Fig. 1D). Neither T2 nor T2DM expresses endogenous class II due to a large homozygous deletion in the MHC. Cells expressing DQ2 dimers on the surface were enriched by MACS. * denotes the site-directed mutation.

FIGURE 1. Rationale for choice of DQA1*0501 and DQB1*0201 mutations. A, Ribbon diagrams (based on crystal structures) of the peptide loading grooves of HLA-DQ2/gliadin (left panels) and HLA-DR3/CLIP (right panels). The structures indicated by highlighted boxes are magnified and shown in the bottom panels. B, The top panel is amino acid alignment of α allele of DQ2.5 (DQA1*0501) and α allele of DQ2.2 (DQA1*0201). The middle panel is the alignment of DQA1*0501 and A1*0101 and A1*0301. Alignment of DQB1*0201, B1*0101, and B1*0401 is shown in the bottom panel. Amino acids of interest are bolded and highlighted by the dots underneath.

C, Summary of rationale for choice of DQA1*0501 and DQB1*0201 mutations. D, Strategies used to construct T2/T2DM cell lines expressing DQ2 WT or mutant. DQ2 α-chain was introduced into T2/T2DM cells using retroviral transduction, followed by subsequent retrovirus-mediated β-chain transfer. Cells expressing surface DQ2 dimer were isolated by MACS. * denotes the site-directed mutation.

<table>
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<th>Mutation</th>
<th>Rationale</th>
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<td>αY22F</td>
<td>mimic the sequence of DQA1*0201</td>
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<tr>
<td>αQ50F</td>
<td>mimic DR3-DM interaction, make an accessible F for DM interaction</td>
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<td>+αS5G</td>
<td>repair the deletion in DQ2 and mimic the sequence of DQA1*0101</td>
</tr>
<tr>
<td>+αS5R</td>
<td>repair the deletion in DQ2 and mimic the sequence of DQA1*0301</td>
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<tr>
<td>βT98K</td>
<td>mimic the sequence of DRB1*0101</td>
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<tr>
<td>βT98E</td>
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| β         | α         | α | β | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | α | alpha_23

THE DQ2 MUTANT α+53G RESTORES DQ2 SUSCEPTIBILITY TO DM

![Diagram](http://www.jimmunol.org/)
Both β-chain mutations (βT98K and βT98E) showed comparable CLIP/DQ2 ratio to WT (data not shown). Therefore, we focused on the α-chain mutations in the following studies.

Expression of the soluble form of WT and mutant DQ2 molecules

WT and mutant sDQ2 molecules, lacking transmembrane and cytoplasmic domains, were expressed in S2 cells. These molecules were engineered with a CLIP peptide tethered to the C-terminal ends of the chains. A CLIP peptide was tethered to the N terminus of the β-chain using a linker containing a thrombin cleavage site and with C-terminal epitope tags followed by complementary acidic and basic zippers (Fig. 2A). sDQ2 proteins were affinity purified from cell-culture supernatants, and dimerization was confirmed by coprecipitation of FLAG-tagged β-chain with His-tagged α-chain (Fig. 2B, top panel), and vice versa (Fig. 2B, bottom panel). Lysate from untransfected S2 cells was included as negative control.

To investigate the effect of mutations on intrinsic peptide exchange, we performed a peptide loading assay using affinity-purified sDQ2 molecules and biotin-labeled peptides that have high binding affinity to DQ2. sDQ2 (200 nM) cleaved by thrombin was incubated with increasing amounts of biotinylated peptide (6.25–100 μM) at pH 4.7, 37°C for 2 h, allowing peptide loading into sDQ2. sDQ2–peptide complexes were then captured by anti-DQ2 Ab SPVL3, and the biotinylated peptide was detected by streptavidin-europium. Peptide exchange in the absence of DM was significantly increased in α+53G, α+53R, or αY22F compared with WT and αQ50F for MHC Ia 49–63 (Fig. 3A). The intrinsic peptide exchange of these three α-chain mutants was increased ~3–6-fold compared with that of WT. Similar results were seen for α+53G and α+53R when another high binding affinity peptide, MB 65KD 243–255, was tested (Fig. 3B). The spontaneous peptide exchange of αQ50F is comparable to WT. This finding indicates that the mutants of α+53G, α+53R, or αY22F decrease the intrinsic stability of DQ2–CLIP complexes, facilitating exchange.

