

Cell-surface MHC density profiling reveals instability of autoimmunity-associated HLA

Hiroko Miyadera,^{1,2} Jun Ohashi,³ Åke Lernmark,⁴ Toshio Kitamura,⁵ and Katsushi Tokunaga¹

¹Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan. ²Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Chiba, Japan. ³Faculty of Medicine, University of Tsukuba, Ibaraki, Japan. ⁴Department of Clinical Sciences, Lund University/CRC, Skåne University Hospital SUS, Malmö, Sweden. ⁵Division of Cellular Therapy and Division of Stem Cell Signaling, Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Sciences, The University of Tokyo, Tokyo, Japan.

Polymorphisms within *HLA* gene loci are strongly associated with susceptibility to autoimmune disorders; however, it is not clear how genetic variations in these loci confer a disease risk. Here, we devised a cell-surface MHC expression assay to detect allelic differences in the intrinsic stability of HLA-DQ proteins. We found extreme variation in cell-surface MHC density among *HLA-DQ* alleles, indicating a dynamic allelic hierarchy in the intrinsic stability of HLA-DQ proteins. Using the case-control data for type 1 diabetes (T1D) for the Swedish and Japanese populations, we determined that T1D risk-associated *HLA-DQ* haplotypes, which also increase risk for autoimmune endocrinopathies and other autoimmune disorders, encode unstable proteins, whereas the T1D-protective haplotypes encode the most stable HLA-DQ proteins. Among the amino acid variants of *HLA-DQ*, alterations in 47 α , the residue that is located on the outside of the peptide-binding groove and acts as a key stability regulator, showed strong association with T1D. Evolutionary analysis suggested that 47 α variants have been the target of positive diversifying selection. Our study demonstrates a steep allelic hierarchy in the intrinsic stability of HLA-DQ that is associated with T1D risk and protection, suggesting that HLA instability mediates the development of autoimmune disorders.

Introduction

HLA (also known as the MHC in other vertebrates) proteins present self- and non-self peptides to the T cell receptor (TCR) to maintain self-tolerance and adapted immunity (ref. 1 and Figure 1A). Certain *HLA-DR-DQ* haplotypes, such as *DR3-DQA1*05-DQB1*02:01* (*DR3-DQ2.5*) and *DR4-DQA1*03-DQB1*03:02* (*DR4-DQ8.3*) in Europeans, confer a risk for autoimmune diseases, including type 1 diabetes (T1D), celiac disease, and autoimmune endocrinopathy (2–7). In East Asian populations (Japanese) *DR9-DQA1*03-DQB1*03:03* (*DR9-DQ9.3*) and *DR4-DQA1*03-DQB1*04:01* (*DR4-DQ4.3*) are the major risk factors for T1D (8–10) and other autoimmune endocrinopathies (11) (Table 1 and see Supplemental Figure 1 for abbreviations for the *DQA1-DQB1* haplotypes [*DQ* haplotype]; supplemental material available online with this article; doi:10.1172/JCI74961DS1). Despite accumulating genetic evidence, the mechanism through which particular *HLA* alleles confer risk for autoimmune diseases has not been fully uncovered.

The *HLA*-autoimmunity association has been generally explained by the allelic differences in self-epitope presentation (see, for instance, refs. 12, 13). However, the binding affinity and specificity in the MHC-self-epitope interaction are highly variable. Studies of T1D, multiple sclerosis, and other autoimmune disorders have found that the disease-relevant self-epitopes interact with the MHC with high or low affinity or in kinetically unfavorable registers (14–29). Some of these self-epitopes bind to both the disease risk and neutral/protective allele products (i.e., promiscuous binders) (15, 30–32). Although self-epitope

presentation is critical in autoimmune pathogenesis, the above findings suggest that additional factors may also contribute to the allele-specific disease risk.

In the 1990s, Kwok's and Unanue's groups and other researchers reported that the T1D risk alleles of *HLA-DQ* and murine *I-A* encode SDS unstable proteins (33–36). The SDS stability measures the migration of non-boiled MHC class II (MHC II) on SDS-PAGE (37) and was initially regarded as an indicator of peptide occupancy. It was later found that SDS stability reflects the stabilization of the peptide-MHC (pMHC) at the P1 and P9 pockets and at the extended peptide residues (38–45). In some of these and other studies, however, SDS stability was not affected by the peptide-binding affinity (41, 42, 46) and was maintained through the peptide-independent stabilization (46). The mechanism of SDS stability, and hence its relevance to the MHC protein function, has remained controversial.

The stability of the pMHC is maintained through the heterodimerization of the α and β subunits and peptide presentation (Supplemental Figure 2). The interaction of the peptide side chain atoms with MHC stabilizes the pMHC in a peptide-specific manner and has been extensively analyzed (1). In this study, we focused on the possibilities that the MHC stability might differ intrinsically among the alleles and that this stability may be associated with autoimmunity. The intrinsic stability of the MHC protein in this study refers to the MHC stability that is formed through the α/β assembly and peptide main chain interactions. The contribution of both the polymorphic and nonpolymorphic residues in the heterodimerization and peptide main chain interactions suggests that MHC stability might differ intrinsically among alleles. However, it has not been possible to measure the intrinsic stability of MHC protein

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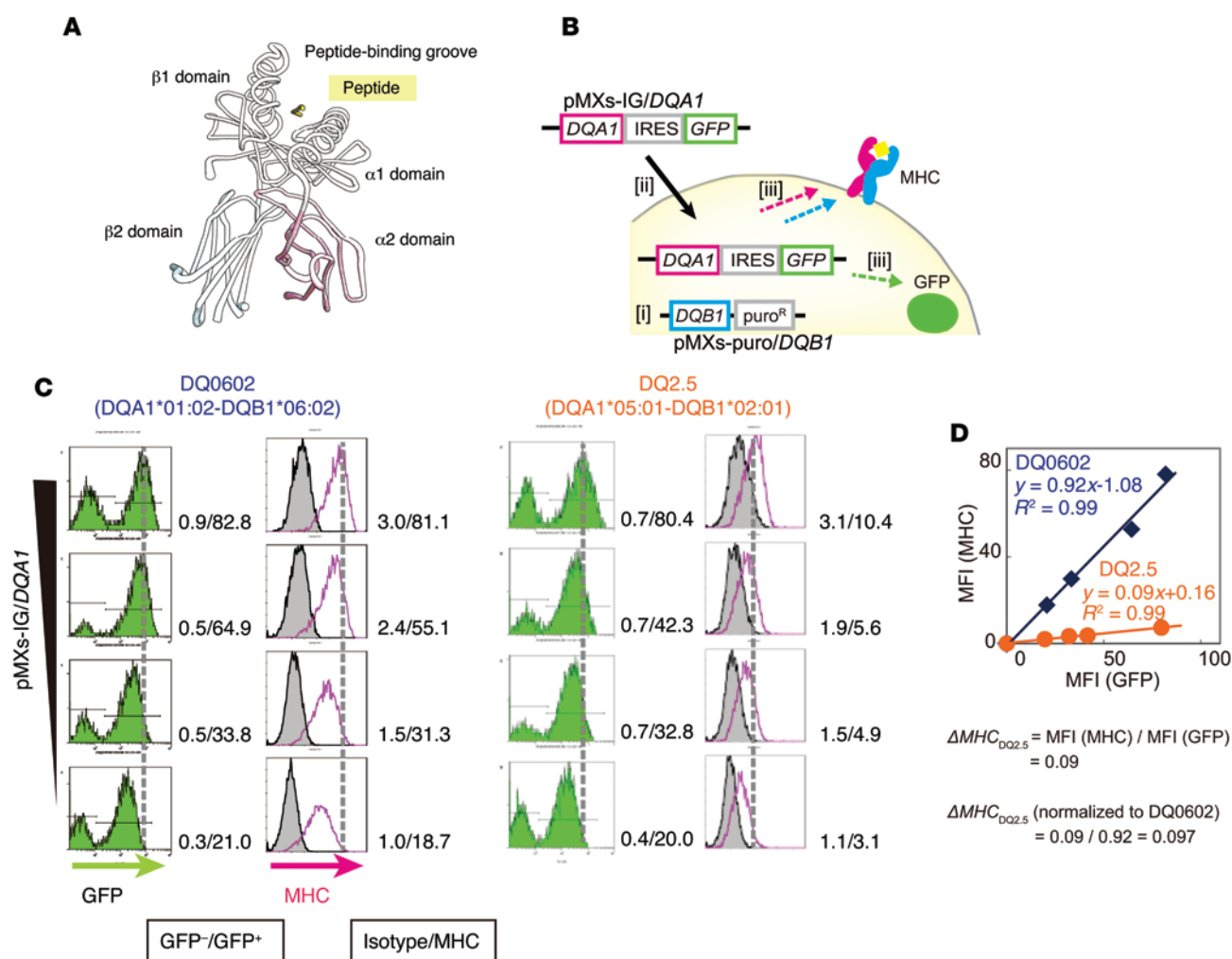


Figure 1. Measurement of Δ MHC. (A) Structure of MHC II (DQ0602 [pdb: 1uvq]) (70). MHC II is a heterodimeric transmembrane glycoprotein that is composed of one α and one β subunit. The $\alpha 1$ and $\beta 1$ domains constitute the peptide-binding groove, and the $\alpha 2$ and $\beta 2$ domains form the constant domain. In the case of HLA-DQ, *HLA-DQA1* and *-DQB1* encode the α and β subunits, respectively. (B) Outline of the Δ MHC assay. The Δ MHC assay measures cell-surface MHC expression levels normalized to the internal control GFP. The *HLA-DQB1*-stable cells (i) were transduced with the retroviral vector pMXs-IG/DQA1 (52) (ii). *HLA-DQ* was expressed on the cell surface in the presence of both the *HLA-DQA1* and *-DQB1*. Cell-surface *HLA-DQ* expression and cytosolic GFP expression were measured by flow cytometry (iii). (C) Representative data from the Δ MHC assay for DQ0602 (left panels) and DQ2.5 (right panels). To quantify cell-surface MHC expression, the *HLA-DQB1*-stable cell line was transduced with a graded concentration of retrovirus containing pMXs-IG/DQA1. Expression levels of both *HLA-DQ* and GFP increased with the concentration of the retrovirus. Numbers indicate the MFI for GFP-negative and -positive cells and for the isotype control and anti-*HLA II* β (WR18). Dashed lines indicate the highest MFI in each sample set. GFP (green), anti-*HLA II* β (WR18) (magenta), and isotype control (black). (D) The increase in cell-surface MHC expression relative to GFP (Δ MHC) was calculated by plotting the MFI (GFP) and MFI (MHC) (Supplemental Figure 3A). The Δ MHC for each *HLA-DQ* allelic pair was normalized to the Δ MHC of DQ0602, which was measured on the same day. The Δ MHC for DQ2.5 and its normalized value are shown. See also Supplemental Figures 3–5 and Methods.

or to demonstrate its allelic differences, because the pMHC is usually stabilized through both the peptide main chain and side chain interactions.

