Mutational analysis of the HLA-DQ3.2 insulin-dependent diabetes mellitus susceptibility gene


Immunology Program, Virginia Mason Research Center, Seattle, WA 98101
Communicated by Hans Neurath, November 7, 1988

ABSTRACT The human major histocompatibility complex includes approximately 14 class II HLA genes within the HLA-D region, most of which exist in multiple allelic forms. One of these genes, the DQ3.2β gene, accounts for the well-documented association of HLA-DR4 with insulin-dependent diabetes mellitus and is the single allele most highly correlated with this disease. We analyzed the amino acid substitutions that lead to the structural differences distinguishing DQ3.2β from its nonidiabetogenic, but closely related, allele, DQ3.1β. Site-directed mutagenesis of the DQ3.2β gene was used to convert key nucleotides into DQ3.1β codons. Subsequent expression studies of these mutated DQ3.2β clones using retroviral vectors defined amino acid 45 as critical for generating serologic epitopes characterizing the DQw3.1β and DQw3.2β molecules.

Genes within the major histocompatibility complex encode polymorphic alleles that are variably associated with autoimmune diseases. The HLA-DQ allele DQ3.2β is a putative susceptibility gene present in approximately 70% of insulin-dependent diabetes mellitus (IDDM) patients (1-4). Although undoubtedly not the only gene conferring susceptibility to diabetes, it is the gene most highly implicated in this disease. The DQ3.2β gene is closely linked to HLA-DR4 and accounts for the nonrelated association of DR4 with IDDM. DQ3.2 differs from the closely related nonidiabetogenic DQ3.1 allele by just six amino acid substitutions, four of which are in the amino-terminal domain important for variation in immune recognition (4, 5). The DQ gene products encoded by the DQ3.1 and DQ3.2β polymorphic alleles can be distinguished by alloantisera and monoclonal antibodies (mAbs). Serologically, the DQ3.1β molecule has a unique epitope, designated T A10 (6, 7), which can be recognized by DQβ-specific mAbs such as 159.1 and SFR20-DQβ5 (8). The DQ3.2β molecule has an epitope not present on DQ3.1, recognized by mAb GSP200.1. The 200.1 epitope is probably identical to IIB3 (3), a public epitope shared by DQ3.2 and some other DQ molecules. Although class II DQ molecules are heterodimers with α and β chains, the β chain determines the DQw3 specificity and related alloepitopes (8). The DQ3.2 and DQ3.1β alleles can also be distinguished by restriction fragment length polymorphisms, two-dimensional protein gel electrophoresis, and oligonucleotide hybridization (4, 6, 8, 9). These methods have been widely used to highlight the strong association of the DQ3.2β gene with IDDM susceptibility (1-6, 9, 10). Since DQ3.1 is not associated with IDDM, analysis of the unique structural characteristics that distinguish DQ3.2β from DQ3.1β should help to pinpoint the molecular contribution of HLA to disease. Site-directed mutagenesis of the DQ3.2β gene was systematically performed to create a panel of intact DQβ genes that carry DQ3.1-like substitutions at the four key codons in the first exon, which are likely to be responsible for DQβ-associated IDDM susceptibility. Expression of these mutagenized DQ3.2 genes in human B-cell lines using retroviral vectors identified mutations at codon 45, but not at codons, 13, 26, or 57, which correlated precisely with expression of the DQβ serologic specificities that distinguish DQ3.1 and DQ3.2.

MATERIALS AND METHODS

Cell Lines and Antibodies. mAbs 159.1 and SFR20-DQβ5 (DQw3.1 specific) (8), mAb 200.1 (anti-DQw3.2 specific), mAb IVD12 (DQw3 specific) (11) and mAb F9 (anti-luteinizing hormone releasing hormone) were prepared from hybridoma culture supernatants or ascites fluid. The Epstein-Barr virus-transformed human B-lymphoblastoid cell line MAT (homozygous DR3,DQw2) was maintained in tissue culture in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum.

