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Allelic variation in class I K gene as candidate for a second component of MHC-linked susceptibility to Type 1 diabetes in non-obese diabetic mice

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Abstract

Aims/hypothesis. Recent studies have revealed that MHC-linked susceptibility to Type 1 diabetes is determined by multiple components. In the non-obese diabetic (NOD) mouse, a second component (*Idd16*) has been mapped to a region adjacent to, but distinct from *Idd1* in the class II region. In this study, we investigated the class I K gene as a candidate gene for *Idd16*.
Methods. We determined the genomic sequences of the class I K gene as well as the reactivity of K molecules with monoclonal antibodies in the NOD mouse, the Cataract Shionogi (CTS) mouse, and the NOD.CTS-*H-2* congenic strain, which possesses a resistance allele to Type 1 diabetes at the *Idd16* on the NOD genetic background genes.
Results. While the K sequence of the NOD mouse was identical to that of K^d type, ten nucleotide substitutions were identified in the CTS mouse compared with the NOD mouse. Of these, three were in exon 4, giving two amino acid substitutions, which were identical to those seen in K^K type. These characteristics were retained in the NOD.CTS-*H-2* congenic strain, which had a lower incidence and delayed onset of Type 1 diabetes owing to a resistance allele at *Idd16*. Lymphocytes from NOD.CTS-*H2* congenic mice reacted with anti-K^d and anti-K^k monoclonal antibodies, reflecting the unique sequence of the K gene. The nucleotide sequence of the K gene in the non-obese non-diabetic (NON) mouse was also unique, consisting of a combination of K^k- and K^b-like sequences.
Conclusions/interpretation. These data suggest that H2-K is unique in CTS and NON mice, and that allelic variation of the class I K gene may be responsible for *Idd16*.

Keywords Congenic mouse · CTS mouse · Gene · H2-K · *Idd16* · Major histocompatibility complex · Monoclonal antibody · Non-obese diabetic mouse · Sequence · Type 1 diabetes

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Abbreviations: CTS, Cataract Shionogi · FACS, fluorescence-activated cell sorter · FITC, fluorescein isothiocyanate · NOD, non-obese diabetic · NON, non-obese non-diabetic · SNP, single nucleotide polymorphism

Introduction

Type 1 diabetes, which is caused by autoimmune destruction of insulin-producing beta cells of the pancreas, is a polygenic disorder involving multiple-susceptibility genes [1, 2, 3]. Of these, MHC-linked susceptibility has been shown to be the strongest component. In humans, class II HLA genes, and in particular *DR* and *DQ* genes, have been shown to confer strong susceptibility. Several recent studies, however, indicated that HLA-linked susceptibility cannot be explained merely by class II *DR* and *DQ* genes, and that a gene or genes adjacent to, but distinct from *DR* and *DQ* genes, such as genes in the class I region, are also involved in susceptibility to the disease [4, 5, 6, 7, 8, 9,

10, 11, 12]. However, the strong linkage disequilibrium across the HLA region makes it difficult to dissect MHC-linked susceptibility to Type 1 diabetes into each component.

The non-obese diabetic (NOD) mouse spontaneously develops autoimmune Type 1 diabetes with marked similarities to human Type 1 diabetes. As in the case of human Type 1 diabetes, inheritance of diabetes in the NOD mouse is polygenic and the strongest susceptibility has been mapped to the MHC region on chromosome 17 [13, 14, 15]. A series of studies using NOD strains congenic for the MHC have shown that the class II MHC contributes to susceptibility to Type 1 diabetes. Both NOD mice congenic for the MHC derived from control strains such as C57BL/10 [16], and NOD-related strains such as non-obese non-diabetic (NON) mice [17] were reported to be completely resistant to Type 1 diabetes, indicating that the NOD MHC is necessary for susceptibility to Type 1 diabetes. In contrast, NOD mice congenic for the MHC from the NOD-related Cataract Shionogi (CTS) strain, which possesses the same class II MHC as the NOD mouse, developed Type 1 diabetes [18, 19], indicating that the CTS MHC confers susceptibility to Type 1 diabetes and that the class II MHC is responsible for MHC-linked susceptibility.