To detect the potential allelic differences in the intrinsic stability of the MHC protein, we used an alternative approach to the conventional stability assays. Specifically, instead of analyzing protein stability itself, we measured the biological outcome, the cell-surface expression of MHC protein. We quantified the amount of cell-surface MHC in engineered conditions and confirmed, through the use of mutagenesis and the model peptides, that the level of cell-surface MHC protein density (referred to herein as the Δ MHC) reflects the intrinsic stability of the

MHC protein. Δ MHC was then used to analyze the relationship between the intrinsic stability of MHC protein and autoimmune disease risk. Δ MHC measures the combined outcomes of the heterodimer assembly, cell-surface transport, and turnover, but not the chemical or physical stability of the MHC protein. However, for simplicity, Δ MHC is used as an equivalent to the protein stability in this article.

In this study, we identified an allelic diversity in the intrinsic stability of HLA-DQ that has been maintained through evolution and is associated with genetic risk for T1D. Our study provides a new framework through which to interpret the *HLA*-autoimmunity association profiles and uncover the mechanism of autoimmunity.

Table 1. Associations of *DR-DQ* haplotypes with autoimmune and other immune disorders

<i>DR-DQA1-DQB1</i> haplotype	Susceptible	Protective
<i>DR3-DQ2.5</i> (<i>DRB1*03-DQA1*05-DQB1*02</i>) ^a	APS type II	
	Celiac disease	
	Selective IgA deficiency	
	MS	
	SLE	
<i>DR4-DQ8.3</i> (<i>DRB1*04-DQA1*03-DQB1*03:02</i>) ^a	T1D	
	APS type II	
	Celiac disease	
	T1D	
<i>DR9-DQ9.3</i> (<i>DRB1*09-DQA1*03-DQB1*03:03</i>) ^b	APS type III	
	T1D	
	Microscopic polyangiitis	
<i>DR4-DQ4.3</i> (<i>DRB1*04-DQA1*03-DQB1*04:01</i>) ^b	APS type III	
	T1D	
<i>DR15-DQ0602</i> (<i>DRB1*15-DQA1*01:02-DQB1*06:02</i>)	Narcolepsy	APS types II and III
	MS	Selective IgA deficiency
	SLE	T1D

^aHaplotypes commonly observed in Europeans. ^bHaplotypes commonly observed in the Japanese population. APS type II (6); celiac disease (56, 137); selective IgA deficiency (5, 138); MS (4, 5, 139); SLE (4, 5); T1D (Europeans) (59, 79, 80); APS type III (11); T1D (Japanese) (10); microscopic polyangiitis (140); narcolepsy (141). MS, multiple sclerosis; SLE, systemic lupus erythematosus.

Results

Measurement of ΔMHC . We generated an MHC expression system using fibroblasts (NIH3T3, murine embryonic fibroblasts) as expression hosts and GFP as an internal control. MHC II is expressed in a functionally intact form in fibroblasts (47, 48) but in inappropriately paired or unassembled forms, the α and β subunits are retained during intracellular transport and are degraded (49, 50). The observation that cell-surface MHC expression on fibroblasts is altered through the gain and loss of hydrogen bond(s) (H-bond) between the MHC and the peptide (51) indicates that subtle changes in the net stability of the pMHC can be detected using cell-surface MHC protein expression levels. H2-DM, which is expressed in antigen-presenting cells (APCs) and stabilizes pMHC, may be absent in this expression system.

We established *HLA-DQB1*-stable cells using the retrovirus vector pMXs-puro and the packaging cell line PLAT-E (52, 53). We then transduced the *HLA-DQB1*-stable cells with a retrovirus containing pMXs-IG/*DQA1* (Figure 1B). Using a graded concentration of retrovirus particles, it was possible to express both the *HLA-DQ* and GFP at several different levels (Figure 1C). Cell-surface *HLA-DQ* and cytosolic GFP were measured by flow cytometry using the pan-*HLA* II β mAb (WR18). The mean fluorescence intensity (MFI) for both the MHC [MFI (MHC)] and the GFP [MFI (GFP)] showed good linear correlation ($R^2 > 0.9$). The increase in MFI (MHC) relative to MFI (GFP) (slope in Figure 1D), which indicates the amount of cell-surface MHC normalized to GFP, was calculated for each *HLA-DQ* allelic pair and was designated as ΔMHC (Figure 1, C and D, and Supplemental Figure 3, A and B). To min-

imize interassay variation, ΔMHC was normalized to ΔMHC for the *DQA1*01:02-DQB1*06:02* haplotype product (DQ0602), which is highly SDS stable (36) and showed one of the highest ΔMHC values among the tested alleles. Hereafter, the ΔMHC values that were normalized to the ΔMHC of DQ0602 are indicated in the figures unless otherwise specified. Representative ΔMHC assay data are presented in Supplemental Figure 4, A and B. ΔMHC was measured for the major *HLA-DQ* alleles in worldwide populations and in their possible *trans* combinations, given that the *trans* *DQA1-DQB1* pair forms heterodimers (54), and certain *trans* combinations are associated with autoimmunity (55–59). In this study, the *HLA* allele and haplotype protein products are indicated using the nonitalic version of the gene name (e.g., DQ0602 represents the *DQ0602* haplotype product).

Figure 2A shows the ΔMHC profile for *HLA-DQ*. ΔMHC varied by nearly 100-fold among the *HLA-DQ* alleles. Consistent with earlier work (60–62), *HLA-DQA1* and *DQB1* alleles of the same evolutionary sublineage (63) expressed *HLA-DQ* on the cell surface (Figure 2, A and B). These sublineages are referred to herein as the subgroups DQ2/3/4 and DQ5/6. *HLA-DQA1*02*, **03*, and **05* and certain *DQB1*06* alleles also expressed *HLA-DQ* on the cell surface (Figure 2A). *HLA-DQ* cell-surface expression was not detectable in the absence of *HLA-DQA1* or in the presence of the incompatible *HLA-DQA1* alleles (Supplemental

Figure 5, A–C, and Supplemental Table 1). The *HLA-DQ* cell-surface expression pattern and the assembly of the DQ α and DQ β subunits were confirmed using stable insect cells (*Drosophila melanogaster* S2) (Supplemental Figure 6, A–D).

Among the major *DQ* haplotypes, *DQ0602* and *DQ9.2* showed the highest ΔMHC , whereas the ΔMHC of the *DQA1*01:04-DQB1*05:01* (*DQ0501*) product was below the threshold (Figure 2, A and C, and Supplemental Table 1). The conserved hierarchy in ΔMHC among the DQ5/6 (*DQA1*01:02* > **01:01*, **01:03* > **01:04* and *DQB1*06:02* > **06:01*, **06:03* > **06:04* > **05:03* > **05:02* > **05:01*) (Figure 2A and Supplemental Figure 7, A–D) indicates that polymorphic variants in each subunit act independently of the variants in the other subunit in the regulation of ΔMHC . In DQ2/3/4, ΔMHC decreased in the order of *DQA1*02* > **03*, **05* > **04* > **06* and *DQB1*03:01*, **03:03*, **04* > **02*, **03:02*, except for the high ΔMHC value of *DQA1*03-DQB1*02* (*DQ2.3*) (Figure 2A and Supplemental Figure 7, E–H). The mechanism that stabilizes DQ2.3 was not identified in this study. The hierarchy in ΔMHC for the major *DQ* haplotype products was similar to the hierarchy in the SDS stability (DQ0602 > DQ0603 > DQ0604, DQ7.3, DQ0501, DQ8.3, DQ2.5) that was measured using the cell lysates of *DQ*-homologous B lymphoblastoid cell lines (B-LCLs) (36). SDS stability may be sensitive to the variants at 57 β , given that DQ9.3 (ΔMHC = 0.12, carries Asp57 β) is SDS stable (43), whereas DQ2.2 (ΔMHC = 0.4–0.6, carries non-Asp57 β) is reported as SDS unstable (43).

The mAb WR18 stains diverse *HLA-DQ* (64, 65), *-DR*, and *-DP* allele products (H. Miyadera, unpublished observations), indicating that the mAb WR18 recognizes a common epitope on *HLA* II β .

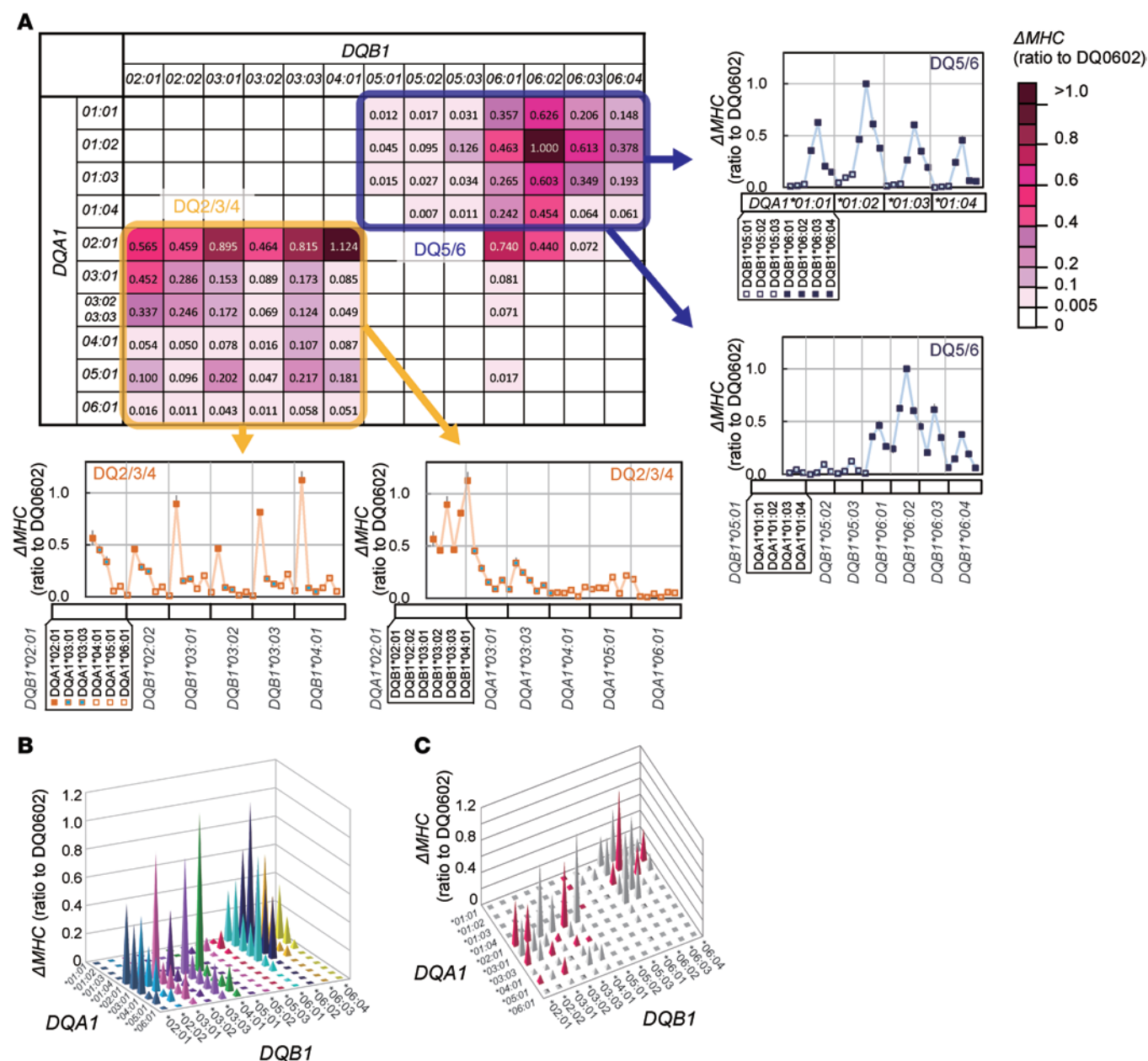


Figure 2. Δ MHC profile for the HLA-DQ allele products. (A) Δ MHC profile for HLA-DQ. Numbers and color scale indicate the Δ MHC (normalized to the Δ MHC of DQ0602). Δ MHC values below the threshold (<0.005 after normalization) are blank. Graphs display the Δ MHC value for each subgroup. Error bars represent the SEM. See also Supplemental Table 1. (B and C) Δ MHC profiles for the major HLA-DQ allele products (B) and the major DQ haplotype products (C). In C, the DQ haplotypes that are present at a haplotype frequency greater than 0.03 in at least 1 of the following populations are magenta colored: Congo Kinshasa Bantu ($n = 90$) (122, 135); Tunisian ($n = 100$) (122); South Korean ($n = 324$) (122); Indian Uttar Pradesh ($n = 202$) (122, 136); and European-American ($n = 1,899$) (133).