Site-Directed Mutagenesis. Oligonucleotide-mediated mutagenesis of the DQ3.2β gene was carried out in a modified protocol using M13 bacteriophage (12). Briefly, a 1.3-kilobase HindIII-Xba I fragment was isolated from the DQ3.2β expression vector (see Fig. 2) and was cloned into M13mp18. One microgram of this plasmid DNA was then mixed with 1 μg of the appropriate mutagenic oligo in 15 μl of 50 mM NaCl/10 mM MgCl2/10 mM Tris-HCl, pH 8. Mutagenic oligomers used were 5'-CATTTAAGGCTATGTTGCTAC-3' for the DQ3.2βm13 mutant, 5'-GCCGTGGTGTTATGTGACCAGA-3' for the DQ3.2βm26 mutant, 5'-AGCCAGCTGGAAGTGTACCGG-3' for the DQ3.2βm45 mutant, and 5'-GGGCCGCCTGATGCGAGCTAC-3' for the DQ3.2βm57 mutant. These oligomers also have additional silent mutation changes compared to DQ3.1 sequences to facilitate screening. The mixture was incubated at 55°C for 5 min and then cooled at 37°C for 15 min. The DNA was then diluted 1:3 with water, used to transform DH5a bacteria, and plated with JM101 cells. Plaques containing the desired mutation were isolated by screening with the appropriate mutagenic oligomers end-labeled with 32P. The frequency of the desired mutation was approximately 0.1%. Nucleotide sequencing of the DQβ coding region of each M13 construct was performed to confirm that the desired mutation was obtained. The HindIII-Xba I DNA fragment from each mutant phage was then reinserted into the expression vector.

HLA Recombinant Retrovirus-Producing Cell Lines and Infection of B-Lymphoblastoid Cells. The parental retroviral construct was pLNL6 (13). The construct pLNCL6 had a 746-base-pair Bal I-Xma III restriction fragment from the human cytomegalovirus genome containing the immediate early enhancer/promoter at the 3' end of the neomycin-resistant gene of the pLN6L6 construct and was provided by R. Hock (Fred Hutchinson Cancer Research Center, Seattle). The construction of the HLA retroviral vector was as

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

Abbreviations: IDDM, insulin-dependent diabetes mellitus; mAb, monoclonal antibody.
described in Fig. 1. HLA retrovirus-producing cell lines were generated by introduction of the retroviral constructs into the amphotropic packaging line PA317 (14). Infection of B-lymphoblastoid cells was carried out by coculturing of $5 \times 10^4$ virus-producing cells with $2 \times 10^6$ B cells in 60-mm plates in the presence of Polybrene ($2 \mu g/ml$) in RPMI 1640 with 10% fetal calf serum. The nonadherent B cells were harvested after 24 hr of co-culture and were grown for another 24 hr before placing them in selective medium containing G418 (1 mg/ml).

**Indirect Immunofluorescence.** Approximately $5 \times 10^5$ cells were incubated with $15 \mu l$ of mAb (1:80 dilution of ascites fluid) for 1 hr at 4°C. The cells were washed with 2.5 ml of isotonic phosphate-buffered saline (PBS) with 1% fetal calf serum and then incubated with 15 $\mu l$ of fluorescein isothiocyanate-labeled sheep anti-mouse immunoglobulin for 1 hr at 4°C. After a second wash in PBS with 1% fetal calf serum, the cells were fixed with 2 ml of PBS with 2% (wt/vol) paraformaldehyde. Approximately $10^5$ cells were then analyzed on a Becton Dickinson FACS IV flow cytometer.