DM-mediated peptide exchange was also increased in α+53G, α+53R, or αY22F

To measure the net effect of DM on peptide exchange, sDQ2 (200 nM) was incubated with increasing amounts of peptide (6.25–100 μM) in the absence or presence of 2.4 μM DM. The results with or without DM were plotted together: the distance between the two curves represents the DM effect. As indicated in Fig. 4A, DM enhanced the exchange of CLIP for MHC Ia 49–63 in sDQ2-A
WT and all of the mutants, but to different extents. For WT and αQ50F, the addition of DM led to a minimal increase in peptide exchange, which was consistent with our previous finding that DQ2 was insensitive to DM function (19). For α+53G, α+53R, or αY22F, however, the net DM effect was significantly higher. Among these three mutants, α+53G had the most evident difference between DM
2 and DM+. Similar patterns were observed for another peptide, MB 65KD 243–255 (Fig. 4B). Our previous studies have demonstrated that the intrinsic dissociation rate is positively correlated to DM-mediated dissociation rate (27). Therefore, the increased peptide exchanged in the presence of DM observed in α+53G, α+53R, or αY22F could be at least partially explained by the faster intrinsic peptide release and the consequent improvement in interaction between DQ2 and DM. The extent of DM enhancement of peptide loading shown on Fig. 4A and 4B also argues that α+53G is more sensitive to DM function than α+53R or αY22F.

A short time-course (0–4 h) experiment was also performed to directly compare the peptide exchange efficiency of WT and the α+53G mutant in the presence or absence of DM. As shown in Supplemental Fig. 1, the peptide exchange of CLIP for MHC Ia peptide was increased in the α+53G mutant at every time point tested (Supplemental Fig. 1).

α+53G is a better substrate of DM

To investigate the possibility that the specifically improved DM–DQ2 interaction contributes to the enhanced DM-mediated peptide exchange, we measured the dissociation rates of biotin-MHC Ia 49–63 in the absence or presence of DM. With or without DM, the peptide dissociation rate was faster in α+53G, α+53R, or αY22F than WT and αQ50F (Fig. 5A). These data confirm that these three mutants decrease the intrinsic stability of these complexes, and this effect contributes to faster peptide dissociation in the presence of DM. Interestingly, although αY22F had the quickest intrinsic dissociation rate, the lowest t1/2 in the presence of DM was detected in α+53G (Fig. 5B). After DM was added, the peptide dissociation from α+53G increased ∼12 times. Although DM also accelerated peptide release from WT and other mutants, the effect was modest (only one to two times). These findings argue that the acceleration of peptide dissociation observed in α+53G is not solely the result of moderately lowered intrinsic stability. Better DQ2–DM interaction is likely caused by this mutation. These two combined effects make α+53G the best substrate of DM among WT and other mutants.

To further assess the DM susceptibility of these five complexes, we calculated the dissociation rate constants, k, of each complex in the presence or absence of DM and plotted them in Fig. 6 together with our previous data in which peptide dissociation rates with or without DM were measured for a panel of DR– or DQ2–peptide complexes with different intrinsic stability. Previously, for most DR–peptide complexes we studied, a positive correlation between the intrinsic dissociation rate constant and DM-mediated dissociation rate constant was observed with a correlation of r = 0.69 (27). All of the six DQ2–peptide complexes (Fig. 6, yellow dots),
however, did not conform to this best-fitting line of correlation (19). Instead, they were all distributed along the line with a slope of 1 through the origin, which indicates no DM effect ($k_{\text{off}} = k_{\text{on}}$). These results imply that DQD2 is resistant to DM function. The square representing αQ50F (Fig. 6, light blue) was partly overlapped with WT (Fig. 6, black square), suggesting this mutant behaves similarly to WT. Both α+53R and αY22F significantly increased the intrinsic off-rate ($k_{\text{off}}$); thus, the squares representing these two mutants (Fig. 6, green for α+53R, purple for αY22F) shifted to the right along the x-axis. Interestingly, WT and all three mutants (αQ50F, α+53R, or αY22F) still followed the no-DM-effect line, suggesting the susceptibility to DM is not altered. α+53G (Fig. 6, red square), however, behaved differently from the other mutants and WT. It slightly shifted to the right along the x-axis due to the moderately lowered intrinsic stability. At the same time, it obviously shifted up along the y-axis and became closer to the line indicating the correlation, reflecting improved DM susceptibility.