The Δ MHC profile was reproducible with pan-HLA II β mAbs BL-IA/6, TDR31.1 (Supplemental Figure 8, A–C), and IVA-12 (H. Miyadera, unpublished observations). The Δ MHC assay was not successful in the B cell lines due to the low efficiency of retroviral transduction (H. Miyadera, unpublished observation).

The effects of peptides on Δ MHC. To confirm that Δ MHC reflects the net stability of the MHC protein, Δ MHC was measured in the presence of high- and low-affinity peptides using the DQB1*06:02 peptide fusion constructs (designed according to ref. 66) (see Methods). Δ MHC increased 3.4-fold in the presence of insulin B_{1–15},

a strong binder of DQ0602 (30), and 1.5-fold in the presence of the class II-associated invariant chain peptide (CLIP)_{81–107} (Figure 3A). Δ MHC decreased 0.6-fold in the presence of insulin B_{9–23}, a weak binder of DQ0602 (30), as well as in the presence of an artificial negative control peptide (GGSGGSGGSGGS) (GGS peptide) (Figure 3A), with which the interaction of the MHC protein with the peptide side chain may be limited. These data confirm that Δ MHC reflects the net stability of the pMHC.

The effect of endogenous peptides on the Δ MHC profile (Figure 2A) was estimated using DQB1-GGS peptide fusion constructs.

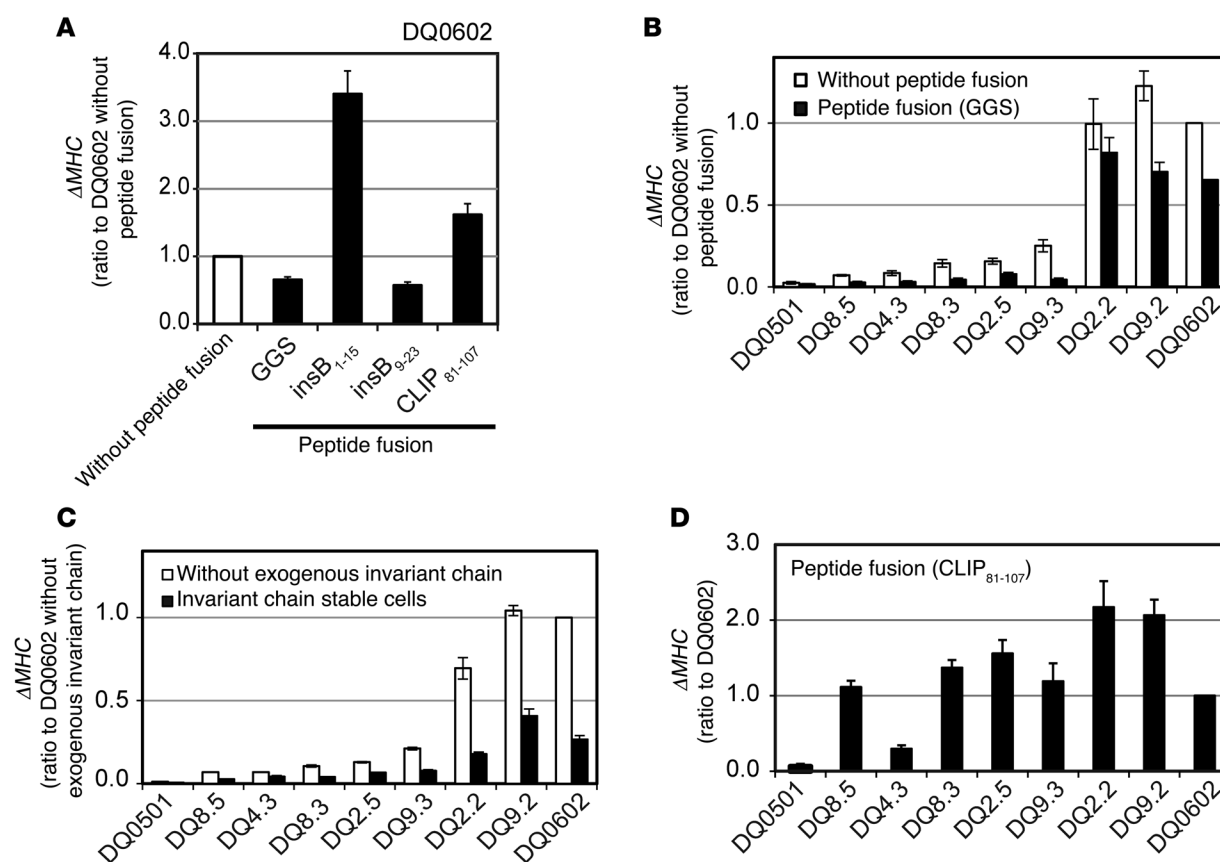


Figure 3. The effect of peptides on the Δ MHC. (A) Δ MHC profile for the DQ0602-peptide fusion constructs. HLA-DQA1*01:02-stable cells were transduced with pMXs-IG/DQB1*06:02, which carries the peptide sequence between the signal and the mature protein sequence (see Methods). (B) Δ MHC profile in the presence and absence of the artificial negative control peptide GGS. HLA-DQA1-stable cells were transduced with pMXs-IG/DQB1 (without peptide fusion) (white bars) or with pMXs-IG/DQB1-GGS peptide (black bars). (C) Δ MHC profile for HLA-DQ in the presence (black bars) and absence (white bars) of the human invariant chain. Δ MHC was measured in NIH3T3 cells that stably expressed the human invariant chain (Supplemental Figure 10) and HLA-DQA1. (D) Δ MHC profile for the DQB1-CLIP₈₁₋₁₀₇ fusion constructs. Error bars represent the SEM ($n \geq 3$).

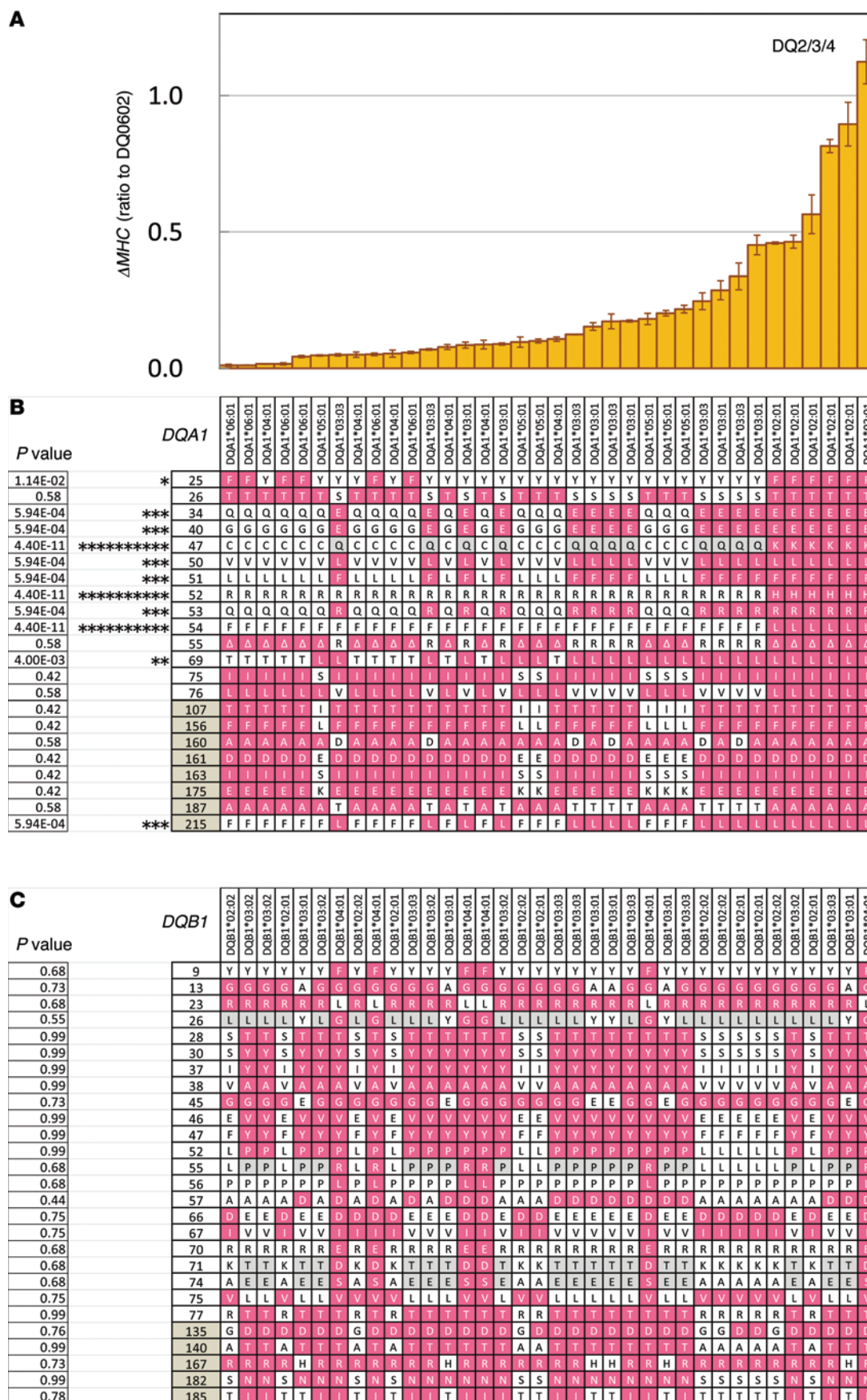
The presence of GGS peptide decreased Δ MHC for nearly all of the tested alleles; however, the overall allelic hierarchy in Δ MHC was maintained in the presence of the GGS peptide (Figure 3B, and Supplemental Figure 9, A and B). These data indicate that the HLA-DQ proteins in Figure 2A were loaded with endogenous peptides and were not empty heterodimers. The high Δ MHC values of DQ0602, DQ9.2, and DQ2.2 indicate that these HLA-DQ proteins are inherently stable. DQ2.2 showed relatively large inter-assay variation with and without GGS peptide (Figure 3B). DQ2.3 showed relatively high Δ MHC values, even in the presence of GGS peptide (Supplemental Figure 9B). It is currently unknown whether the GGS peptide occupies the peptide-binding groove of HLA-DQ.

