# IDENTIFICATION OF AN IMMUNODOMINANT REGION ON THE I-A $\beta$ CHAIN USING SITE-DIRECTED MUTAGENESIS AND DNA-MEDIATED GENE TRANSFER

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Class II MHC molecules (Ia) are transmembrane glycoproteins that function as cell recognition structures during the initiation of an antigen-specific immune response (1). T lymphocytes of the helper–inducer lineage (Th cells) express receptors that recognize foreign antigens in association with the Ia glycoproteins. The binding of this complex ligand to the TCR is required for the activation of Th cells, an essential step in the promotion of antibody and cell mediated immune responses. Although the sequences of many Ia alleles and TCR molecules are now known (2, 3), the molecular details of the interaction that occurs between Ia, foreign antigen and the T cell receptor remains an enigma.

The murine Ia molecules, designated A ($A_\alpha$ $A_\beta$) and E ($E_\alpha$ $E_\beta$), are transmembrane glycoproteins, each consisting of a 33-kD $\alpha$ chain noncovalently associated with a 29-kD $\beta$ chain. Both the $\alpha$ and the $\beta$ chains contain two extracellular domains, designated $\alpha_1$ and $\alpha_2$, $\beta_1$ and $\beta_2$, respectively (4). Sequence analysis of allelic $\alpha$ and $\beta$ chain genes revealed that most polymorphic residues are located in the $NH_2$-terminal $\alpha_1$ and $\beta_1$ domains (5–8). These polymorphic residues, which tend to be clustered in three or four regions of the primary structure of the $NH_2$-terminal domains, are believed to be responsible for determining allele specific antibody binding sites and T cell recognition properties of the Ia molecules (9–11).

Allospecific antisera and mAbs directed against the Ia glycoproteins have been important tools for the characterization of MHC alleles (HLA typing in the human), H-2 recombinant mouse strains, and the biochemical purification of Ia molecules (12–14). Since these reagents specifically block the activation of T cells in antigen presentation assays, they have been used for evaluating Ia structure–function relationships (15). Recently, there has been increasing interest in the use of anti-Ia mAbs in an attempt to modulate the immune system and as therapeutic reagents in certain autoimmune diseases (16–18). Thus, the identification of residues comprising the Ia antigenic determinants will contribute to the identification of the functionally important regions on the Ia molecule.

Several advances have been made toward identifying antibody binding sites

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on Ia antigens. The chain specificity of anti-Ia mAbs and the relationship of different antibody binding sites have been addressed in the analysis of in vitro immunoselected Ia bearing cell lines and L cells transfected with Ia genes (19–23). Exon-shuffling experiments further mapped the binding sites of the various $A^k_\beta$-reactive antibodies to the $\beta_1$-domain of the $A^k_\beta$ polypeptide (9). Recently, antibody binding sites within the $\alpha_1$ and $\beta_1$ domains were investigated through the use of cell lines expressing genes that contained a limited number of mutations introduced by site-directed mutagenesis (11, 24, 25). These results, together with the sequence analysis of the in vivo, spontaneous mutant bm12 (26), suggested that the residues in the polymorphic region around position 66 in the $\beta_1$ domain were involved in determining antibody binding sites on the Ia molecule.

We have undertaken a comprehensive analysis of the structural basis of antibody binding sites on the $\beta_1$ domain of the $A^k_\beta$ and $A^d_\beta$ polypeptides. We constructed variant $A^k_\beta$ genes that encoded single or multiple residues of the $A^d_\beta$ polypeptide at 14 polymorphic positions in the $\beta_1$ domain. The mutant $\beta$ chain polypeptides were expressed in combination with either the $A^d_\alpha$ or $A^k_\alpha$ polypeptides after transfection of genes into the B lymphoma cell line M12.C3. Analysis of the transfected cell lines using a panel of $A^k_\beta$- and $A^d_\beta$-reactive mAbs has enabled us to identify the polymorphic residues that are involved in determining these antibody binding sites.

## Materials and Methods

*Oligonucleotides.* The following oligonucleotides were used for mutagenesis: 5 CATTTCGTGGTCCAGTTC 3 (pos.9); 5 CACCAGTTCAAGCCC 3 (pos.12); 5 GTTCCAGGGCTTCTG 3 (pos.13); 5 CCAGCCCGAGTGCTACT 3 (pos.14); 5 TGCTACTACACCAACGG 3 (pos.17); 5 (CTTCTGACCAGATACA 3 (pos.28); 5 TGCGCTACGACAGC 3 (pos.40); 5 ACTGGAATAGCCAGTACC 3 (pos.63); 5 GGAATAAGCAGCCGGAGATCCTGGAGCGAA 3 (pos. 65–67); 5 GGCCGAGGTGGACACG 3 (pos.75); 5 GACACGGCGTGCAGAC 3 (pos.78); 5 CTxCGAGGGGACGGAG 3 (pos.85); 5 CGAGAAGCCGAGA 3 (pos.86); 5 GAGACCAGCACCTCC 3 (pos.89); 5 TTTCGTGGTCCAGTTCAAGGCGAGTGCTACTACACCAAC 3 (region A); 5 ACTGGAATAGCCAGCCGGAGATCCTGGAGCGA 3 (region B); 5 GGGCCGAGGTGGACACGGCGTGCAGACA 3 (region C); 5 CAACTACGAGGGGCCGGAGACCAGCACCTCCCT 3 (region D). The oligonucleotide intended to change position 87 and region A were purchased from SYNTEK AB, Umea, Sweden, and used without further purification. All other oligonucleotides were synthesized with a DNA synthesizer (model 380A; Applied Biosystems, Inc., Foster City, CA) using the phosphoramidite method and were purified by polyacrylamide gel electrophoresis.