The effect of mutations on CLIP phenotype

To evaluate the functional effect of α+53G, WT, or α+53G in cells, the mutant cDNAs were stably expressed in T2 and T2DM cell lines, which are human T cell leukemia/B cell line hybrids that have no endogenous MHC II molecules (Fig. 1D). In addition to its critical role in peptide exchange, DM also functions as a chaperone and conformational editor of MHC II (28–30). Previous studies have suggested that DM can increase the cell-surface level of some class II molecules, in particular those with low affinity for CLIP (28, 31–34). Because of the poor interaction between DQ2 and DM, DQ2 resisted DM-mediated conformation change, and cellular abundance of DQ2 was not influenced by DM (19). Due to the better sensitivity of α+53G to DM function, we expected to see more DQ2 abundance on α+53G-expressing cells. To test this, we measured cell-surface abundance of DQ2 using three different anti-DQ2 Abs, 2E11.12, Ia3, and SPVL3, at saturating concentrations. Fold change in mean fluorescence intensity (MFI) of DM- compared with DM+ cells was calculated and plotted in Fig. 7A. As we expected, coexpression of DM significantly increased surface expression of α+53G by approximately two to three times, with a <1.5-fold increase in WT (Fig. 7A).

To assess the effect of α+53G on CLIP accumulation at the cell surface, transfected T2 and T2DM cells were stained for DQ2 and CLIP with PE-conjugated anti-DQ2 Ab (Ia3) and FITC-conjugated anti-CLIP Ab (CerCLIP; BD Biosciences). The percentage of cells positive for both DQ2 and CLIP in α+53G-expressing T2 cells was significantly lower than that of WT (6.44 versus 62.1%; Fig. 7B, top panels), which likely is due to the low stability of α+53G–CLIP complexes, allowing exchange of CLIP for other peptides. When DM is present, it catalyzes CLIP exchange for other peptides in endocytic compartments. The percentage of double-positive cells (DQ2+CLIP+) in WT decreased from 62.1 to 11.6% in the presence of DM. The sensitivity of WT DQ2 to DM effect is probably caused by the high expression of DM in transfected cell lines, the absence of the normal competition between DQ2 and DR3 for interaction with DM that occurs in cells expressing the physiological (DR3/DQ2) haplotype (35), and the resulting increased molar ratio of DM to DQ2. The percentage of double-positive cells in α+53G-expressing cells was nearly equivalent after transfection of DM (from 6.44 to 5.49%). This is likely explained at least in part (see Discussion) by the rapid dissociation of α+53G–CLIP complexes in the absence of DM, which leaves no window to measure DM–α+53G interaction using CLIP (Fig. 7B, bottom panels).

The influence of α+53G on gliadin presentation to T cell hybridomas

Altered intrinsic stability and/or DM interaction may influence the T cell stimulatory capacity of antigenic peptides. We tested the presentation of α-gliadin 57–73 Q65E (QLQPFPQPELPYPQPQPS) by irradiated T2 and T2DM expressing DQ2 WT, α+53G, or αY22F to DQ2-restricted mouse T cell hybridomas HH8 or DB4. The peptide α-gliadin 57–73 Q65E contains the partially over-