MHC II is usually coexpressed with the invariant chain, which influences peptide loading and trafficking of the MHC II protein (67). To examine whether the lack of the human invariant chain affected cell-surface HLA-DQ expression, we measured Δ MHC in cells that abundantly expressed the human invariant chain (Supplemental Figure 10). In the presence of the human invariant chain, Δ MHC decreased by nearly half, possibly due to insufficiencies in cathepsins and HLA-DM. We found that the Δ MHC hierarchy was maintained in both the presence and absence of the human invariant chain (Figure 3C).

We analyzed the effect of CLIP on Δ MHC using the DQB1-CLIP₈₁₋₁₀₇ fusion constructs. The fusion of CLIP₈₁₋₁₀₇ greatly increased Δ MHC for certain alleles including DQ2.5 (Figure 3D), which is consistent with the high affinity of CLIP₉₄₋₁₀₄ for DQ2.5 (68). The variable effects of CLIP₈₁₋₁₀₇ on Δ MHC of the tested alleles indicate that the Δ MHC profile (Figure 2A) was not affected by CLIP-mediated stabilization.

Collectively, the Δ MHC profile (Figure 2A) does not appear to be biased by endogenous peptides, invariant chain, or CLIP. These observations support the possibility that the Δ MHC profile represents the allelic hierarchy in intrinsic HLA-DQ protein stability.

Polymorphic residues that regulate the Δ MHC of DQ5/6. We next searched for polymorphic sites that regulate Δ MHC. Supplemental Figures 11 and 12 show the pairwise comparisons of Δ MHC and the polymorphisms between the representative alleles (Supplemental Figure 11, A and B, and Supplemental Figure 12, A and B). For the highly conserved DQ5/6 subgroup, the responsible residues were identified through mutagenesis. For the highly polymorphic DQ2/3/4 subgroup, the major regulators of Δ MHC were identified through association analysis (see Supplemental Figure 13, A and B, for the amino acid sequence alignments of HLA-DQ).



Figure

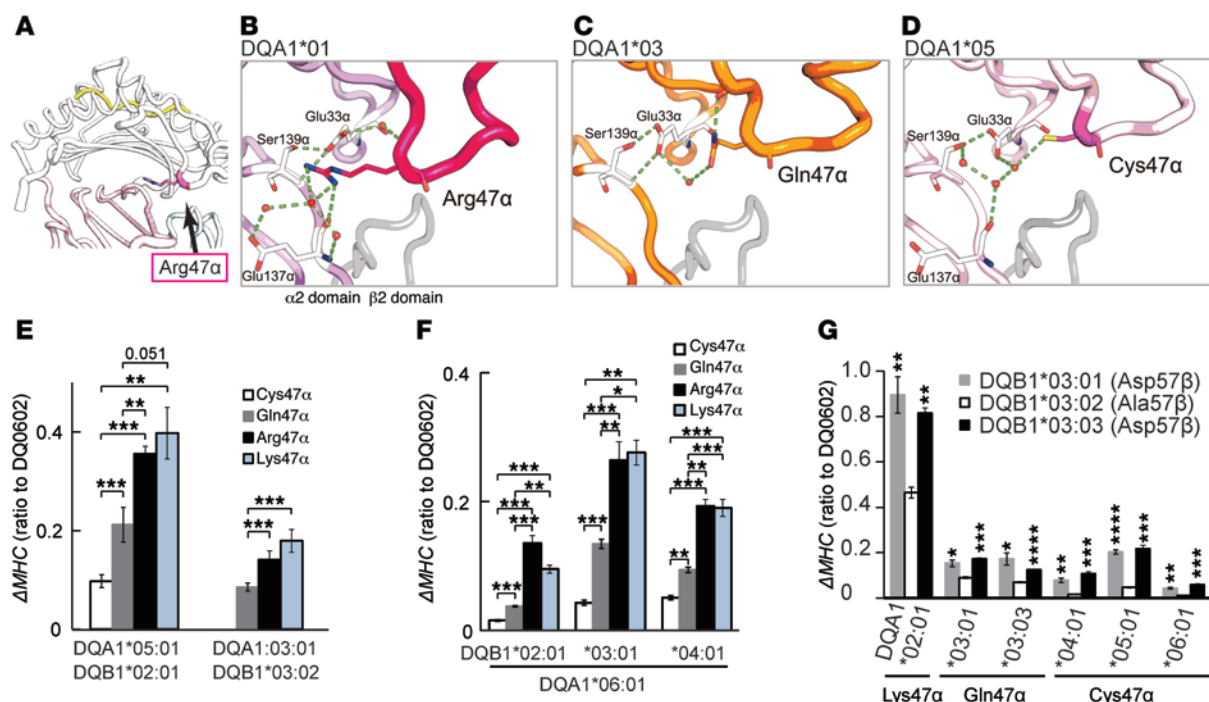


Figure 5. Stabilization/destabilization of HLA-DQ protein by 47 α and 57 β . (A) The location of Arg47 α (magenta) in the protein structure of DQ0602 (PDB: 1uvq) (70). α 1 domain (white), α 2 domain (pink), and bound peptide (yellow). (B–D) Interdomain H-bonds formed between the α 1 and α 2/ β 2 domains in the presence of Arg47 α in DQA1*01:02 (DQ0602) (PDB: 1uvq) (70) (B), Gln47 α in DQA1*03 (DQ8.3) (PDB: 2nna) (69) (C), and Cys47 α in DQA1*05 (DQ2.5) (PDB: 1s9v) (72) (D). α 1 and α 2 domains (magenta in B, orange in C, and pink in D); β 2 domains (gray); water molecules (red spheres); and distances of less than 3.4 Å (green dots). (E) Effects of variants at 47 α on the Δ MHC values of DQ2.5 and DQ8.3. (F) Effects of variants at 47 α on the Δ MHC values of DQA1*06:01. (G) Differences in Δ MHC between DQB1*03:02 and *03:03, which differ at 57 β , and between HLA-DQB1*03:01 and *03:03, which differ at 13 β , 26 β , and 45 β in the β 1 domain (Supplemental Figure 12B and 13B). * P < 0.05, ** P < 0.01, *** P < 0.001, and **** P < 0.0001 by 2-tailed t test. Error bars represent the SEM ($n \geq 3$).

In DQ5/6, the HLA-DQA1*01 alleles differ at Asp/Gly2 α , Tyr/Phe25 α , Gln/Glu34 α , and Lys/Arg41 α . Of these, variants at 2 α , 25 α , and 34 α , but not at 41 α , significantly altered Δ MHC (Supplemental Figure 14, A–D). Tyr/Phe25 α also altered Δ MHC between DQA1*04:01 and *06:01 (Supplemental Figure 14E). Asp2 α , which is located outside of the peptide-binding groove, stabilized the HLA-DQ protein, possibly through the formation of interdomain H-bonds (Supplemental Figure 14, F and G). Tyr25 α and Gln34 α stabilized the HLA-DQ through the interdomain/intersubunit H-bonds and interactions with the peptide main chain (Supplemental Figure 14, H and I). The hierarchy in Δ MHC among DQA1*01 alleles is perfectly explained by the stabilizing and destabilizing effects of 2 α , 25 α , and 34 α (Supplemental Figure 14J).

For DQB1*06, we analyzed the effect of polymorphic residue on Δ MHC through mutagenesis at the sites that differed between DQB1*06:02 and *06:04 (9 β , 30 β , 57 β , 70 β , and 87 β) (Supplemental Figure 14, K and L). The replacement of Tyr30 β with His30 β in DQB1*06:02 and of Tyr9 β with Phe9 β in DQB1*06:04 decreased Δ MHC. However, the substitution of Phe9 β with Tyr9 β in the presence of Tyr30 β (in DQB1*06:02) and the substitution of His30 β with Tyr30 β in the presence of Tyr9 β (in DQB1*06:04) did not affect Δ MHC (Supplemental Figure 14, K and L), indicating that Tyr9 β and Tyr30 β act complementarily to increase the Δ MHC. Tyr9 β forms an H-bond with Asn72 α , which interacts with the peptide main chain (Ser7p O and Glu9p N) in DQ8.3 (ref. 69 and Supplemental Figure 14M). Tyr30 β interacts with the peptide main chain (Ser7p N) in

DQ8.3 (ref. 69 and Supplemental Figure 14N). The higher Δ MHC of DQB1*06:02 compared with that of DQB1*06:03 suggests a greater stabilizing effect of Phe9 β -Tyr30 β than of Tyr9 β -His30 β (Supplemental Figure 11B). Asp57 β mediates an H-bond/salt bridge with Arg79 α and H-bonds with Ala10p N and Tyr37 β (ref. 70 and Supplemental Figure 14O). Arg70 β , which is predicted to interact with the p6 residue (71), increased Δ MHC relative to Gly70 β in DQB1*06:04, but not in DQB1*06:02 (Supplemental Figure 14, K and L). Tyr/Phe87 β did not affect Δ MHC (Supplemental Figure 14, K and L). Collectively, the hierarchy in Δ MHC among DQB1*06:02, *06:03, and *06:04 is shaped mainly through the variants at 9 β , 30 β , and 57 β , the residues that alter the intrinsic stability of the MHC protein.

The large difference in Δ MHC between DQB1*05 and *06 is consistent with their difference in SDS stability (36) and was replicated with pan-HLA II β mAb IVA-12 and the DQw1-specific mAb Genox3.53 (H. Miyadera, unpublished observations). We determined that 14 β (Leu in DQB1*05 and Met in DQB1*06), which projects its side chain toward the interface of the α 1 and α 2/ β 2 domains, is one of the responsible variants that diversifies Δ MHC between DQB1*05 and *06 (H. Miyadera, unpublished observations). Other variants that also contribute to the low Δ MHC value of DQB1*05 have not been fully elucidated in this study.