While the class II MHC of the NOD mouse is involved in susceptibility, the class II MHC is not sufficient for full expression of the Type 1 diabetes phenotype in the NOD mouse, because NOD mice congenic for the CTS MHC had a markedly lower incidence and delayed onset of Type 1 diabetes, despite having the same class II MHC as the NOD mouse [18, 19]. These data indicate that the strong susceptibility linked to the MHC is due to the combined effect of *Idd1* in the class II region and another gene or genes outside the class II region. The second component of MHC-linked susceptibility, designated *Idd16*, was mapped to the <10.5 cM region adjacent to, but distinct from the class II MHC region containing class I genes [20].

The class I K gene, located centromeric to the class II region, is syntenic to HLA class I genes A, B and C in humans. MHC class I molecules are important for presenting antigens in target cells to cytotoxic T cells responsible for immune attack, leading to destruction of target cells. In humans and animals MHC class I molecules are divergent among individuals, and this variation, determined by genetic polymorphism, is involved in the divergent immune responses against foreign and self antigens in each animal. In a previous study with serological typing of class I K molecules with monoclonal antibodies, we found that the NOD mouse showed the same reactivity as K^d, whereas the CTS mouse showed a unique reactivity (which was different from any previously known K molecules), reacting not only with anti-K^d, but also with some anti-K^k monoclonal antibodies [21]. Given the role of class I molecules in antigen presentation in the autoimmune process, the class I K gene is a strong functional and positional candidate gene for *Idd16*. In fact, recent studies using a recombinant MHC between NOD and a wild-derived strain with high recombination frequency within the MHC mapped a second component of MHC-linked susceptibility to the 1.1 cM region that is proximal to class II and contains the class I K gene [22, 23].

To clarify the genetic basis of the unique reactivity of class I K molecules in NOD-related strains and the possible contribution of allelic variation in the class I K gene to MHC-linked susceptibility to Type 1 diabetes, we determined the genomic sequences of all exons, exon–intron junctions and the promoter region of the class I K gene in NOD mice and their sister strains, CTS and NON, as well as in NOD.CTS-*H2* congenic mice, which were used for mapping of *Idd16*.

Materials and methods

Animals. We used NOD/Shi, NON/Shi and CTS/Shi mice, which were maintained in the colony of Shionogi Aburahi Laboratories (Shiga, Japan). This is the colony from which NOD and CTS mice for congenic mapping [19] and MHC typing [18, 21] were derived.

DNA. Genomic DNA was extracted from liver tissue of NOD, NON, CTS and NOD.CTS-*H2* congenic mice by a standard method. A total of 13 pairs of primers were designed using Primer3 Input (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi), so that the promoter region (approximately 700 bp upstream from transcription initiation site), the 8 exons, and the 3′-UTR of the class I K gene were covered by 13 segments (primer sequences available upon request). Genomic DNA was amplified by PCR with these primers. The PCR products were refined using a Qia quick gel extraction kit (Qiagen, Tokyo, Japan) and subjected to direct sequencing, utilising dye termination chemistry with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan). To minimise the sequencing artifact, we did direct sequencing of the PCR products with both strands sequenced at least twice in each strain. The sequences were compared with the published sequences of class I K^b (Accession no. M11847) [24, 25], K^d (Accession no. X01815, U47329) [26], and K^k (Accession no. X01652) [27], as well as K from NOD/Lt (Accession no. L36065) [28]. To search for transcription binding sites in the promoter region, we used TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCHJ.html).