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**FIGURE 5.** MHC Ia 49–63 dissociation from WT or mutant DQ2 molecules in vitro. Thrombin-digested sDQ2 molecules were preloaded with biotin-MHC Ia 49–63 overnight. The unbound peptide was removed, and dissociation of labeled peptide was followed in the absence (filled circles) or presence (filled squares) of DM (1.25 μM) for different periods of time (A). The signals at each time point were normalized to the starting counts for each molecule, and single-exponential decay curves were fit in Prism5 (GraphPad) to calculate $t_{1/2}$ (B). The experiment was repeated twice independently. There was a significant difference in DM-induced fold change of $t_{1/2}$ between α+53G and WT or any other α-chain mutant (B).
levels of WT and the differences in DQ2 expression level, we measured cell-surface hybridoma activation induced by WT and abundant peptides. To exclude the possibility that the differential change on the cell surface can be achieved in the presence of HH8; when a high concentration of peptide was used (10 kM for DQ2 WT and mutants was 1.25 M, are represented by squares of different colors. The concentration of DM is calculated and plotted as described above. Data from DQ2 WT and mutants are represented by squares of different colors. The concentration of DM used for DQ2 WT and mutants was 1.25 M.

Interestingly, the difference between WT and +53G was overexpressed in T2DM cells (+53G-expressing T2-DM cells 95% of total cells were DM positive, and DM levels in WT, +α53G, and αY22F-expressing cells were comparable (Fig. 8C).

Suppressed processing and presentation of gluten by α+53G-expressing T2-DM cells

High-m.w. fraction of pepsin- and trypsin/chymotrypsin-digested gluten is the high molecular mass (>10 kDa) fraction of processed gluten protein fragments, generated by pepsin and trypsin/chymotrypsin digestion and treated with transglutaminase. It has been shown that the presentation of PT-gluten requires internal processing (37), and we also saw that T cell proliferation was dramatically inhibited when APCs were fixed (data not shown). It is known that DM plays an important role in selection of endosomally generated peptides for presentation. To better understand the effect of DQ2 mutants on this functional interaction with DM, we measured T cell hybridoma activation in response to T2-DM cells led with PT-gluten. Interestingly, activation of both HH8 and DB4 was significantly compromised when +α53G was coexpressed with DM (Fig. 9). DM+ cells with DQ2 WT more

lapping α-I (QLQFPFPQELPY) and α-II (PQPELPYPQQL) gliadin epitopes, both of which have been identified as dominant epitopes recognized by T cells present in the small intestine after in vivo gluten challenge in HLA-DQ2+ patients with CD (36).

Both hybridomas express the markers of human CD4, murine CD4, and murine CD3 and are specific for the α-II gliadin epitope (residues 62–70) (22) (A.L. de Kauwe and J. McCluskey, unpublished observations). In the presence of low concentration of peptide (0.5–5 μM for HH8; 0.05–0.5 μM for DB4), T cell activation was more efficient with T2–α+53G (Fig. 8A, 8B, left panels). This finding likely reflects the more efficient CLIP replacement by exogenous gliadin peptide on the surface of α+53G-expressing T2 cells. In contrast, decreased hybridoma activation was observed when cells were cocultured with T2DM–α+53G (Fig. 8A, 8B, right panels) with low concentration of peptide. A likely explanation is the more effective editing of DQ2–peptide complexes in these cells, restricting peptide surface exchange. Interestingly, the difference between WT and +α53G was overcome when a high concentration of peptide was used (10 μM for HH8; 1 μM for DB4), indicating that comparable peptide exchange on the cell surface can be achieved in the presence of abundant peptides. To exclude the possibility that the differential hybridoma activation induced by WT and +α53G was caused by the differences in DQ2 expression level, we measured cell-surface levels of WT and +α53G DQ2 by FACS. As shown in Fig. 8C, +α53G expression on the T2 surface was lower than WT, whereas +α53G was expressed at higher levels than WT in T2DM cells. Thus, surface expression levels do not explain the pattern of hybridoma activation induced by +α53G. The αY22F mutant was not able to efficiently activate either T cell hybridoma, perhaps because the αY22F mutation substantially decreases the stability of peptide–class II complexes (26) and accelerates release of gliadin peptide from surface DQ molecules. In all of the T2DM cells, >95% of total cells were DM positive, and DM levels in WT, +α53G, and αY22F-expressing cells were comparable (Fig. 8C).
efficiently presented the epitope α-gliadin 57–73 Q65E than DM− cells (Supplemental Fig. 2A, 2B, left panels). This difference between DM− and DM+ was modest (for HH8) or even reversed (for DB4) for cells containing α+53G (Supplemental Fig. 2A, 2B, right panels). Considering the improved sensitivity of α+53G to the DM effect as demonstrated above, these findings suggest that the presentation of epitope α-gliadin 57–73 Q65E is suppressed by DM in endocytic compartments.