Polymorphic residues that regulate the Δ MHC of DQ2/3/4. To identify the residues that affect the Δ MHC value of DQ2/3/4, we conducted an association analysis between each amino acid variant and Δ MHC (Figure 4, A–C). In HLA-DQA1, variants at 47 α

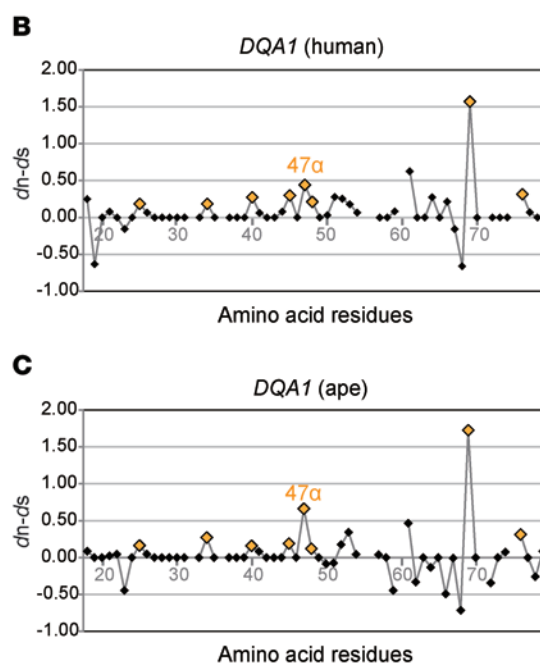
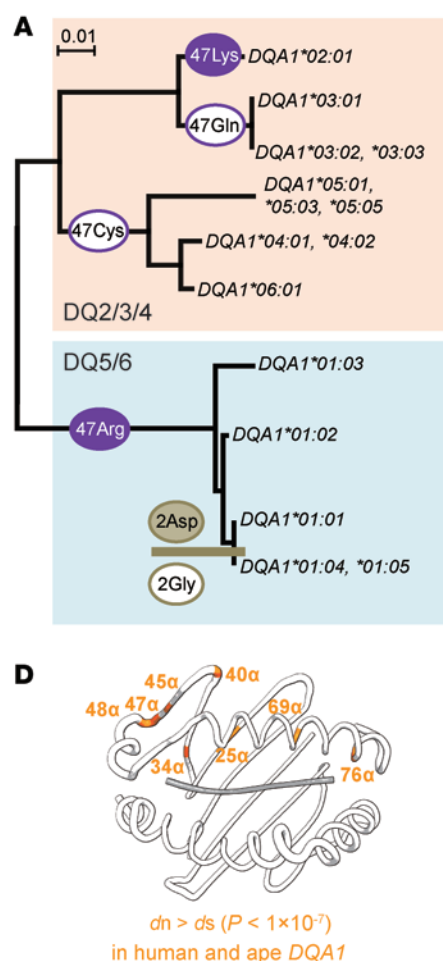


Figure 6. Evolutionary diversification of the stability regulatory sites. (A) Neighbor-joining tree for *HLA-DQA1* showing the amino acid substitutions at 2 α and 47 α . (B and C) *dn-ds* for codons in *HLA-DQA1* (14 alleles) (B), and ape *DQA1* (20 alleles) (C). The x axis indicates the amino acid residue numbers starting at 18 α and ending at 79 α . The y axis indicates the *dn-ds* at each codon, as calculated by the Nei-Gojobori method (124) using SNAP (126) (<http://www.hiv.lanl.gov>). The codons with a positive *dn-ds* ($P < 1 \times 10^{-7}$, calculated for *pn* and *ps*) are plotted in orange. (D) Location of the amino acid residues that showed a positive *dn-ds* ($P < 1 \times 10^{-7}$) in both human and ape *DQA1*. See Supplemental Tables 3 and 4 for the association tables.

(Lys vs. Cys and Gln), 52 α (His vs. Arg), and 54 α (Leu vs. Phe) were most strongly associated with Δ MHC ($P = 4.4 \times 10^{-11}$ by 2-tailed *t* test) (Figure 4B and Supplemental Table 2). In the structures of DQ2.5 and DQ8.3 (69, 72), 47 α is located at the interface of the α 1 and α 2/ β 2 domains and appears to affect heterodimer stability. Arg52 α , which does not participate in interdomain interaction, and Phe54 α , which projects its side chain toward the outer surface or to the β 1 domain, does not seem to explain the lower Δ MHC values of DQA1*03 and *05 compared with those of DQA1*02.

47 α , which encodes Arg, Lys, Gln, and Cys, is the most variable site in *HLA-DQA1* (Supplemental Figure 13A). Unlike typical MHC polymorphisms, 47 α is located outside the peptide-binding groove and TCR-recognition surface (Figure 5A and Supplemental Figure 14F). In DQA1*01:02, Arg47 α forms extensive H-bonds with the α 2 domain (70), which may be partially maintained by Lys47 α (in DQA1*02), but not by Gln47 α in DQ8.3 (69, 73) or by Cys47 α in DQ2.5 (ref. 72 and Figure 5, B–D). The substitution of Cys47 α with Lys47 α in DQ2.5 increased the Δ MHC value by 4.1-fold (from 0.10 to 0.40) (Figure 5E), accounting for approximately 69% to 86% of the Δ MHC of DQ2.2 (Δ MHC = 0.46–0.57). The substitution of Gln47 α with Lys47 α in DQ8.3 increased the Δ MHC value by 2.1-fold (from 0.09 to 0.18) (Figure 5E), accounting for approximately 39% of the Δ MHC of DQ8.2 (DQA1*02:01–DQB1*03:02) (Δ MHC = 0.46). Mutagenesis at 47 α in the least stable DQA1*06:01 confirmed greater stabilizing effects of Lys47 α and Arg47 α compared with

those of Gln47 α and Cys47 α (Figure 5F). Therefore, DQ2.2 and DQ9.2, which carry Lys47 α , are intrinsically more stable than the heterodimers formed by DQA1*03–*06.

In *HLA-DQB1**02, *03, and *04, none of the variants were associated with Δ MHC (Figure 4C and Supplemental Table 2). Non-Asp/Asp57 β is responsible for the differences in the Δ MHC within DQB1*03, but not the entire DQ2/3/4 that is attributable to *HLA-DQA1* (Figure 5G).

In both DQ5/6 and DQ2/3/4, the hierarchies in Δ MHC are generated mainly through stabilization and destabilization at the polymorphic sites that mediate the intersubunit or interdomain interactions or the interaction with the peptide main chain. These findings demonstrate that the major factor that determines the Δ MHC is the intrinsic stability of the MHC protein. Based on these and earlier findings, the Δ MHC values in Figure 2A were used as indicators of the intrinsic stability of HLA-DQ.

Evolutional divergence at 47 α . The extensive variation at the 47 α stability regulatory site (Supplemental Figure 13A) suggests that 47 α may have been the target of positive natural selection, as has been observed for the peptide-binding sites (74, 75). The variants at 47 α may have appeared before or at the time of the divergence of the *HLA-DQA1* sublineages (Figure 6A). To determine whether positive selection has been operating at 47 α , we compared the number of nonsynonymous substitutions per nonsynonymous sites (*dn*) with the number of synonymous substitutions per synonymous sites (*ds*)

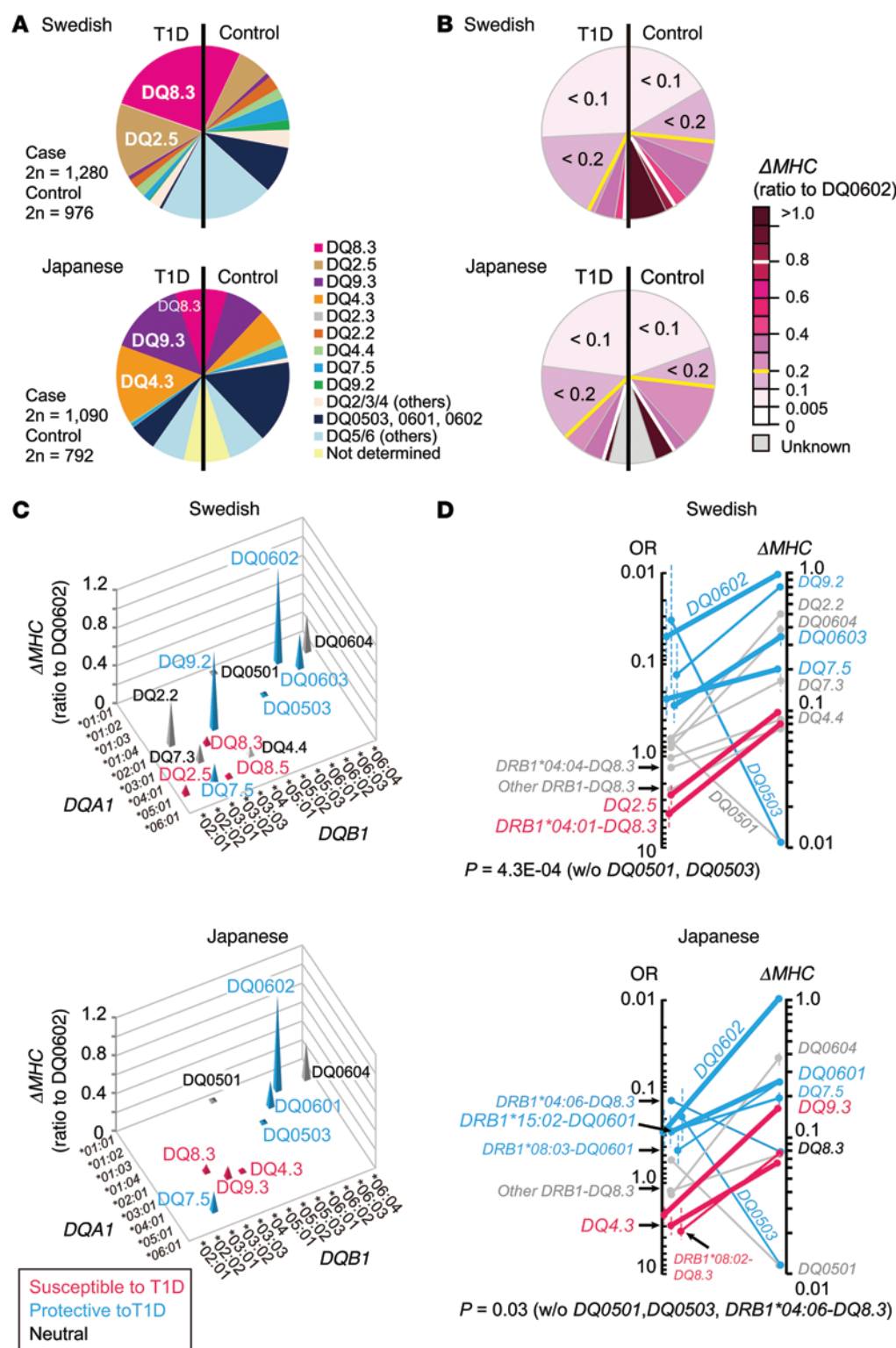


Figure 7. Association between the Δ MHC and genetic risk for T1D.

(A) Frequencies of *DQ* haplotypes in T1D cases and controls in the Swedish (77, 78) and Japanese populations (10). See Supplemental Table 5 for the association table. (B) Frequencies of *DQ* haplotypes of various Δ MHC levels in the Swedish (77, 78) and Japanese populations (10). Lines indicate the boundaries for the Δ MHC less than 0.2 (yellow) and the Δ MHC greater than 0.8 (white). (C) Δ MHC profiles for the major *DQ* haplotypes (haplotype frequency >0.03) in the European (133) and Japanese populations (134). Colors indicate the *DQ* haplotypes that confer risk (magenta), protection (blue), or neutrality (gray) with regard to T1D in the Swedish (77, 78) and Japanese populations (10). (D) Relationship between the Δ MHC and the ORs for the major *DQ* haplotypes (haplotype frequency >0.03) in the Swedish and Japanese populations. The left axis indicates the OR, and the right axis indicates the Δ MHC. The dashed lines indicate a 95% CI for the ORs or standard errors for the Δ MHC. T1D risk (magenta), protective (blue), and neutral (gray) haplotypes. Haplotypes that are most strongly associated with susceptibility to or protection against T1D in each population are indicated by bold lines. *P* values indicate the association between the rankings in the Δ MHC and the ORs (Spearman's rank test).

for each codon in the *DQA1* of human, ape, and other mammals. The comparison of *dn* with *ds* detects a positive selection operating on each codon or gene. Under a null hypothesis of selective neutrality, the *dn* = *ds* is expected (ref. 76 and references therein).