**Oligonucleotide Hybridization.** The DQ5 locus-specific oligonucleotide probe (5'GCCCTTAAACTGCTACAGGAAATCTC-3') and the DQ3.2 allele-specific oligonucleotide probe (5'CGGCGATAACCCAGTCGTC-3') were end-labeled by polynucleotide kinase and $2 \mu l$ of [y-32P]ATP (specific activity 7000 Ci/mmol; 1 Ci = 37 GBq) and 49 ng of T4 polynucleotide kinase (New England Biolabs) per 500 ng of probe, to a specific activity of $10^8$ cpm/$\mu l$. After labeling, the oligonucleotides were passed through Sephadex G-50 in 0.1x SET (1x SET = 1% NaDODSO4, 5 mM EDTA/10 mM Tris-HCl, pH 7.5). Genomic DNA was digested with BsaI restriction endonuclease and electrophoresed on 1% agarose gels at 40 V/cm for 16–18 hr. The gels were denatured, neutralized, and dried on Whatman 3MM filter paper. The gels were then soaked in water to remove the filter paper, prehybridized for 2 hr at 50°C in 6x NET (0.5 M/0.18 M Tris-HCl, pH 8.0/6 mM EDTA) containing RNA (250 $\mu g/ml$), then hybridized for 3 hr at 50°C in 6x NET/10% (wt/vol) dextran sulfate/5x Denhardt’s solution/5 mM EDTA/0.1% NaDODSO4/0.05% Nonidet P-40 containing RNA (250 $\mu g/ml$) and oligonucleotides (10$^5$ cpm/ml). (a Denhardt’s solution = 0.02% polyvinylpyrrolidone/0.02% Ficol/0.02% bovine serum albumin.) Gels were washed in 6x SSC (1x SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) twice for 10 min at room temperature and then three times for 20 min at 55°C.

were then exposed to Kodak XAR-5 film with an intensifying screen at $-70^\circ$C for 2–5 days.

**RESULTS**

**Oligonucleotide-Mediated Mutagenesis.** DNA sequence comparisons show that there are six codon differences in the coding region distinguishing between the DQ3.1 and DQ3.2 molecules, mutant DQ3.2B genes were generated by oligonucleotide-directed mutagenesis. We systematically altered each of the 4 specific DQ3.2B amino acids to the corresponding DQ3.1B amino acid, as illustrated in Fig. 2. The mutant DQ3.2b mRNA encodes a molecule identical to DQ3.2B except that glycine at position 13 was replaced by alanine corresponding to the DQ3.1 molecule at that position. Similarly, substitutions at position 26, 45, and 57 of DQ3.2B generated the mutants DQ3.2b mRNA, DQ3.2b mRNA, and DQ3.2b mRNA, respectively. The first 83 amino acids of these DQ3.2B mutants, as well as DQ3.1B and DQ3.2B, are depicted in Fig. 2. All mutations were verified by resequencing.

**Expression of Mutagenized DQ3.2B Genes in Human B-Lymphoblastoid Cells.** To analyze the serologic specificities encoded by this panel of mutant DQ8 genes, retroviral vectors with the *in vitro*-mutagenized DQ3.2 clones were transferred into a human B-lymphoblastoid cell line. The structure of the vector used is described in Fig. 1. A cytomegalovirus immediate gene enhancer/promoter was used to drive the HLA gene, which was inserted distal to a neomycin-resistance gene. Virus-producing cells were generated from these vectors with either the normal DQ3.2 gene or the *in vitro*-mutagenized DQ3.2B genes. Infection was carried out by coculturing $2 \times 10^6$ B-lymphoblastoid cells with $5 \times 10^5$ virus-producing fibroblasts for 24 hr, followed by exposure in the presence of G418 (1 mg/ml). Resistant cells were then analyzed by cytofluorometry with anti-HLA mAbs.

Analysis of the various DQ3.2B constructs expressed in a DQ2 homozygous B-lymphoblastoid cell line is shown in Fig. 3. None of the antibodies to DQw3-related epitopes reacted with uninfected B-lymphoblastoid cells, while both the DQw3 and DQw3.2-specific mAb gave high levels of immuno-fluorescent reactivity on B-lymphoblastoid cells infected with DQ3.2B. We have also expressed the unmodified DQ3.2B gene in a DQ3.1 homozygous LCL with results comparable to those shown in Fig. 3. To evaluate the effects of DQ3.1-like substitutions on DQ8 epitopes, analysis of mutagenized DQ3.2B genes was performed in a DQ2-positive B-lymphoblastoid cell line: DQ3.1-like amino acid substitutions and residues 13, 26, or 57 did not change serologic reactivity patterns with a panel of anti-class II antibodies as compared to the wild-type DQ3.2 gene. However, a glycine to glutamic acid interchange at codon 45 abolished reactivity with anti-DQ3.2 mAb GSP200.1. This same mutation at codon 45 also led to acquisition of reactivity with the DQ3.1-specific mAbs 159.1 and SFR20-DQ8 (Fig. 3). The D3.1 specificity, also known as TA10 and recently renamed DQw7 (15), has frequently been used as a marker to discriminate among DQ8 molecules associated with type I diabetes.