Discussion
It is generally accepted that DQ2 is a strong, inherited risk factor for several autoimmune diseases, especially CD (38, 39). Currently, the susceptibility of DQ2 to CD is understood to reflect the preferential binding of gliadin peptide to the DQ2 molecule (19, 23, 40). One of the structural characteristics of DQ2 that makes it able to accommodate the proline-rich gliadin peptide is the single residue deletion at position α53 (23, 40). A hydrogen bond formed between the amide carboxylic oxygen of the residue α53 and the amide hydrogen of the P1 residue is a general feature of most MHC II–peptide interaction (41, 42). Due to the lack of an α53 residue, proline can be accommodated by DQ2 at the P1 pocket without energetic cost. It is possible that insertion of an extra G or R in α53 influences the accommodation of proline at the P1 pocket of DQ2, although the gliadin-derived peptide α-gliadin 57–73 Q65E still binds sufficiently to stimulate the T hybridomas tested. Also, according to the crystal structure of the DQ2–α-I-
gliadin complex, a hydrogen bond is formed between the amide hydrogen of α52R of DQ2 and the amide carboxylic oxygen of the P-2 residue (23), which may also be affected by the addition of G or R at α53. These two factors may explain the accelerated spontaneous release of DQ-binding peptides from these two mutants (Figs. 5, 6). As for the mutant αY22F, recently reported by Dr. Ludvig Sollid’s group (26), it disrupts a water-mediated hydrogen bond formed between α22Y and the bound peptide, leading to a decrease in the overall peptide-binding stability. These three α-chain mutations also have increased peptide exchange in the absence of DM (Fig. 3), which is caused, we think, mainly by the increased off rate of CLIP.

A positive correlation between intrinsic peptide/class II and DM-mediated dissociation rate has been reported (27). Most DR–peptide complexes in our previous studies showed the predicted behavior (Fig. 6); however, all six DQ2–peptide complexes with varied levels of intrinsic stability (Fig. 6, yellow dots) were away from the best-fitting straight correlation line. In fact, they were all around the line with a slope of 1 through the origin, which indicates no DM effect ($k_{obs} = k_{on}$). These findings imply that DQ2 is resistant to DM effect, probably due to the poor interaction between these two molecules. If the mutants of DQ2 only reduce the intrinsic peptide dissociation rate, they should shift to the right along the x-axis, but still follow the no-DM-effect line, which is what we saw with α53R or αY22F (Fig. 6). The mutant α53G, however, had a shift both to the right and up, which makes it closer to the line of correlation, similar to other class II molecules that interact with DM (Fig. 6). This result indicates that the faster peptide dissociation from α53G in the presence of DM is not only because of the accelerated spontaneous peptide release, but also because of the increased susceptibility of α53G to DM effect.

The DM-interacting site on DQ2 has not been mapped yet. Our previous mutational analysis revealed that αF51 in DR3 plays a critical role in DM interaction (24). This residue is located on the interface of DR3 that interacts with DM and may function as a lever to move the extended strand including residues 51–53 (24). Because the structural homolog of αF51 in DQ2 is sterically blocked (Fig. 1A), we mutated αQ50 to F to provide an interaction site for DM. This mutation did not improve the DQ2 susceptibility to DM (Figs. 4, 5). In fact, the recombinant αQ50F protein is more sensitive to freezing and thawing than WT and other mutants (data not shown), probably because the mutation alters the local charge and influences the protein stability. The point deletion at α53 is also a special characteristic of DQA1*0501 that is not found in DQA1*0101 and DQA1*0301, which are both sensitive to DM function. Although the insertion of G or R both accelerated intrinsically peptide release, only α+53G has improved DQ2 susceptibility to DM. This difference probably is due to the different charge of G and R. The repulsion between the inserted α53R and the endogenous α52R in DQ2 may cause local conformational change that does not occur when uncharged G is inserted.