In human and ape *DQA1*, the codons for 69a showed the highest *dn*-*ds* value (*dn*-*ds* = 1.573, *P* = 5.17×10^{-12} in human *DQA1*; *P* value calculated by the Wilcoxon signed-rank test for the proportion of

positive diversifying selection. The codons for 47a in swine *DQA1* also showed a significantly positive *dn*-*ds* value (H. Miyadera, unpublished observations). The other residues that showed significantly positive *dn*-*ds* values (*P* < 1.0×10^{-7}) in both human and ape *DQA1* were 25a, 34a, 40a, 45a, 48a, 69a, and 76a (Figure 6, B-D, and Supplemental Tables 3 and 4). Of these, 25a and 34a participate in peptide-binding and protein stability regulation, and 69a and 76a

synonymous differences per synonymous site [*ps*] and the proportion of nonsynonymous differences per nonsynonymous site [*pn*]; see Methods). The codons for 47a showed a *dn*-*ds* value that was one of the highest in *DQA1* (*dn*-*ds* = 0.443, *P* = 1.63×10^{-11} in human *DQA1*; *dn*-*ds* = 0.664, *P* = 1.43×10^{-23} in ape *DQA1*) (Figure 6, B and C, and Supplemental Tables 3 and 4), indicating that 47a has been subjected to positive

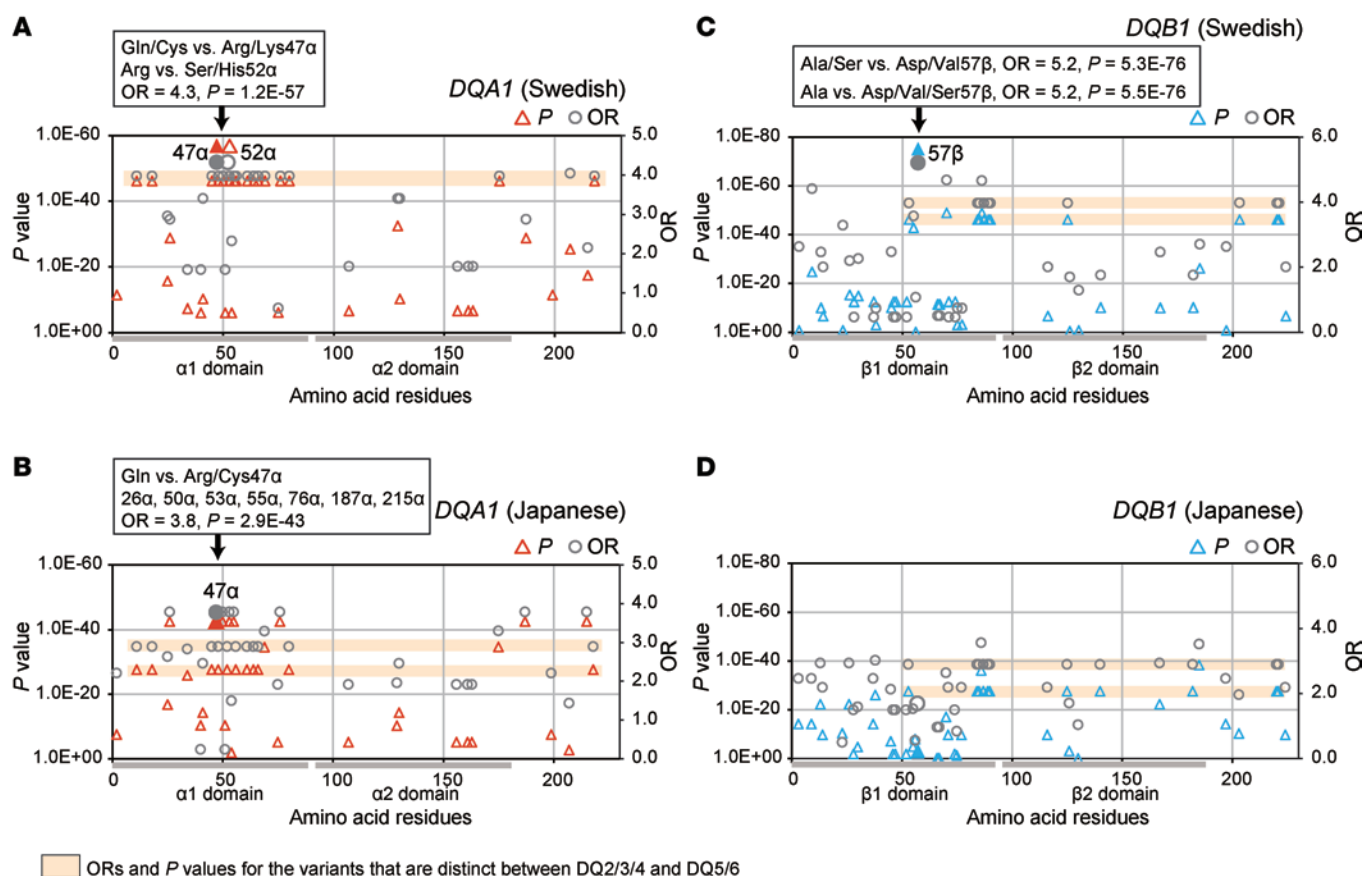


Figure 8. Association analyses between the amino acid variants in HLA-DQ and susceptibility to T1D. (A–D) Associations between the amino acid variants in HLA-DQA1 (A and B) and -DQB1 (C and D) and susceptibility to T1D in the Swedish (A and C) and Japanese populations (B and D). The ORs (circles) and the lowest P value (triangle) at each site (χ^2 test). ORs and P values for the variants that are distinct between DQ2/3/4 and DQ5/6 are shaded in orange. ORs for 2α and 199α in the Swedish population are not presented. See Supplemental Tables 6 and 7 for the association tables.

constitute the peptide-binding pockets. The significant excess of d_n versus d_s at 47α was not due to positive selection operating on antigen-binding sites, because the variants at 47α were not in linkage disequilibrium with the variants at the peptide-binding sites, such as 25α and 69α. The signature of positive selection at 47α independently of antigen-binding sites suggests that HLA-DQ has been evolving in favor of diversification in protein stability, in addition to increasing variations in the peptide-binding spectrum.

Autoimmune-susceptible DQ haplotypes encode unstable proteins. We next analyzed the relationship between Δ MHC and genetic risk for autoimmunity using the Δ MHC profile (Figure 2A) and case-control data for T1D in Swedish (77, 78) and Japanese populations (10). T1D is caused by the autoimmune destruction of insulin-producing β cells in the pancreas. Both HLA-DR and HLA-DQ confer a predisposition to T1D. The association of DR-DQ haplotypes with T1D has been extensively studied, and the risk hierarchy among the haplotypes has been established (59, 79, 80). In European and African-American populations, DR3-DQ2.5 and DR4-DQ8.3 are the risk haplotypes for T1D (59, 79–81). In the Japanese population, DR9-DQ9.3 and DR4-DQ4.3 are most strongly associated with T1D (8–10). DR9-DQ9.3 confers a risk for T1D in the Filipino and Korean populations (8, 82, 83). DQ0602 confers protection against T1D in Swedes and Japanese, among other populations (ref. 79, Figure 7A, and Supplemental Table 5).

The composition of the DR-DQ haplotypes differs greatly between the Swedish and Japanese populations (Figure 7A); however, the frequency of the DQ haplotypes is similar in the 2 populations when the haplotypes are subgrouped by Δ MHC (Figure 7B). The DQ haplotypes with low Δ MHC values (Δ MHC < 0.2) are predominant in T1D cases, whereas those with high Δ MHC values (Δ MHC > 0.8) are present at higher frequencies in controls than in T1D cases (Figure 7B). The steep hierarchy in Δ MHC among the DQ haplotypes appears to be correlated with the risk and protection for T1D (Figure 7C and Supplemental Figure 15, A and B).

Indeed, a clear inverse relationship exists between Δ MHC and susceptibility to T1D (estimated by the odds ratio [OR]) for the majority of DQ haplotypes, including those haplotypes that are most predisposing (DQ2.5, DQ8.3, DQ9.3, and DQ4.3) and protective (DQ0602, DQ0603, and DQ9.2). In the absence of DQ0501 and DQ0503 (in the Swedish and Japanese populations) and DRB1*04:06-DQ8.3 (in the Japanese population), which encode unstable HLA-DQ proteins and are neutral or protective of T1D, the rankings in Δ MHC and in the ORs were inversely associated ($P = 4.3 \times 10^{-4}$ [Swedish]; $P = 0.03$ [Japanese], Spearman's rank correlation test) (Figure 7D). Although the CIs for the ORs are large, these data confirm an overall inverse relation between Δ MHC and genetic risk for T1D. The protective phenotype of the DRB1*04:06-DQ8.3 haplotype may be ascribed to DRB1*04:06

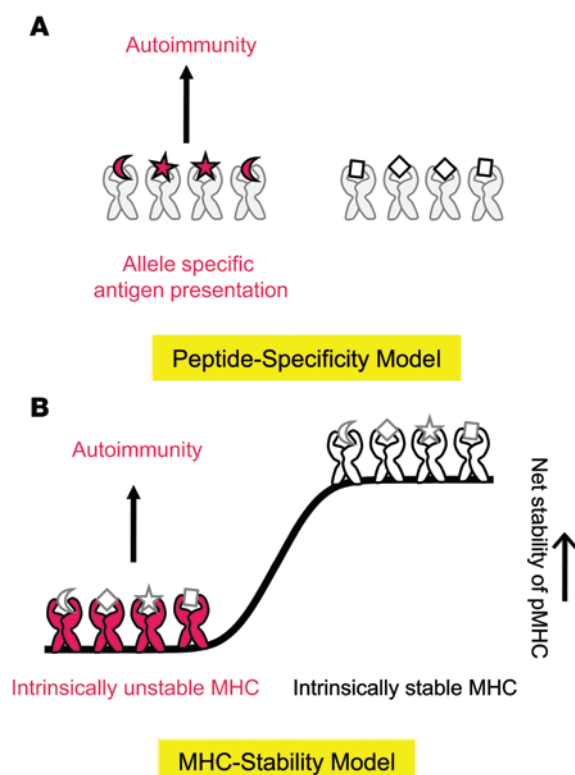


Figure 9. Hypothetical mechanisms of the HLA-autoimmunity association. (A) Peptide specificity model. This model postulates that the peptide-binding spectrum of the MHC determines the genetic association of HLA with autoimmunity. According to this model, the HLAs that are able to present the disease-relevant peptides (magenta) confer a risk for autoimmunity. Colors indicate the disease-relevant (magenta) and irrelevant peptides (white). (B) MHC stability model. This model postulates that intrinsically unstable MHC proteins (magenta), which form unstable MHC-self-epitope complexes through presentation of diverse self-peptides and are more likely to form unstable pMHC than intrinsically stable MHC, confer a risk for autoimmunity.

the TCR (95). The protein-destabilizing effect of non-Asp57 β may also contribute to T1D. However, the neutral phenotype of DQ2.2 (non-Asp57 β , $\Delta MHC = 0.46$ –0.57) (59, 80) and the susceptible phenotypes of DQ9.3 and DQ4.3 (Asp57 β , $\Delta MHC = 0.05$ –0.12) suggest that the low ΔMHC value, rather than non-Asp57 β , is indispensable to the shared mechanism of T1D risk across ethnicities.

