DNA SEQUENCE ANALYSIS OF I-A$_\beta^k$ MUTANTS REVEALS SEROLOGICALLY IMMUNODOMINANT REGION

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The class II MHC-encoded molecules, expressed primarily by B lymphocytes, macrophages, and certain other cell types, serve as target molecules for recognition by T helper/inducer lymphocytes. The T helper/inducer cells play a critical role in regulating both cellular and humoral immunity, and thus, the antigen-specific activation of these cells triggered by the engagement of the receptor on the T cell with its ligand, an ill-defined complex of processed antigen and class II MHC molecule, is one of the key events in the activation of an immune response. It is clear that the extensive natural polymorphism of class II molecules has some relevance to their function. In the mouse, the failure of some strains to respond to certain antigens is a property of the class II alleles, and in man, susceptibility to certain disease states correlates significantly with HLA class II type. To explore the relationship between the sequence diversity and function of murine class II I-A molecules, we have produced a series of in vitro serologically selected cell lines expressing mutant I-A^k molecules (1). In this report we will describe the DNA sequence analysis of four of our collection of mutant cell lines. Each of the major serologic epitopes on the A$_\beta^k$ polypeptide has been altered in one or more of the four mutants described in this report. In addition, these four mutants exhibit a broad spectrum of functional defects when used to stimulate a panel of T hybridomas of various specificities. The DNA sequence analysis of the I-A$_\beta^k$ gene from each mutant has revealed that each has sustained a single amino acid substitution and that all four independent substitutions have occurred within or near only one of the four variable regions defined in the β_1 domain of the A$_\beta$ polypeptide by allelic comparisons. These data strongly suggest that this one variable region is the major determinant of alloantigenicity on the A$_\beta^k$ polypeptide.

Materials and Methods

Cell Lines. Mutants were obtained by a protocol of negative and positive selection using a panel of anti-I-A^k mAbs after ethylmethanesulfonate mutagenesis of the parental TA3 cells, as has been described (1). All cell lines have been cloned and subsequently maintained in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 10^{-4} M 2-ME, 10 mM Hepes, and antibiotics at 37°C in a humidified atmosphere of 5% CO_2 in air.
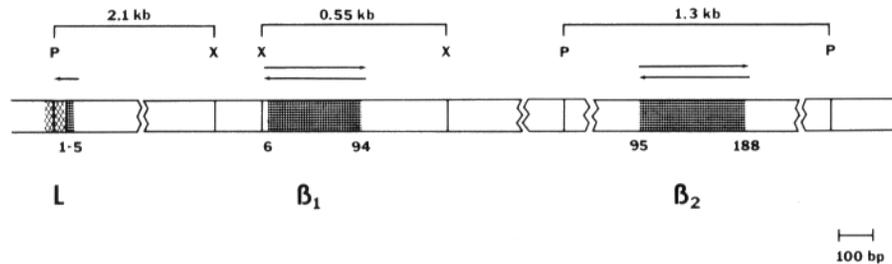
FIGURE 1.   The sequencing strategy for the exons of the A^k_β gene encoding the external domains of the A^k_β polypeptide. Only that portion of the gene encoding the external domains of the mature A^k_β polypeptide is pictured. Regions encoding sequence present in the mature protein are denoted by heavy shading and the region encoding the signal peptide is denoted by hatched shading. Numbers beneath the heavily shaded areas refer to the amino acid residues encoded by each region. The restriction sites cut by Pst I (P) and Xma I (X) are indicated along with the approximate sizes of the restriction fragments cloned for sequencing.

Serologic Phenotypes.   Serologic phenotypes of the cell lines were analyzed by indirect immunofluorescence on a flow cytometer (FACS IV) after staining under saturating conditions with various I-A^k-reactive mAbs as previously described (1). Fluorescence intensity was measured using a logarithmic amplifier and quantitated by the comparison of peak channel numbers. Antibodies 10-2.16 and 11-3.25 were derived by Oi et al. (2); 4-2.1 and 4-2.3 by Harmon et al. (3); and 39B, 39E, 39J, 40A, 40M, 40F, and 40L by Pierres et al. (4).