The point deletion at the position 53 of DQ α-chain is also found in other DQ alleles, including all the DQA1*05 alleles, DQA1*0601, A1*0602, A1*0401, and A1*0404. The relationship between some of these alleles and autoimmune diseases has been reported. For example, DQ7.6 (DQA1*0601:DQB1*0301) is associated with asthma in Chinese population (43) and pauciarticular juvenile chronic arthritis without anti-nuclear Abs (44). The allele of DQA1*0401 has been found associated with primary chronic progressive multiple sclerosis among the Ashkenazi patients (45). It is possible that the interaction between these alleles and DM is also defective, similar to what we have observed in DQ2, and it will be interesting to determine whether our finding that the insertion of glycine restores the DM susceptibility also applies to these DQ alleles.

Our previous data show that DQ2 purified from B cells is associated with two different groups of CLIP peptides, the traditional CLIP peptides (CLIP1) and the unusual CLIP peptides (CLIP2), which bind to DQ2 in overlapped but distinct binding registers (19). One of the major functions of DM is to replace CLIP with antigenic peptides in endosomal compartments (16–18). Because the available anti-CLIP Ab (CerCLIP; BD Biosciences) can only recognize the traditional CLIP1, but not the unusual CLIP2 peptide, which is the predominant one associated with DQ2, FACS staining with CerCLIP is not able to reveal the complete CLIP profile binding to WT or mutants. It is thus possible that the mutation of α+53G changes the ratio of CLIP1 and CLIP2 presenting on the cell surface. MALDI-TOF analysis of CLIP peptides eluted from DQ2 will be needed to assess this possibility.

Because DM edits the peptide repertoire presented by MHC II to CD4+ cells, the sensitivity to DM function may have a profound effect on T cell-mediated immune response. Even in the presence of DM, the peptide repertoire bound to WT DQ2 is not edited. Due to the sensitivity of α+53G to DM-mediated peptide editing, α+53G is loaded in endosomal compartments with the help of DM with peptides of high kinetic stability, which are eventually presented on the cell surface. The high stability of α+53G–peptide complexes only allows limited peptide exchange on the cell surface when exogenous gliadin peptide is provided. As a result, the presentation of the α-gliadin epitope to T cell hybridomas is inhibited (Fig. 8A, 8B, right panels). In the absence of DM editing, the lower intrinsic stability of α+53G–peptide complexes
facilitates peptide exchange at the cell surface, and the subsequent activation of T cell hybridomas by gliadin peptide is thus increased (Fig. 8A, SB, left panels).