Except for 57 β in the Swedish population, we found that no variant in the peptide-binding sites showed an association signal that was stronger than the signals observed for the sublineage-specific variants (i.e., variants that differ between DQ2/3/4 and DQ5/6) (Figure 8, A–D). These results indicate that (a) a certain combination of the peptide-binding variants confers a risk for T1D, or (b) the peptide-binding spectrum is not strongly associated with T1D. The observations that the peptide-binding spectra of DQ2/3/4 partially overlap and are partially unique (96–98) permit both mechanisms.

Collectively, the absence of a common association signal for T1D in the peptide-binding sites confirms the predisposing effect of HLA-DQ protein instability. Among the multiple factors that might contribute to T1D risk, instability of HLA-DQ may be one of the major components that determines the pathogenic potential of each DR-DQ haplotype.

Discussion

To uncover the mechanism of the HLA-autoimmunity association, the most logical approach would be to identify the functional variation(s) among HLA alleles that are correlated with disease risk. The present study revealed an inverse association between ΔMHC and T1D and confirmed the strong association signals for T1D at the protein-destabilizing variants. These results suggest a potential causal link between the intrinsic instability of HLA-DQ and T1D.

The HLA-autoimmunity association has been explained for decades by the allele-specific presentation of disease-relevant self-peptides (referred to as the peptide specificity model) (Figure 9A). Our study indicates that the intrinsic instability of the MHC protein may also be a major functional component of autoimmune susceptibility (i.e., the MHC stability model) (Figure 9B). The 2 mechanisms are not mutually exclusive, and neither mechanism alone sufficiently explains the DR-DQ haplotype association with T1D. The combination of the 2 parameters might generate the high level of allele specificity in the autoimmune disease risk.

The simplest mechanism by which an unstable MHC protein might confer autoimmune disease risk would be the contribution of MHC protein instability to the incomplete thymic negative selection. This possibility is consistent with the established concept of autoimmunity, in which the formation of an unstable pMHC

(59, 79, 80). The protective association of DQ0503 may be attributable to DR14 (84).

Destabilizing variants at 47 α confer a predisposition to T1D. To address whether the association between ΔMHC and T1D is affected by the peptide-binding variants, we analyzed the association between each amino acid variant and T1D. In HLA-DQA1, the strongest association signals with T1D in the Swedish population were detected at 47 α (Gln and Cys vs. Arg and Lys) and 52 α (Arg vs. Ser and His) (OR = 4.33, $P = 1.2 \times 10^{-57}$, χ^2 test), which are the variants that differ between HLA-DQA1*01, *02, and *03–*06 (Figure 8A and Supplemental Table 6). The association of Arg52 α with T1D has been noted previously (85, 86), but Arg52 α may not affect the stability or peptide binding in the structures of DQ2.5 and DQ8.3 (69, 72). In the Japanese population, variants at 26 α (Ser vs. Thr), 47 α (Gln vs. Arg and Cys), 50 α (Leu vs. Glu and Val), 53 α (Arg vs. Lys and Gln), 55 α (Arg vs. Gly and Del), 76 α (Val vs. Leu and Met), 187 α (Thr vs. Ala), and 215 α (Leu vs. Phe), which distinguish HLA-DQA1*03 from other alleles, were the most strongly associated with T1D (OR = 3.79, $P = 2.9 \times 10^{-43}$) (Figure 8B and Supplemental Table 6). The 2 populations share the risk variant Gln47 α . Cys47 α is not associated with T1D in the Japanese population due to the absence of DQ2.5 (risk haplotype) and the presence of DQ7.5 (protective haplotype).

Non-Asp57 β has been known as a T1D risk variant in Europeans (87, 88) and in murine models (89). As expected from earlier studies, variants at 57 β were strongly associated with T1D in the Swedish population (Ala and Ser vs. Asp and Val, OR = 5.22, $P = 5.3 \times 10^{-76}$; non-Asp vs. Asp, OR = 5.23, $P = 6.1 \times 10^{-68}$) but not in the Japanese population (Figure 8, C and D, and Supplemental Table 7). Non-Asp57 β may confer a risk for T1D through an alteration in the binding preference for the p9 residue (90–94) and for

by low-affinity peptides permits thymic escape of self-reactive T cells (99, 100). Upon weak self-epitope presentation, intrinsically unstable MHC may form MHC-self-epitope complexes in the low-stability range and confer a risk for autoimmunity. The ability of intrinsically unstable MHC to form unstable pMHC with diverse self-epitopes, including the promiscuous binders, may explain the association of T1D risk *DR-DQ* haplotypes with a variety of autoimmune disorders (Table 1), as well as the involvement of promiscuous self-epitopes in allele-specific disease pathogenesis.

The intrinsically unstable MHC protein may be expressed either at a basal level or maximum level depending on the availability of high-affinity peptides and accessory molecules. The intrinsically unstable MHC protein is also expected to preferentially present high-affinity peptides on the cell surface. In contrast, intrinsically stable MHC may be expressed relatively constantly on the cell surface and present the peptides with wider affinity ranges. The potential variation in the expression patterns of each MHC type might affect the outcomes of thymic selection, peripheral activation, and subset development of T cells (101–105), processes that are controlled by cell-surface MHC density. The actual mechanism that links MHC instability to autoimmunity should involve diverse immunological processes and could be highly complex.

The Δ MHC profile provides a clue for dissecting the mechanism of the *DQ* haplotype association with T1D risk. The high risk for T1D of *DQ2.5/DQ8.3* heterozygotes (58, 59, 79, 80) may be ascribed to the instability of *DQA1*05-DQB1*03:02* (*DQ8.5*) (Δ MHC = 0.05) (Figure 7C and Supplemental Figure 15, A and B) and its unique peptide-binding spectrum (106, 107).

DQ0602 and *DQ9.2* may confer protective effects through abundant or sustained MHC expression. The high stability of *DQ0602* and its ability to bind diabetogenic self-epitopes (30–32) are compatible with the proposed mechanisms of protection, such as the thymic deletion of self-reactive T cells (108) and the “affinity model” or “determinant capture” (106, 109, 110). The protective associations of *DRB1*15:01-DQB1*06:02* with T1D, autoimmune polyglandular syndrome (APS) types II and III, and selective IgA deficiency (Table 1) suggest a shared mechanism of protection among these disorders.

The neutral and protective phenotypes of *DQ0501*, *DQ0503*, *DQ7.3*, and *DQ7.5*, which encode unstable HLA-DQ proteins (Figure 7D and Supplemental Figure 15B), are not explained by the Δ MHC value. As established for the *DRB1*04-DQ8.3* haplotypes (10, 59, 79–81), the predisposing and protective phenotypes of *DR-DQ* haplotypes can be largely influenced by the *DR* allele. The instability of HLA-DQ could be the condition that permits the linked *DR* to exert pathogenic or protective effects. The increased risk for T1D of *DQ8.3/DQ0501* and *DQ8.3/DQ0604* heterozygotes (79, 80, 111, 112) (*DR4/DR13* in the Japanese population; refs. 8–10), which do not generate *trans* DQ heterodimers (Figure 2A), also remains difficult to explain by the existing hypothesis.

The dependency of MHC II on the invariant chain, CLIP, and HLA-DM are proposed as the risk factors for T1D (113–117). The affinity of HLA-DQ with CLIP_{81–107} appears to be variable among the alleles (Figure 3D). The dependency of HLA-DQ on HLA-DM may also be variable due to polymorphisms at the 47 α –56 α segment (117). It will be intriguing to speculate how these interrelated parameters and their combinations might affect T1D risk.

In this study, Δ MHC was used as an indicator of the intrinsic stability of the MHC protein. Several lines of direct and indirect evidence, including the mutagenesis studies, have confirmed that the intrinsic stability of the MHC protein is the major factor in determining Δ MHC. In fibroblasts, the stabilization of MHC II promotes rapid transport of pMHC into the Golgi apparatus (118), and the MHC II proteins that weakly interact with peptides are protease sensitive and are degraded in the endosomes (50). In B cells, unassembled HLA-DR subunits can be degraded rapidly during intracellular transport (119, 120). Presumably, intrinsically less stable MHC proteins that are inefficient in both α and β subunit assembly and in the formation of the pMHC may generate a substantial amount of unassembled or unfolded subunits that are sensitive to proteolysis, resulting in reductions in both the number and lifetime of the pMHCs that reach the cell surface. However, because Δ MHC is measured by the cell-based assay, the possibility that Δ MHC is biased by unknown cellular components cannot be completely ruled out.

It should also be noted that the Δ MHC profile, which is measured in an engineered condition, does not represent the expression profile of HLA-DQ on professional APCs. As observed in B-LCLs, the *HLA-DQ* alleles that showed low Δ MHC values can be expressed at a high level (36), possibly through transcriptional upregulation and/or stabilization by high-affinity peptides.

Our study provides new insights into the molecular evolution of the MHC, which has been explained by diversification of the peptide-binding spectrum (ref. 121 and references therein). The *DQ* haplotypes that encode unstable and stable proteins are maintained at a high frequency in a variety of populations (allele-frequencies.net; ref. 122), indicating that the alleles encoding unstable HLA-DQ heterodimers are not selectively disadvantageous. Presumably, both the stable and unstable MHC may have functional advantages against important pathogens. The stability of HLA-DR may also be diverse (123); however, the scale of variation may be limited by the monomorphic nature of *HLA-DRA*.

Collectively, our study reveals an additional layer of functional hierarchy among the *HLA* alleles that has been generated through evolution and is associated with autoimmunity. These findings complement and extend the existing model of the *HLA*-autoimmunity association and suggest a mechanistic basis of autoimmune susceptibility.