Functional Phenotypes.   The ability of the various cell lines to present antigen to a panel of T hybridomas was assayed by quantitating antigen-specific IL-2 release. The panel of T hybridomas used included both I-A^k-specific auto- and alloreactive T cells and I-A^k-restricted antigen-specific T cells. The antigen-reactive hybridomas recognized either KLH, hen egg lysozyme (HEL)[1] or OVA. Most of these data have been published previously (1). Additional functional studies have been carried out by Drs. E. Rosloniec and J. Freed, using lysozyme-reactive, I-A^k-restricted T hybridomas (unpublished data). In all cases, either the supernatants were titrated for IL-2 activity or supernatants from titrated numbers of stimulator cells were assayed for IL-2 activity.

I-A^k_β Gene Cloning and Sequencing.   A bacteriophage lambda clone of the 17-kb Eco RI fragment containing the A^k_β gene (5) from each mutant was isolated from a size-selected (16–20 kb) Eco RI genomic library prepared in either Charon 4 or EMBL4. The A_β insert-containing phage were identified by hybridization with either the pIA$_\beta$-1 (6) or the pCA12 plasmid probe kindly provided by Dr. J. Seidman (Harvard Medical School, Boston, MA) and Dr. L. Hood (California Institute of Technology, Pasadena, CA), respectively. The pCA12 plasmid consists of the 2.4-kb Hind III fragment of the A^d_β gene that contains exons 1 and 2 and the intervening sequence (7) subcloned into pBR325. The mutant A^k_β genes were digested with Pst I or Xma I or both and the fragments were cloned into M13mp18 or 19 for nucleotide sequencing by the dideoxy chain termination method (8). The approximate sizes of the fragments cloned are indicated in Fig. 1. The desired recombinants were identified by hybridization with synthetic oligonucleotides complementary to intron sequences directly adjacent to the particular exons, based on published sequence (9). The same oligonucleotides subsequently were used as primers in the sequencing reactions. The exons encoding the β_1 and β_2 domains were sequenced in both directions and the leader exon sequenced only in the 3' to 5' direction, as indicated in Fig. 1.

Transfection Analysis of Mutant A^k_β Genes.   The plasmid constructions for the transfections were as follows. Since Eco RI cuts in the middle of the 3' untranslated exon of the A^k_β gene, the 17-kb Eco RI fragment containing the A^k_β gene from the mutant cell lines

TABLE I

Serologic Phenotypes of A_β^k Mutants

Cell line	Allospecificity:	Ia.17			Ia.18	Ia.1				I-A^{k,f,u}	
	mAb: 10-2.16	11-3.25	4-2.1	4-2.3	39B	39E	40A	40M	40F	40L
TA3 (parent)	++	++	++	++	++	++	++	++	++	++
LD3*	±	−	+	−	±	−	−	−	+	+
G1	±	++	++	++	++	++	++	++	++	++
K5	++	++	++	++	±	−	−	−	++	++
F16	++	++	++	−	++	++	++	++	−	−

Reactivity with these A_β^k-reactive mAbs was analyzed by indirect immunofluorescence as previously described (1, 32). Reactivity levels are designated as follows: −, no detectable reactivity above background; ±, weak reactivity (2–10% of control TA3 level); +, intermediate reactivity (10–30% of control TA3 level); ++, parental-level reactivity (30–100% of control TA3 level).

* The LD3 mutant appears to have a lower overall expression of I-A^k, in addition to the indicated specific alterations in serologic reactivity, as evidenced by only an intermediate level of reactivity with all A_α^k-specific mAbs (data not shown).