Reactivity of MHC molecules. The reactivity of MHC molecules with monoclonal antibodies was studied by fluores-

Fig. 1. Comparison of nucleotide sequences of promoter regions of K^d, K^k, NOD and CTS mice. The nucleotide differences between NOD (non-obese diabetic) and CTS (Cataract Shionogi) mice are shown by bold characters at positions: –217 (G→C), –201 (A→G), –137 (A→T), –101 (T→A) and –92 (T→A). The TATA box and CAAT box are underlined. Details on sequences and numbering, see Methods section (references [28] and [29])

```
                                                  -217            -201
Kd                                                            gccaggcagtgaggtcaggggtggggaagcccagggctgg
Kk    tgagagtttctggatcagaactcggagacgacagcacagggttcaggcaaagtcttagtt-------g--------------------------------
NOD   ---------------------------------------------------g---------------a--------------------------------
CTS   ---------------------------------------------------c---------------g--------------------------------

                                -137                               -101     -92
Kd    ggattccccatctccacagtttcacttctgcacctaacctgggtcaggtcctcctgtccggacactgttgacgcgctgtcagctcttacccccattgggt
Kk    -------------------------------t-----------------------------------t--------a-----------------------
NOD   -------------------------------a-----------------------------------t--------t-----------------------
CTS   -------------------------------t-----------------------------------a--------a-----------------------

                                                                      +1
Kd    ggcgcgatcaccaagaaccaatcagtgtcgccgcggacgctggctataaagtccacgcagcccgcggaactcagaagtcgctaatcgccgacaggtgcg
Kk    ---------------------------------------------------------------------------------------------------
NOD   ---------------------------------------------------------------------------------------------------
CTS   ---------------------------------------------------------------------------------------------------
```

cence-activated cell sorter (FACS) using lymphocytes from NOD/Shi, C3H/He and NOD.CTS-*H2* congenic mice. The following monoclonal antibodies were used: 16-1-11N (anti-K^k), SF1-1.1 (anti-K^d) and RPC.5.4 (negative control). Fluorescein isothiocyanate (FITC) goat anti-mouse IgG was used as a second antibody. These antibodies were provided by M. Hattori (Joslin Diabetes Center, Boston, Mass., USA). Approximately 10^6 lymphocytes suspended in buffer (RPMI-1640 with 5% FCS) were incubated for 30 min at 4 °C with monoclonal antibodies. The lymphocytes were then washed and incubated for 30 min at 4 °C with FITC-goat anti-mouse IgG. The counts of positive cells were determined by FACS scan (Becton Dickinson, San Jose, Calif., USA).

Results

The nucleotide sequences in the NOD/Shi mouse (Accession no. AB114273–114280) were identical to those reported for the promoter and all exons in BALB/c (K^d type) [26] and to the cDNA sequences in the NOD/Lt mouse [28]. We found ten single nucleotide polymorphisms (SNPs) between NOD and CTS (Accession no. AB114281–114288) (Fig. 1, Fig. 2). Of these SNPs, five were in the promoter region at position −217 (G→C), −201 (A→G), −137 (A→T), −101 (T→A), and −92 (T→A) (Fig. 1); one was in exon 2 at position +533 (A→G); and the other four were in exon 4 at position +2767 (T→C), +2788 (G→A), +2800 (T→A), and +2842 (T→C). According to TFSEARCH, the SNPs in the promoter region did not correspond with the binding sites of known transcription factors.

While the SNP in exon 2 (A533G) and one SNP (T→C at position +2842) in exon 4 were silent polymorphisms, the other three SNPs in exon 4 (T→C at position +2767, GAT→AAA at position +2788 and +2800) were expected to cause two amino acid substitutions, Tyr^{191} (NOD)→His^{191} (CTS) and Asp^{198} (NOD)→Lys^{198} (CTS) in the α3 domain. These two amino acids, histidine at position 191 and lysine at position 198, are seen in K^K (C3H mice), but not K^d (Fig. 2). Except for these 2 amino acids, the other amino acids were identical to those in K^d and the NOD mouse.

To confirm that NOD.CTS-*H2* congenic mice, which showed a lower incidence and delayed onset of Type 1 diabetes, retain this unique K molecule, we determined the K sequence in this strain, finding that the K sequence of the congenic mouse (Accession no. AB121050) is identical to that in the CTS mouse and different from that in the NOD mouse.

In our previous study, the K molecule of the NON mouse also showed unique reactivity to anti-K monoclonal antibodies, reacting with both anti-K^b and anti-K^k antibodies [21]. We therefore determined the K sequence of the NON mouse (Accession no. AB114279–114296) which was partially identical to K^b and partially identical to K^k.