Methods

Measurement of Δ MHC. Full-length cDNAs for *HLA-DQA1* and *DQB1* were cloned from *HLA*-typed cell lines or peripheral blood samples from healthy individuals. The cDNAs were inserted into the retroviral vectors pMXs-puro or pMXs-IG (52) with an *EcoRI* site and a Kozak sequence in the 5' terminus and a *Strep*-tag II (IBA GmbH) and a His-tag in the 3' terminus of *HLA-DQA1* and *-DQB1*, respectively. The first nucleotide of the second amino acid of *HLA-DQA1* and *-DQB1* was changed to guanine to introduce a Kozak sequence. The *HLA-DQA1-Strep*-tag II and *HLA-DQB1*-His-tag were inserted into the pMXs-puro and pMXs-IG vectors, respectively, using *EcoRI* and *NotI* sites. To generate the retroviruses containing pMXs-puro/*DQB1* and pMXs-IG/*DQA1*, approximately $0.5\text{--}1 \times 10^6$ PLAT-E cells (53) were transfected with 1.5 μ g plasmid and 4.5 μ l Fu-GENE reagent (Roche Diagnostics), according to the manu-

facturer's instructions. *HLA-DQB1*-stable cells were established through the transduction of NIH3T3 cells with a retrovirus containing pMXs-puro/*DQB1* and selection with puromycin (6 μ g/ml). To measure Δ MHC, the *HLA-DQB1*-stable cells were seeded at approximately 2×10^5 cells per well in a 12- or 24-well plate and cultured overnight. The cells were transduced with a retrovirus containing pMXs-IG/*DQA1* using 5–60 μ l PLAT-E medium. Forty-eight hours after transduction, GFP and cell-surface MHC (in GFP-positive cells) levels were measured by flow cytometry (EPICS-XL; Beckman Coulter) using anti-HLA II β mAb (WR18) (65) (MorphoSys AG) or isotype control (mouse IgG2a [6H3]; Medical & Biological Laboratories Co. Ltd.) with phycoerythrin-conjugated anti-mouse IgG (Rockland Immunochemicals or Southern Biotechnology Associates Inc.). The MFI (GFP) was defined as the MFI for GFP-positive cells minus MFI for GFP-negative cells. The MFI (MHC) was defined as the MFI for anti-HLA II β [WR18] minus the MFI for the isotype control of the GFP-positive cells (Supplemental Figure 3A). The MFI (GFP) and MFI (MHC) were plotted to calculate the increase in the MHC relative to GFP (Δ MHC) (Figure 1D and Supplemental Figure 3B). To minimize interassay variation, the Δ MHC for each DQ heterodimer was normalized with the Δ MHC value of DQ0602, which was measured on the same day (Figure 1D and Supplemental Figure 4, A and B). The lower detection threshold of Δ MHC was set at 0.005 (after normalization to DQ0602). The assay was performed more than 3 times for each *HLA-DQA1* and *-DQB1* allelic pair. Site-directed mutagenesis was performed with QuikChange II (Agilent Technologies), according to the manufacturer's instructions. The following anti-HLA mAbs were purchased: BL-IA/6 (Santa Cruz Biotechnology Inc.) and TDR31.1 (Ancell Corporation). The mAb IVA12 was a gift of S. Kawai (Wakunaga Pharmaceutical Co.). The NIH3T3 cell line was obtained from the RIKEN Cell Bank.

The *HLA-DQB1*-peptide fusion construct was designed as described in Kozono et al. (66), with modification. Briefly, the sequences for insulin B_{1–15}, insulin B_{9–23}, CLIP_{81–107}, and an artificial GGS peptide (GGSGSGSGSGS) were inserted between the signal sequence of *HLA-DQB1**06:02 and the mature protein region of *HLA-DQB1* via linkers (SGG and GGGSIEGRGGGSGSA at the N and C termini of the peptide, respectively). The fragments that encode the signal sequence and the peptide sequences were synthesized as double-stranded DNA and were ligated to the mature *DQB1* sequence using the *AccIII* site. For the *DQB1*-peptide fusion constructs, Δ MHC was measured in *HLA-DQA1*-stable cells.

To establish NIH3T3 cells that stably express the human invariant chain, the full-length cDNA for the invariant chain (isoform b) was cloned from the B-LCL of the healthy individual and was inserted into pMXs-neo using *EcoRI* and *Not I* at the 5' and 3' termini of the invariant chain, respectively. NIH3T3 cells were transduced with the pMXs-neo/invariant chain and were selected with G418 (2 mg/ml) for 2 weeks. The invariant chain-stable cells were then transduced with pMXs-puro/*HLA-DQA1* and were selected with puromycin (6 μ g/ml). Expression of the invariant chain was detected with anti-invariant chain mAb (LN2) (BioLegend). Intracellular staining was performed using the FIX & PERM Cell Fixation and Cell Permeabilization Kit (Invitrogen), according to the manufacturer's instructions.

Expression of HLA-DQ protein in insect cells. Full-length cDNAs for *HLA-DQA1* and *-DQB1* were inserted into pMT-V5/His (Invitrogen) with a Kozak sequence at the 5' terminus and a *Strep*-tag II (IBA

GmbH) and His-tag at the 3' terminus of *HLA-DQA1* and *-DQB1*, respectively. The expression plasmids were cotransfected with pCoBlast (Invitrogen) into the *Drosophila melanogaster* Schneider cell line (S2) via calcium phosphate transfection (Invitrogen) or lipofection using Effectene (QIAGEN), according to the manufacturer's instructions. Stable polyclonal cells were obtained in the presence of blasticidin (25 μ g/ml). For each *HLA-DQA1* and *-DQB1* allelic pair, stable cells were obtained from more than 3 independent transfections. The expression of HLA-DQ proteins was induced with 0.5 mM CuSO₄ for 48 hours and measured by flow cytometry (EPICS-XL; Beckman Coulter) with an anti-HLA II β mAb (WR18, FITC conjugate) (MorphoSys AG) or an isotype control (mouse IgG2a-FITC) (Beckman Coulter). 3' Rapid amplification of cDNA ends (3'-RACE) was performed with the 3'-Full RACE Core Set (Takara Bio) using total RNA (1 μ g) from the stable S2 cells as a template. Total RNA was primed for reverse transcription at 42°C with the Oligo dT-3 Sites Adaptor Primer (Takara Bio). 3'-RACE PCR was performed according to the manufacturer's instructions. Specifically, the internal primers for *HLA-DQA1* (p-50: 5'-ACTCTACCGCTGC-TACCAATG-3') and *HLA-DQB1* (p-48: 5'-ACGGTGTGCAGACA-CACTAC-3') were used as the 5' primers, and the Oligo dT-3 Sites Adaptor Primer was used as the 3' primer. The *Drosophila* ribosomal protein rp49 was amplified as a positive control with the primers p-266 (5'-ATGACCATCCGCCAGCATAC-3') and p-267 (5'-TGT-GTATTCCGACCAGGTAC-3'). For the purification of HLA-DQ proteins and Western blotting, the stably transfected S2 cells were harvested 48 hours after induction and stored at –80°C. The cells were thawed on ice and lysed in 30 mM sodium phosphate buffer (pH 8.0, 150 mM NaCl, 0.5% NP-40) in the presence of a protease inhibitor cocktail (Sigma-Aldrich) and Benzonase Nuclease (EMD Millipore, Merck KGaA) for 1 hour at 4°C. The lysate was centrifuged at 2,000 g for 10 minutes at 4°C, and the supernatant was subjected to purification. Purification by *Strep*-tag II and His-tag was performed with a *Strep*-Tactin Spin Column (IBA GmbH) and MagExtractor (Toyobo Co., Ltd.), respectively, according to the manufacturers' instructions. For Western blotting, the elution fractions were subjected to denaturation and boiling in the presence of SDS (2%) and reducing agents. *Strep*-Tactin-AP (IBA GmbH) and anti-His6 mAb-HRP (Roche Diagnostics) were used for the detection of DQ α and DQ β , respectively.

Evolutionary analysis. The *dn-ds* for each codon was calculated in a pairwise manner by the Nei-Gojobori method (124) with Jukes-Cantor correction (125) using SNAP (126) (<http://www.hiv.lanl.gov>). The amino acids 18 α –79 α of the *DQA1* for humans (*HLA-DQA1*, 14 alleles) and apes (*Gogo-DQA1* [*Gorilla gorilla*], *Patr-DQA1* [*Pan troglodytes*], *Hyla-DQA1* [*Hylobates lar*], and *Popy-DQA1* [*Pongo pygmaeus*], 20 alleles), which represent all of the alleles that are registered in the IMGT/HLA and IMGT/IPD databases (EMBL-EBI) (127–130) and which were nonredundant in the 18 α –79 α region (i.e., at least 1 codon was different from the others), were used for the analyses. The codons with a deletion or insertion (55 α and 56 α in the human and ape *DQA1*) were excluded from the analysis. For the sequence pairs with codons encoding Met or Trp (48 α , 66 α , and 76 α in human and ape *DQA1*), the *ps* was assumed to be 0 and was used to calculate the *ds*. For sequence pairs showing a *ps* greater than or equal to 0.75 (32 α , 36 α , 60 α , 71 α , and 75 α in human and ape *DQA1*), the *ds* was not calculated (at these codons, the *ps* was greater than the *pn*, and

there were no codons with a $pn \geq 0.75$) (Supplemental Tables 3 and 4). To assess the possibility of positive selection (i.e., diversifying selection), the differences between the ps and pn as well as between the ds and dn were statistically tested for each codon using the Wilcoxon signed-rank test. A neighbor-joining phylogenetic tree was constructed for the nucleotide sequences of exon 2 of the *HLA-DQA1* alleles using the alleles with frequencies greater than 0.02 in world-wide populations (131). Molecular Evolutionary Genetics Analysis (MEGA) version 4 software (132) was used for the analysis.

Association analysis between the ΔMHC and genetic risk for T1D. The case-control data for T1D in the Swedish population (77) were collected and genotyped by the T1D component of the 13th International Histocompatibility Workshop (78) and were deposited in the dbMHC database (<http://www.ncbi.nlm.nih.gov/projects/gv/mhc/>). For the Japanese population, published case-control data (10), which were collected and genotyped by the Committee on T1D of the Japanese Diabetes Society, were used for the analysis. All of the T1D subtypes in the Japanese population (acute, fulminant, and slowly progressive) (10) were combined. The *HLA-DQA1* alleles were manually estimated from the *DRB1-DQB1* haplotype data based on the *DRB1-DQA1-DQB1* haplotype frequency in the general European-American (133) and Japanese (134) populations. In the Japanese population, all of the *DRB1*08:02-DQB1*03:02* haplotypes were assumed to carry *HLA-DQA1*03*. The rare *DRB1-DQB1* haplotypes and *DRB1-DQB1* haplotypes that are linked to more than 2 *HLA-DQA1* alleles were excluded from the analysis. Closely related alleles, such as *HLA-DQA1*01:04* and **01:05*; *HLA-DQA1*05:01*, **05:03*, and **05:05*; and *HLA-DQB1*04:01* and **04:02*, were assumed to have identical ΔMHC values. Because not all of the *HLA-DQA1*03* and *-DQB1*02* alleles were genotyped at a 4-digit resolution in the case-control data, the ΔMHC values of *DQA1*03* and of *DQB1*02* were assumed to be the average of the ΔMHC values for *DQA1*03:01*, **03:02*, and **03:03*, and of *DQB1*02:01* and **02:02*, respectively, and were used for the association analyses. For the analysis of the association between the amino acid variants and genetic risk for T1D, the variants at 160 α , which differ between *HLA-DQA1*03:01* and **03:03*, and at 135 β , which differ between *HLA-DQB1*02:01* and **02:02*, were excluded from the analysis.

Statistics. Allelic differences in the ΔMHC values as well as associations between the amino acid variants and ΔMHC were analyzed by

a 2-tailed t test. Differences between ps and pn and between ds and dn were analyzed by the Wilcoxon signed-rank test. Associations between the *DQ* haplotype frequency and susceptibility to T1D were analyzed by the χ^2 test with Bonferroni's correction. Spearman's rank test was used to evaluate associations between the ranking in the ΔMHC and ORs. A χ^2 test was used to analyze associations between the amino acid variants and genetic risk for T1D. A P value of less than 0.05 was considered statistically significant.

Study approval. The use of cDNAs and cell lines from healthy individuals was approved by the ethics committee of the Graduate School of Medicine, The University of Tokyo, and written informed consent was obtained from all participants.

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Address correspondence to: Hiroko Miyadera, Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. Phone: 81.3.5841.3653; E-mail: miyadera-h@umin.net.

Jun Ohashi's present address is: Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Tokyo, Japan.

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