**Fig. 1.** Structure of the DQ3.2B expression vector. The DQ3.2B cDNA was isolated as a 860-base-pair PsI–EcoRI fragment from a DQ3.2B cDNA clone generated from the B-lymphoblastoid cell line TAS. This 860-base-pair cDNA fragment included the 5’ ATG initiation codon, but lacked a 3’ polyadenylation signal. This fragment was cloned into the 3’ end of the cytomegalovirus immediate early (CMV IE) enhancer/promoter within the neomycin-resistance (NEO) retroviral construct pLNCL6. pLNCL6 was a derivative of pLN6L (13). LTR, long terminal repeat.

**Fig. 2.** Sequence comparison of amino acid residues 1–83 of DQ3.2, DQ3.2B mutants, and DQ3.1. Amino acid sequences of the first exon as deduced from nucleotide sequences are shown. The numbers above the sequence indicate the amino acid position.
Immunology: Kwok et al.

Monoclonal Antibody used for Staining

IV D12  GSP200.1  159.1
(DQw3 specific)  (DQw3.2 specific)  (DQw3.1 specific)

Transfected Gene

None

DQ3.2β

DQ3.2βm13

DQ3.2βm26

DQ3.2βm45

DQ3.2βm57

Log Fluorescence

Cell Number

FIG. 3. Flow-cytometric analyses of MAT cells transfected with either native or mutant DQ3.2β retroviruses. Indirect immunofluorescence was used to detect binding of mAb F9 (specific for luteinizing hormone releasing hormone), which was used as a negative control, of IVD12 (DQw3 specific), of GSP200.1 (DQw1/DQw3.2 specific), and of 159.1 (DQw3.1/DQw7 specific) to various B-lymphoblastoid cell lines. Cell lines analyzed included untransfected MAT (DQw2) and MAT transfected with the following native and mutant DQ3.2β retrovirus constructs: DQ3.2β, DQ3.2βm13, DQ3.2βm26, DQ3.2βm45, and DQ3.2βm57. Cells were analyzed for fluorescence with a Becton Dickinson FACS IV flow cytometer, using a logarithmic amplifier to give the histograms shown. The dashed lines indicate staining with negative control mAb F9, and the solid lines indicate staining with the appropriate class II mAb as stated in the top of each column. mAb SFR20-DQ8 (DQw3.1 specific) gave similar results as mAb 159.1 (data not shown).

(1, 7-10); these results map the residue responsible for this specificity to codon 45 of the DQ3.1β gene. This mutation did not alter reactivity with the DQw3-specific mAb IVD12, which indicates that the location of the DQw3 epitope is distinct from the TA10 (DQw7) and 200.1 epitopes. Levels of cell-surface expression of each of the mutant genes were comparable to the control levels, indicating that none of these point mutations prevented adequate dimer formation or expression.

An oligonucleotide probe to the DNA sequence corresponding to seven codons of DQ3.2 centered on codon 45 was used for hybridization to genomic DNA. As expected, this probe discriminated DQ3.1 from DQ3.2 (Fig. 4) and also failed to hybridize to DQ1 or DQ2 genes. As reported (10, 16) for probes corresponding to other DQB polymorphic sequences, such as at codon 57, therefore, such oligonucleotide hybridization serves as a useful marker for detection of putative susceptibility alleles in the population.