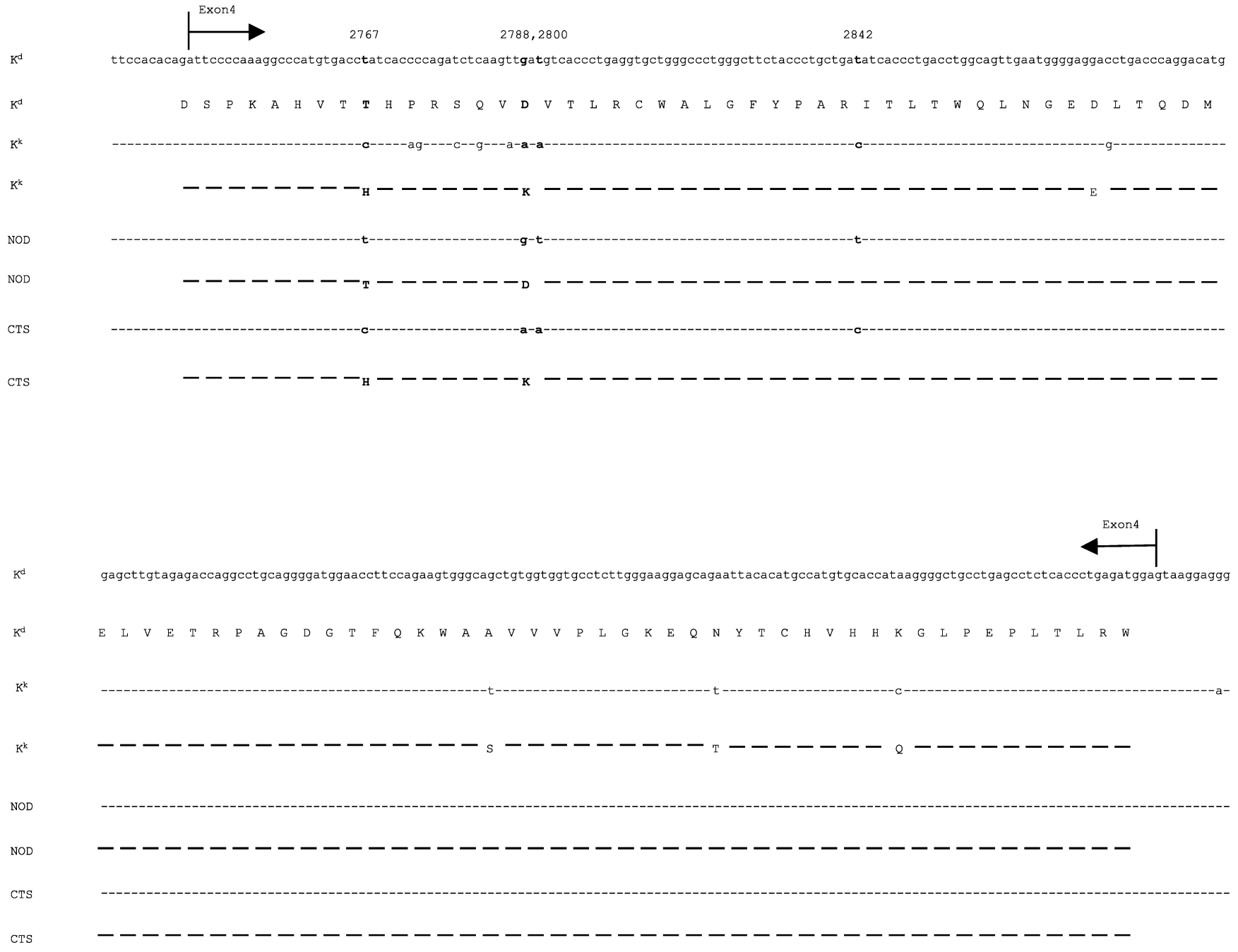
To confirm that the unique sequences of the CTS mouse are reflected in the corresponding molecules, we studied the reactivity of splenocytes with monoclonal antibodies to class I molecules in NOD and CTS mice. The reactivity of K molecules with monoclonal antibodies correlated with the predicted amino acid sequences of K, in that splenocytes of CTS mice reacted with anti-K^k (16-1-11 N) as well as anti-K^d (SF1–1.1).