LD3, G1, and F16 was cloned into a pBR327 derived plasmid containing a 2.5-kb Bam HI fragment encoding the complete 3' untranslated region of the A_β^k gene (10). For the mutant K5, the 352-bp Sst II fragment containing the second exon (β_1 domain) was cloned into pUC18 containing the wild-type A_β^k gene on a 16-kb Hind III fragment from the cosmid H-2k1.1 (11), from which the corresponding wild-type Sst II fragment had been deleted. The A_α^k and pMSV-neo (12) plasmids were kindly provided by L. Glimcher, Harvard School of Public Health, Boston, MA. The plasmid DNAs were transfected into the M12.C3 cells (13) by electroporation (14) carried out with an apparatus obtained from Prototype Design Services (Madison, WI).

Results

The TA3 cell line, derived from the fusion of (BALB/c × A/J)F$_1$ spleen cells with the BALB/c B lymphoma cell line M12.4.1 (15), and thus haploid for the I-A^k genes, was used as the parent for the isolation of cell lines expressing mutant I-A^k molecules (1). Several different A_α^k- and A_β^k-reactive mAbs have been used for both the negative and positive selection steps, thus generating an extensive series of mutant cell lines expressing serologically altered I-A^k molecules. This selection scheme favors the isolation of variant cell lines expressing A^k molecules with only limited alterations in either the A_β^k or A_α^k polypeptide (16).

In this report we describe the sequence analysis of four mutants that have distinct serologic and functional phenotypes. Each of these mutants expresses a serologically altered A_β^k polypeptide in association with a wild-type A_α^k polypeptide. With the exception of mAb 4-2.1, reactivity with each antibody in an extensive panel of 10 A_β^k-reactive mAbs has been reduced or eliminated in one or more of the four mutant cell lines (Table I). Each cell line retains the capacity to react at wild-type or near wild-type levels with a panel of A_α^k reactive mAbs (data not shown). When analyzed with a panel of A^k-reactive and A^k-restricted T hybridomas (summarized in Table II), each of the four mutant cell lines has a distinct functional phenotype, ranging from nearly wild-type (F16 and G1) to almost completely defective (K5) (reference 1 and Rosloniec, E., J. Freed, B. N. Beck, and D. J. McKean, unpublished data). All of these mutant cell lines do retain the capacity to stimulate E^k-restricted T hybridomas at wild-type levels (data not shown).

TABLE II

Functional Phenotypes of A^k_β Mutants

Cell line	Stimulation of A^k-reactive and A^k-restricted T hybridomas*	
	Number tested	Number positive
TA3 (parent)	36	36
LD3	35	20
G1	36	33
K5	23	2
F16	19	17

* The stimulatory capacity of the cell lines was determined as described in Materials and Methods. In almost all cases, the mutant cell lines were either clearly negative or virtually identical to the parent TA3 cell line in stimulatory capacity. The chief exception is the LD3 cell line, which occasionally stimulated weak but clearly positive responses as described in the original report (1).

To ascertain the structural basis for the mutant serologic and functional phenotypes, a genomic clone of the A^k_β gene from each mutant cell line was obtained in bacteriophage lambda and appropriate restriction fragments were subcloned into bacteriophage M13 for sequencing. A partial map of the A^k_β gene is given in Fig. 1. Only those exons encoding the external domains of the polypeptide were sequenced. DNA-mediated gene transfer studies (17) have shown that the alloantigenicity of the A_β polypeptide is determined within this region of the gene. Sequence analysis of the first three exons of the A^k_β genomic clones revealed that each mutant A^k_β gene had sustained a single nucleotide substitution resulting in a single amino acid substitution in the β_1 domain. LD3 has a Gln to Pro substitution at residue 64, G1 a Gln to Arg also at 64, K5 an Arg to Gln at 70, and F16 a Glu to Asp at 59 (Fig. 2).