DISCUSSION

Among HLA DQB sequences, a glutamic acid residue at codon 45 is unique to DQ3.1 (5, 10). The finding that this codon determines the TA10 (DQw7) epitope pinpoints the structural milieu around codon 45 as a likely site for immune recognition; this conclusion is supported by the finding that codon 45 is also critical for the DQ3.2 epitope. The demonstration, using site-directed mutagenesis and retroviral-mediated gene transfer, that a single codon substitution contributes both a gain and a loss of a major allospecificity also suggests the possibility that this residue is important for immune recognition and may contribute directly to the function of the DQ B molecule.

Substitutions at positions other than codon 45, although not accounting for the DQ8 allospecificity, may, of course, contribute to immune recognition. The substitutions at codons 13 and 26, however, are both conservative replacements. Position 57 represents a substitution of an aspartic acid for an alanine and, although not detected by our experiments, might be expected to contribute to the formation of recognition epitopes. Based on a nucleotide sequence comparison of the human DQ3.2 gene with other DQ genes and with the I-Aβ gene of NOD (diabetes-prone) mice, Todd et al. (10) have suggested that codon 57 is important for the pathogenesis of IDDM. They reported that the presence of an aspartic acid at codon 57 is negatively associated with IDDM, while a small noncharged residue at that position is positively associated with the disease. However, numerous exceptions to this rule are reported. For example, DR7.DQ2 individuals, with Ala57 are not positively associated with IDDM, and DR4, DQ4 individuals, with Asp57, are highly associated with this disease (16). These data suggest that it is most likely that codon 57 is not the only amino acid residue in the DQB molecule involved in the pathogenesis of IDDM.

Our data indicate that codon 45 is an important serological epitope and should contribute to the structural characteristics of the DQ B molecule. It is possible that the DQ8 segment encompassing residues 45–57 of the DQw3 molecule interacts with either an antigenic peptide and/or cell receptor crucial for the autoimmune process leading to IDDM. One possible interpretation is that although residue 45 is key to alloepitope formation and, by inference, to immune recognition, residue 57 may be intimately involved in class II interactions with antigen. This model would be consistent with the observation that HLA class I molecules contain residues that, both spatially and functionally, appear to interact with antigen and T cells at distinct sites (17). Since the precise function of the DQ3.2 molecule in IDDM predisposition is unknown, mechanisms involving inappropriate, or defective, antigen presentation cannot yet be distinguished from mechanisms involving inappropriate, or defective, self-recognition. It is apparent that detailed expression and mutagenesis studies, in addition to nucleotide sequence comparisons, are necessary to resolve this issue.
The mutated DQ3.2β genes were introduced into the human lymphoblastoid cell line MAT (DQw2 homozygous) for these expression studies. The high level of DQw3 expression achieved in all cases indicates that an endogenous class II α chain was efficiently recruited for dimer formation and that the substitutions of variable residues at codons 13, 26, 45, and 57 did not interfere with this heterodimer formation. This DQ2 cell expressing the transfected DQ3.2β gene also provides an interesting model for IDDM immunogenetics: DR3/DR4 (DQ2/DQ3) heterozygous individuals have the highest relative risk for IDDM (18), suggesting some synergy between the HLA genetic contributions of these two haplotypes. In the present study, expression of the transfected DQ3.2β gene implies heterodimer formation between the endogenous DQ2α chain and the inserted DQ3.2β chain. Such DQ2α/DQ3.2β heterodimers have been detected in diabetic patients (19), implying that these mixed dimers may represent a distinct immune recognition molecule that can contribute to the immune responsiveness of an individual and the immune cascade that leads to IDDM.

We thank Phillip Thurtle for technical assistance; Peter Gregersen for the TAS DQβ cDNA; Susan Radka and Karen Nelson for mAbs 159.1, GSP200.1, and SFR20-Dqβ5; Randy Hock for retroviral construct pLNCL6; and Harriet Langsford for preparation of the manuscript. This work was supported by Grants AR 37296 from the National Institutes of Health and IM-450 from the American Cancer Society.