To confirm that the unique reactivity with anti-K monoclonal antibodies is retained in NOD.CTS-*H2* congenic mice whose genome is identical to the NOD mouse except for the *Idd16* region, lymphocytes from NOD.CTS-*H2* congenic mice were stained with anti-K monoclonal antibodies (Fig. 3). Lymphocytes from NOD.CTS-*H2* congenic mice reacted with anti-K^d and anti-K^k monoclonal antibodies, indicating that the unique characteristics of the K molecule of the CTS mouse are retained in NOD.CTS-*H2* congenic mice and reflecting the unique sequence of the K gene. The fluorescence intensity of lymphocytes stained with anti-K^d monoclonal antibody in NOD.CTS-*H2* congenic mice was similar to that in NOD mice. This suggests that expression of K molecules is similar between the two strains and that nucleotide substitutions in the promoter region of the CTS allele may not have affected expression of K molecules.

Discussion

In this study, we determined the genomic sequences of all eight exons, exon–intron junctions and the promoter region of the MHC class I K gene as a candidate gene for *Idd16*. This was done in the NOD mouse and the CTS mouse, which have susceptible and resistant alleles respectively at *Idd16*. The nucleotide sequence in the NOD mouse was identical to that reported in BALB/c (K^d), whereas that in the CTS mouse was different at ten positions from that in the NOD mouse. In particular, three nucleotide substitutions in exon 4 were predicted to cause two amino acid substitutions in the α3 domain. These two amino acids were identical to those found in K^k, while the rest of the molecule was completely identical to K^d. These characteristics of nucleotide sequences were comparable to the char-

Fig. 2. Comparison of nucleotide and predicted amino acid sequences of H2-K gene in exon 4 of K^d, K^k, NOD and CTS mice. The amino acid sequence is below the nucleotide sequence. Differences in nucleotides and amino acids between NOD (non-obese diabetic) and CTS (Cataract Shionogi) mice are shown by bold characters at positions: +2767 (T→C), +2788 (G→A), +2800 (T→A) and +2842 (T→C). Nucleotide substitutions at +2767, +2788 and +2800 are predicted to cause two amino acid substitutions, Tyr (T) (NOD)→His (H) (CTS) at residue 191, and Asp (D) (NOD)→Lys (K) (CTS) at residue 198. Details on sequences and numbering, see Methods section (references [28] and [29])


Exon4
2767
2788,2800
2842
Kd
Kd
Kk
Kk
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Exon4
Kd
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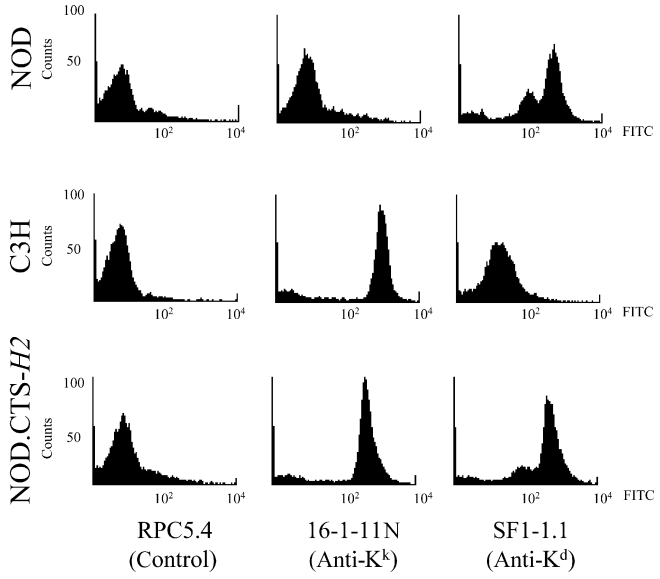