To be certain that the mutant gene isolated for sequence analysis was responsible for the mutant phenotype of the cells, the A^k_β gene from each mutant was subcloned into a plasmid vector and subsequently cotransfected with a plasmid containing the wild-type A^k_α gene and the pMSV-neo plasmid (12) into the Ia⁻ M12.C3 cells (13) by electroporation. Transfectants were selected for resistance to G418 and subsequently cloned. The I-A⁺ clones were analyzed for reactivity with the panel of I-A^k-reactive mAbs by immunofluorescence on a flow cytometer. Fig. 3 shows representative data from a comparison of the parent TA3 cells, the original F16 mutant cells, and the M12.C3 cells transfected with the A^k_β gene from F16 and the wild-type A^k_α gene (Tf.F16). No differences were detected between the original F16 mutant cells and Tf.F16 using any of the antibodies in the panel. In addition, we have obtained completely concordant serologic results with each of the other A^k_β mutants and their respective transfectants (data not shown). These results indicate that the substitutions in the A^k_β genes detected by DNA sequence analysis are responsible for the mutant serologic phenotypes.

Discussion

A comparison of the predicted amino acid sequences based on either gene or cDNA sequences (9, 18) of seven A_β alleles reveals four clusters of variable
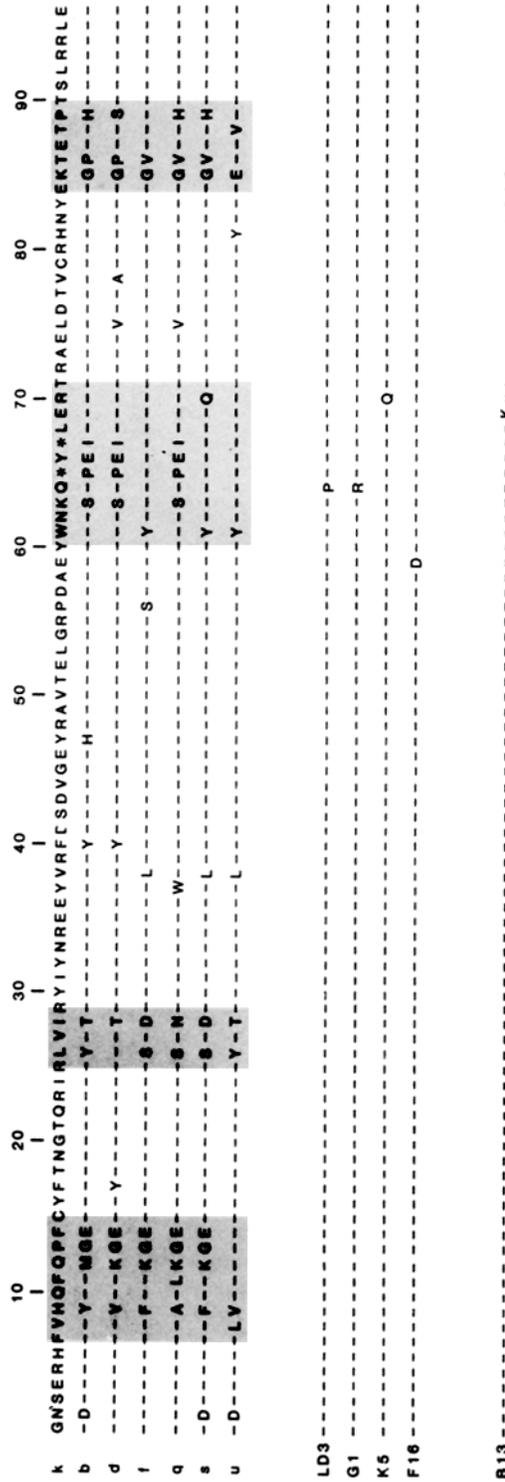
FIGURE 2. Comparison of the β_1 domain amino acid sequences of seven A$_\beta$ alleles (9, 18) and the four in vitro serologically selected A$_\beta$ mutants described in this paper and the B13 mutant derived by Glimcher et al. (20) and sequenced by Brown et al. (5). The residues are numbered according to the positions in the b, d, and q haplotypes and will be so referred to in the text. The k, f, s, and u alleles each have a deletion of two residues in the region 65–67. The shaded areas indicate the variable regions that can be defined by allelic comparisons. The codon substitutions in the four in vitro mutants described in this paper are: LD3, CAG → CCG; G1, CAG → CGG; K5, CGA → CAA; and F16, GAG → GAT.
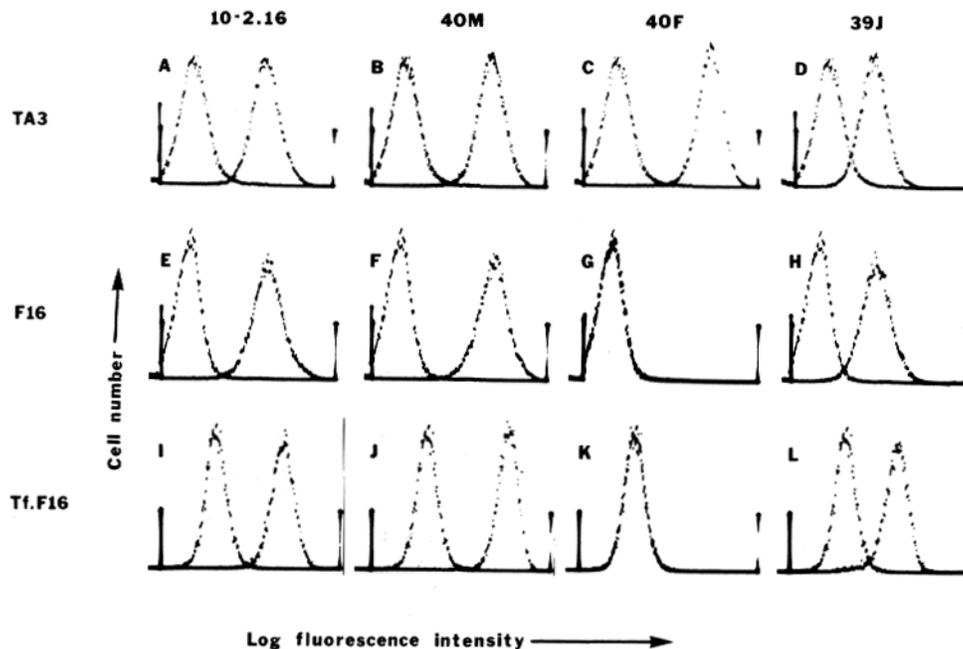
FIGURE 3.   Immunofluorescence analysis of TA3, F16, and Tf.F16. TA3 (A–D), F16 (E–H), and Tf.F16 (I–L) were stained with the indicated mAb as previously described (1), and with FITC–protein A. The left peak in each panel is the fluorescence profile of each population stained with the control mAb and FITC–protein A. Antibodies 10-2.16, 40M, and 40F detect A$_\beta^k$ epitopes and antibody 39J detects an A$_\alpha^k$ epitope.