Epitopes of an intact Ag that elicit potent T cell activation are classified as immunodominant epitopes. By contrast, epitopes that fail to trigger a T cell response, but can bind to class II, are called cryptic epitopes. It has been proposed that DM determines the immunodominant and cryptic fate of CD4\(^+\) T cell epitopes (34, 46–48). DM removes cryptic peptides from the loading groove of class II in endosomal compartments and thereby antagonizes or completely eliminates the presentation of cryptic epitopes. The cryptic fate of α-gliadin epitopes was initially implied by work with DR-transfected murine fibroblasts (DM-negative cells) that presented naturally processed α-gliadin protein (whole gliadin derived from unbleached flour) (49) to T cell clones more efficiently than EBV-transformed cell lines (DM\(^+\)) (50). The naturally processed peptide containing the α-gliadin II epitope is not efficiently removed by DM in endosomal compartments, at least in part because of the poor interaction between WT DQ2 and DM, leading to presentation of this epitope (Supplemental Fig. 2, left panels). However, the DQ2 WT DM\(^+\) cells have modestly higher levels of surface DQ2 WT than the DM-null DQ2 WT cells (Fig. 7A), perhaps due to variation in the (highly oligoclonal) transfectant lines and/or to some DM chaperone effect at the high DM/DQ2 molar ratio, although in DR3/DQ2/DM\(^+\) lymphoblastoid lines, DM is not an effective chaperone for DQ2 (19). This increase likely enhances presentation of the epitope and contributes to the increased activation of T cell hybridomas by the DQ2/DM\(^+\) cells. The difference in DQ2 surface level between DM\(^-\) and DM\(^+\) cells is even more evident for α+53G, likely due to chaperone effects (Fig. 7A). However, the T cell activation is not significantly increased or even suppressed by coexpression of DM (Supplemental Fig. 2, right panels). This likely reflects the sensitivity of α+53G to DM editing, which results in crypticity of the α-gliadin 57–73 Q65E epitope in the context of the α+53G mutant. Thus, an attractive hypothesis is that the resistance of WT DQ2 to DM changes the fate of α-gliadin 57–73 Q65E from a cryptic epitope to an immunodominant epitope, influencing the generation of a gliadin-induced immune response in patients expressing the DQ2 allele. The two hybridomas, HH8 and DB4, used in this study both specifically recognize the α-II-gliadin epitope (residues 62–70) from α-gliadin 57–73 Q65E (22 and unpublished observations from Prof. James McCluskey’s laboratory). We are currently analyzing other disease-related gliadin epitopes (51) to determine the generality of our finding. As α+53G mutation effects both the peptide binding behavior and the DM interaction of the protein, we are studying other DQ2 mutants for an ideal one that only improves DM interaction without influencing the peptide-binding groove. We predict that presentation of the gliadin epitopes will be suppressed by such a mutant, as their affinity for the DQ2 binding groove is lower than that of CLIP (19), which is sensitive to DM when the low affinity of DM for DQ2 is overcome with high levels of DM (19).

We have previously hypothesized that disease susceptibility of certain MHC II alleles may be highly related to alterations in events surrounding peptide loading and editing in endosomes, with consequences for Ag presentation events in the thymus and periphery that facilitate loss of self-tolerance and the development of autoimmune disease (52–55). It has been demonstrated that in mice lacking H2-DM molecules on certain H2 backgrounds, such as H-2\(^b\), class II is predominantly occupied by CLIP (56–58). A very narrow spectrum of class II-associated peptides leads to a reduction in the number of CD4\(^+\) T cells to ~30–50% of that in normal mice (56–59), and though positive selection occurs, it clearly restricts the diversity of the repertoire (58, 60). However, the population of CD4\(^+\) T cells from these DM-knockout mice is tolerized by CLIP-class II complexes rather than a broad range of self-peptides during negative selection. As a consequence, a large proportion of H2-DM\(^+\) CD4\(^+\) T cells show broad (self-) reactivity to APC from a DM\(^+\) mouse of the same (I-A\(^\beta\)) class II haplotype (56–59). In the case of DQ2, the predominance of (unexchanged) CLIP peptides associated with DQ2 likely affects positive selection in the thymus, likely reducing their number and diversity. Further, these peripheral DQ2-restricted T cells are likely to be potentially self-reactive, having been negatively selected primarily on DQ2/CLIP. This potential for autoreactivity would be revealed if/when conditions allow other-self-peptides to be presented, as may happen at sites of inflammation where self-peptides are generated extracellularly by proteases and where the pH of the microenvironment drops, favoring peptide exchange (61, 62). In addition, in peripheral APC, reduced DQ2–DM interaction and sufficient DQ2/CLIP affinity allows DQ2/CLIP to accumulate on the cell surface; these complexes more easily undergo peptide exchange than tightly bound peptides that have been subject to and survived DM editing.

In this study, we investigated the structural basis for the poor DQ2–DM interaction and identified one mutant, α+53G, which is able to restore DQ2 sensitivity to DM editing. Our results provide important mechanistic insights into the unique features of DQ2 interaction with the Ag presentation machinery. This may ultimately lead to improved understanding of the association of DQ2 with autoimmunity and may suggest new therapeutic approaches for DQ2-associated autoimmune disorders.

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