Fig. 3. Comparison of the reactivity of lymphocytes with anti-K monoclonal antibodies in NOD.CTS-*H2* congenic and non-obese diabetic (NOD) and C3H mice. Lymphocytes from NOD.CTS-*H2* congenic mice reacted with anti-K^d and anti-K^k monoclonal antibodies, while those from NOD mice reacted with anti-K^d and those from C3H mice with anti-K^k. The fluorescence intensity of lymphocytes stained with anti-K^d monoclonal antibodies in NOD.CTS-*H2* congenic mice was similar to that in NOD mice. FITC, fluorescein isothiocyanate goat anti-mouse IgG

acteristics of K molecules of the CTS mouse in their reactivity with monoclonal antibodies in this, as well as previous studies in our [21] and other laboratories [29]. The same unique sequence was also retained in NOD.CTS-*H2* congenic mice, which had a lower incidence and delayed onset of Type 1 diabetes. The unique reactivity to anti-K monoclonal antibodies correlated with the unique sequence of the K gene in NOD.CTS-*H2* congenic mice, as well.

Recently, another congenic strain was used for genetic dissection of MHC-linked susceptibility to Type 1 diabetes. In this strain a recombinational hotspot from the B10.A (R209) mouse was introduced to the region between class I K and class II A genes of the NOD mouse [22]. The recombination between class I K and class II A genes created a recombinant MHC with a NOD-derived distal region containing class II A and E genes, and an R209-derived proximal region containing the class I K gene. The frequency of Type 1 diabetes in NOD mice congenic for this recombinant MHC was markedly reduced, despite the presence of the NOD-derived MHC at *Idd1* (class II A and E genes) [22], indicating that the proximal MHC, containing the class I K gene, contributes to susceptibility to Type 1 diabetes. In a study using subcongenic strains, this region was recently narrowed down to the 1.1 cM region containing the K gene [23]. Together, these data suggest that the class I K gene is a strong positional candidate for *Idd16*.

The functional contribution of the class I MHC to the development of Type 1 diabetes was clearly demonstrated by the fact that the NOD mouse lacking class I molecules is completely protected from insulitis and diabetes due to targeted disruption of the β2 microglobulin gene [30, 31, 32, 33]. Among class I molecules expressed in the NOD mouse, class I K^d (but not D^b) appears to be important because almost all the $CD8^+$ T cells isolated from NOD islets recognise islet antigens in the context of K^d molecules [34, 35, 36, 37, 38]. In addition, Type 1 diabetes of the NOD mouse was reported to be prevented by anti-K^d monoclonal antibody, but not by anti-D^b monoclonal antibody [39]. Recently, transgenic expression in NOD mice of K^{R209}, the K gene from the B10.A (R209) strain, was also shown to reduce the incidence and delay the onset of Type 1 diabetes [23]. This was shown in the case of NOD.CTS-*H2* congenic mice [19] and intra-MHC recombinant NOD mice, which possess the class I K region from R209 [22, 23]. These data, together with the substitutions of K^d observed here in the NOD mouse, with K^k-like residues in NOD.CTS-*H2* congenic mice, suggest that class I K^d is both a functional and a positional candidate for *Idd16*.

Although class I K gene is a strong candidate for *Idd16*, other genes in the MHC could be responsible for *Idd16* effect. A recent study, for example, reported that NOD mice congenic for a 6-cM interval proximal to H2-K from C57BL/6 strain had a markedly reduced incidence and delayed onset of Type 1 diabetes [40]. Another study reported that at least two, and probably more, distinct genes centromeric to class II genes contribute to diabetes-resistance in NOD mice congenic for the MHC proximal to class II genes from the B10.A (R209) strain [23]. In addition to genes in proximal MHC, genes in distal MHC such as *Tnf* [41] and class I *D* [29] have also been suggested as candidates for *Idd16*. It is possible, therefore, that *Idd16* consists of multiple components and that class I K gene is a candidate for one component.