residues in the β_1 domain (Fig. 2). The distribution among these clusters of the substitutions detected in the four serologically selected A$_\beta^k$ mutants LD3, G1, K5, and F16, is quite striking. All of the substitutions fall near or within the third of the four clusters of allelicly variable residues. Since the four mutants analyzed have among them sustained alterations such that the reactivity with all but 1 of the panel of 10 A$_\beta^k$-reactive mAbs has been affected, it was surprising to find that the mutations are clustered into only one of the four variable regions. This observation strongly suggests that this region of the molecule is serologically dominant. Consistent with this observation are the results previously reported on the sequence analysis of the A$_\beta^k$ mutant B13 (5) (also derived in vitro from TA3) and the A$_\beta^b$ spontaneous in vivo mutant B6.C-H-2^{bm12} (bm12) (19). B13 was independently derived by Glimcher et al. (20) using a selection scheme similar to that used for LD3. The LD3 and B13 mutants are serologically indistinguishable except for their reactivities with mAb 4-2.3 (our unpublished data). B13 has a Glu to Lys substitution at position 69 (5). The A$_\beta^{bm12}$ polypeptide has three amino acid substitutions at positions 67, 70, and 71 (19). Also consistent with the notion that this region is serologically dominant are the results reported in a study of three site-directed mutants. Cohn et al. (21) created three A$_\beta^b$ mutants in which they substituted the k residues for the b residues at amino acid position 9 or 13 in the first allelicly defined variable region, or replaced the b residues at

positions 65, 66, 67 (in the third allelicly variable region) with the k haplotype tyrosine. (The A_β^k polypeptide has a deletion of two residues in this group of three). Neither of the substitutions in the first variable region changes the serologic reactivity of the molecule, whereas the alteration of residues 65–67 leads to the failure of the molecule to be recognized by any of the three A_β^b-reactive mAbs tested. Thus, all of the available data suggest that the region of the polypeptide encompassing the variable residues 61–70 is the major determinant of the serologic alloantigenicity.

Attempts have been made to assign alloantigenic epitopes to particular amino acid residues by comparing allelic sequences and allospecificity patterns. However, several observations suggest that the structure of alloantigenic epitopes is complex and that there is not a simple one-to-one correspondence between alloantigenic epitopes and polymorphic residues. Landais et al. (22) compared the serology and sequence of A_α polypeptides and predicted that the allospecificities Ia.2 and Ia.19 would be mapped to Arg-57 and Glu-75, respectively, of the A_α^k polypeptide. Subsequent analysis of A_α^k site-directed mutants and in vitro-selected mutants revealed that both Ia.2 and Ia.19 are determined in large part by Glu-75, but also, that in the presence of Glu-75, the alteration of two other residues can result in the loss of both the Ia.2 and Ia.19 epitopes (23). Clearly, the structure of the Ia.2 and Ia.19 epitopes is determined by the interaction of several neighboring residues. Of the group of five serologically selected A_β^k mutants (four described in this report and the B13 mutant described by Glimcher et al. [20]), only one (K5) has a substitution at a residue that is polymorphic among the seven sequenced A_β alleles. However, these A_β^k substitutions are all clustered in a region of the polypeptide that displays considerable allelic variability, indicating the importance of this region in determining the alloantigenicity of the polypeptide. The complexity of the serologic epitopes is further revealed by the observation that reactivity with several of the mAbs can be altered by single substitutions at two or more positions. Reactivity with the mAb 10-2.16 (Ia.17) can be altered by substitution at position 64 (LD3 and G1) or at position 69 (B13). Reactivity with the mAbs of allospecificity Ia.1 has been affected by substitution at 64 (LD3), 69 (B13), or 70 (K5). Reactivity with mAb 4-2.3 (Ia.18) has been affected by substitution at 59 (F16) or 64 (LD3). Furthermore, in three mutants, more than one allodeterminant has been altered by the single amino acid substitution. In LD3, Ia.17, Ia.18, and Ia.1 have been altered; in B13, Ia.17 and Ia.1; and in F16, Ia.18 and the 40F, 40L epitope. Thus, it is clear that an alloantigenic epitope is created by the interaction of several residues. In addition, our data indicate that serologically distinct epitopes may overlap, perhaps by sharing some contact residues. Alternatively, substitutions in this one region may induce conformational alterations of distinct epitopes either locally or at a distance. The selection scheme used to produce each of these mutants relied on random chemical mutagenesis followed by selection against one of several serologic epitopes. It seems unlikely that we would recover multiple independent substitutions only in the third allelicly variable region if the major effect of these substitutions were the induction of conformational changes at distant epitopes on the molecule. Thus, we favor the interpretation that the serologic epitopes detected by our panel of mAbs are determined largely by the region of the

polypeptide encompassing the third allelicly variable segment, and that the substitutions that have occurred in the mutant cell lines either have occurred at contact residues and/or have caused local conformational changes.