In human Type 1 diabetes mellitus, we previously reported that the HLA class II region is strongly associated with disease susceptibility, whereas age at disease onset is linked to the class I region [5]. Subsequent studies in several ethnic groups also suggested that a gene or genes in the class I region confer or modify susceptibility to Type 1 diabetes [42, 43, 44]. Given the strong effect of HLA gene polymorphisms per se on immune responses, it is possible that HLA class I gene polymorphism modifies autoimmunity through its effect on autoantigen presentation to effector T cells. However, identification of a susceptibility gene outside the class II region in humans is difficult owing to the strong linkage disequilibrium across the HLA region. As a second component of the MHC-linked gene has been mapped to the class I region in the NOD mouse, as in humans, the identification of

non-class II susceptibility genes in the NOD mouse will contribute to a better understanding of the causes of human Type 1 diabetes.

Class I MHC molecules, which consist of a heavy chain (α1, α2 and α3 domains) and a light chain (β2 microglobulin), bind short peptide fragments and present them as target antigens to cytotoxic T cells. The two amino acid substitutions found by comparing the sequences of the class I K gene in NOD and CTS mice, Tyr^{191} (NOD)→His^{191} (CTS) and Asp^{198} (NOD)→Lys^{198} (CTS), are located in the α3 domain. Substitutions of positively charged aspartate with negatively charged lysine and the aromatic amino acid tyrosine with the basic amino acid histidine may affect the interaction of the α3 domain with β2 microglobulin, leading to changes in the three-dimensional structure of the overall molecule. Provided that one amino acid substitution in MHC molecules affects immune responses [45, 46, 47], it is possible that these amino acid substitutions affect the autoimmune response to pancreatic beta cells, leading to a lower incidence and delayed onset of Type 1 diabetes in NOD.CTS-*H2* congenic mice.

In addition to amino acid substitutions in exon 4, nucleotide substitutions were found in the promoter region. These may also affect expression of K molecules. None of the substitutions, however, corresponded to the binding site of known transcription factors. In addition, the similar fluorescence intensity in NOD and NOD.CTS-*H2* lymphocytes stained with anti-K^d monoclonal antibody suggests that nucleotide substitutions in the promoter have little, if any, effect on expression of K molecules.

The CTS mouse was previously reported to have the same cDNA sequences of the K gene as the NOD mouse, which has K^d type [29]. In our study, the CTS/Shi mouse from the original colony turned out to have a unique nucleotide sequence that is different from the NOD sequence, leading to two amino acid substitutions. These substituted amino acids are identical to those found in the K^k molecule. This result is compatible with the reactivity of H-2 K molecules with monoclonal antibodies in the present study as well as in previous studies from our [21] and other laboratories [29], in that splenocytes from CTS mice reacted with an anti-K^k monoclonal antibody in addition to an anti-K^d monoclonal antibody.

The reason for the discrepancies between the sequence in the present study and those reported by Mathews et al. is unknown. One possibility is difference in strains. In the present study, sequences were determined in the CTS/Shi mouse obtained from the original colony at Shionogi Aburahi laboratories, which is the colony from which the CTS mice for original studies on monoclonal antibody reactivity and also for congenic mapping of *Idd16* were derived. Another possibility is the difference in the template used for sequence analysis. Genomic DNA was used in the present study, whereas cDNA constructed from splenic RNA was used by Mathews et al. Whatever the reason for the difference in sequences, the sequence found by us is more likely to reflect the real sequence of the CTS/Shi possessing resistant allele at *Idd16* because the data are obtained directly from the NOD.CTS-*H-2* congenic strain that is used for congenic mapping and identification of *Idd16*. Also, our results are able to explain the difference in reactivity with anti-K monoclonal antibodies seen in NOD and CTS mice in this and previous studies.

In summary, our nucleotide sequence analysis of the MHC class I K gene revealed that, while the NOD mouse has an identical sequence to K^d type, the sequence in the CTS mouse was different from K^d type at two amino acids that are seen in K^k type. These sequence data are compatible with the immunoreactivity of the K molecule in this and previous studies. Given the function and the position of class I K gene, together with recent reports on the position and function of a second diabetogenic gene in the MHC, these data support the possibility that the class I K gene itself may be responsible for the *Idd16* effect.

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