The effect of the various amino acid substitutions on the ability of the cell lines expressing the mutant molecules to stimulate T cells appears to be more complex than the effect on antibody binding. The four mutants described in this paper exhibit a range of functional phenotypes from nearly wild-type (F16 and G1) to nearly completely defective (K5) (Table II, reference 1, and Rosloniec, E., J. Freed, B. N. Beck, D. J. McKean, unpublished data). As yet, no pattern of reactivities has emerged that correlates with either the structural data or the serologic phenotypes. The limited functional alterations exhibited by the F16 cells may be due to the chemically conservative nature of the substitution in this mutant (Glu to Asp). Although both the LD3 and G1 mutants have substitutions at residue 64, LD3 cells fail to stimulate approximately half of the T hybrids tested, while G1 cells stimulate nearly all. The LD3 Gln to Pro substitution might be expected to have significant impact on the secondary structure of the poly-peptide due to the imide bond introduced by the proline residue. The K5 substitution has the most dramatic effect on T cell activation: only 2 of the 23 T hybrids assayed are stimulated by these mutant cells. The K5 substitution at residue 70 (Arg to Gln) appears to be in an important location (directly or indirectly) for T cell antigen recognition. The T hybrid panel used for this analysis largely comprised antigen-reactive and autoreactive T cells (1). A detailed evaluation of the ability of the mutants to activate a large panel of T hybrids with specificity for either a defined peptide antigen or for the A^k molecule as alloantigen has not yet been completed. In a study of the T cell response to HEL, using a panel of 10 I-A^k-restricted HEL-specific T hybridomas and a panel of 8 serologically selected A$_\beta^k$ and A$_\alpha^k$ mutant cell lines, five T hybridomas specific for the HEL peptide 46–61 exhibited four different patterns of stimulation by the mutant cell lines (24). These data suggest that multiple determinants can be formed by the association of even a sixteen-residue peptide and the I-A^k molecule, indirectly indicating the existence of several T cell recognition sites on the I-A^k molecule. Several earlier studies (25–28) had shown that the *bm12* mutation (at residues 67, 70, and 71 of the A$_\beta^b$ polypeptide) had a substantial impact on antigen-presenting capability and in vivo immune responses. Direct comparisons of the ability of B6 and *bm12* cells to present antigen to either B6- or *bm12*-derived T cell clones indirectly indicated the existence of at least two T cell recognition sites on the I-A^b molecule (29, 30). Functional studies of a limited number of site-directed A$_\beta^b$ mutants (20) have revealed that even those substitutions that failed to alter serologic reactivity of the molecule did affect the ability of some T cells to recognize the molecule. Lechler et al. (31) have examined the separate contributions of the NH$_2$- and COOH-terminal halves of the A$_{\beta 1}$ domain to the formation of epitopes required for recognition of antigen by a panel of I-A-restricted T hybridomas. They found that both halves of the A$_{\beta 1}$ domain influenced the antigen-presentation capability of the I-A molecule. Thus, it appears either that recognition by T cells is more sensitive to structural alterations than recognition by antibodies or that at least some T cell epitopes comprised portions of the I-A molecule not directly involved in recognition by antibodies.

The absence of x-ray crystallographic and other physicochemical data on the secondary and tertiary structure of MHC class II molecules limits the scope of the inferences that can be drawn on the effects of these mutations on the structure and function of the I-A molecule. Analysis of these serologically selected mutants has demonstrated that the region encompassing positions 61–70 of the A_β^k polypeptide is serologically dominant. Substitutions in this region also can have dramatic effects on the ability of the molecule to be recognized by T cells. Studies of variant cell lines generated by site-directed mutagenesis and DNA-mediated gene transfer will be useful to examine the extent to which the other allelically variable regions are involved in determining either serologically or functionally important sites on the I-A molecule.

Summary

We have produced a series of in vitro serologically selected cell lines that express mutant I-A^k molecules. In this report we describe the DNA sequence analysis of the A_β^k gene of four cell lines that express serologically altered A_β^k polypeptides in association with wild-type A_α^k polypeptides. Each of the major serologic epitopes on the A_β^k polypeptide has been altered in one or more of the four mutants. In addition, the four mutants exhibit a broad spectrum of functional defects when used to stimulate a panel of T hybridomas of various specificities. The DNA sequence analysis revealed that each mutant had sustained a single nucleotide substitution resulting in a single amino acid substitution. All four independent substitutions occurred within or near the third of the four variable regions defined in the β_1 domain of the A_β polypeptide by allelic comparisons. These data strongly suggest that the third variable region is the major determinant of alloantigenicity on the A_β^k polypeptide.

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