*Mutagenesis.* The method for site-directed mutagenesis used in this study was described previously (27). The second exon of the $A^k_\beta$ gene was cloned into M13 mp10am (Pharmacia Fine Chemicals, Piscataway, NJ) after a partial digestion of Cos. 1.1 (kindly provided by Dr. Lee Hood, California Institute of Technology, Pasadena, CA) with the endonuclease Xcy I. The M13 clone contained a 731-bp insert with two Sst II endonuclease restriction sites closely flanking the second exon. The heteroduplex was formed using 2 $\mu$g of SSDNA from this clone together with 0.5 $\mu$g of double-stranded M13 mp10wild (Amersham Corp., Arlington Heights, IL) that was double digested with the endonucleases Eco RI and Sma I in the polylinker region. 2–30 nmol of each oligonucleotide were used to introduce mutations and up to four different oligonucleotides were annealed to the heteroduplex in a single reaction. Filters were lifted from plates containing foci of infected MK-30 su-bacteria (all the bacterial strains required for the experiments were generously provided by Dr. Hans-Joachim Fritz, Max-Planck Institute for Biochemistry, Martinsried,

Federal Republic of Germany) and were hybridized with the mutagenic oligonucleotides. M13 clones showing preferential hybridization were expanded and their entire second exon of the $A_\beta$ gene was sequenced by the dideoxy chain termination method (28).

*Construction of Mutant $A^k_\beta$ Genes.* Replicative form of M13 clones containing the desired mutations was digested with endonuclease Sst II and ligated into an $A^k_\beta$ gene construct that lacked the Sst II fragment. The $A^k_\beta$ gene was originally cloned from the Cos.1.1 into puc 18 as a 12-kb Hind III/Hpa I fragment. Recombinant bacterial colonies were screened by an oligonucleotide that covers the Sst II site 5′ to the second exon. The uptake of only one Sst II second exon fragment was confirmed by a partial restriction map of the isolated plasmids with endonuclease Sma I. Before transfection preferential hybridization of the mutant $A_\beta$ gene constructs with the mutagenic oligonucleotides was checked by Southern blot analysis. The $A^{d/k}_\beta$ gene was constructed by ligating the second exon of $A^d_\beta$ contained in pCA12 (provided by Dr. Lee Hood, California Institute of Technology) into an $A^k_\beta$ gene construct that had the second exon deleted by Sst II digestion.

*DNA-mediated Gene Transfer of M12.C3 Cells.* The M12.C3 cell line, which was given to us by Dr. Laurie Glimcher, Harvard University, Cambridge, MA, was grown in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.1 mM 2-ME and buffered to pH 7.3 with 10 mM Hepes. 5–10 × $10^6$ cells were mixed with 40 μg of pRSVneo plasmid (29) that was linearized with the endonuclease Bam HI, 25 μg of mutant $A_\beta$ gene plasmid, and 40 μg of $A^k_\alpha$ gene plasmid (both linearized with the endonuclease Hind III) in 0.6 ml medium containing 140 mM NaCl, 25 mM Hepes, 0.75 mM $Na_2HPO_4$, pH 7.4 (the $A^k_\alpha$ gene, a gift from Dr. Laurie Glimcher, was recloned into puc 18 as a Hind III fragment). Electroporation (30, 31) was carried out at 480 V/cm using the PDS Model ZA 1000 (Prototype Design Services, Madison, WI). The treated cells were resuspended in 12 ml of the culture medium described above and distributed to 12 wells of a 24-well Costar (Cambridge, MA) plate. After 16 hr, 1 ml selective medium (culture medium plus 600 μg/ml G-418 (Gibco Laboratories) was added to each well. 2–3 wk later G-418 resistant cells were subcloned by limiting dilution. Subclones were screened using a cell ELISA (19) with mAbs reactive to $A^k_\beta$ or $A^d_\beta$. Positive subclones were expanded for further analysis.

*Quantitative Immunofluorescence.* The mAbs used are listed in Tables I and IV. The staining procedure has been described previously (19). All antibodies were used in form of culture supernatants. FITC–protein A (Pharmacia Fine Chemicals) was the developing reagent for IgG mAbs, and FITC–goat anti–mouse IgM (Meloy Laboratories Inc., Springfield, VA) for IgM mAbs. Control antibodies of the appropriate isotype were used to quantitate nonspecific binding. Cytofluorometric analyses were measured on a logarithmic scale and peak channel values were subsequently converted to linear values for calculating comparative immunofluorescence values.

## Results

The $A^k_\beta$ and $A^d_\beta$ polypeptides differ from each other by single amino acids at 13 positions in the $\beta_1$ domain. In addition, the $A^d_\beta$ polypeptide contains three amino acids at positions 65, 66, and 67 (designated 65–67) compared with a single amino acid present in the $A^k_\beta$ polypeptide (Fig. 1 *a*). Using site-directed mutagenesis, we separately introduced codons characteristic of the d allele into the $A^k_\beta$ gene sequence at each of the polymorphic positions (Fig. 1 *b*). The DNA sequence of the second exon, which encodes residues 6–96 of the $\beta_1$ domain was determined for each of the mutant genes. The mutant $A^k_\beta$ genes were cotransfected with the wild-type $A^k_\alpha$ gene and a neomycin resistance gene into the B lymphoma line M12.C3. As described previously, this B lymphoma line does not express its endogeneous $A^d$ molecule on the cell surface (32). The lack of $A^d$ expression results from the absence of $A^d_\beta$ mRNA, although functional $A^d_\alpha$ mRNA

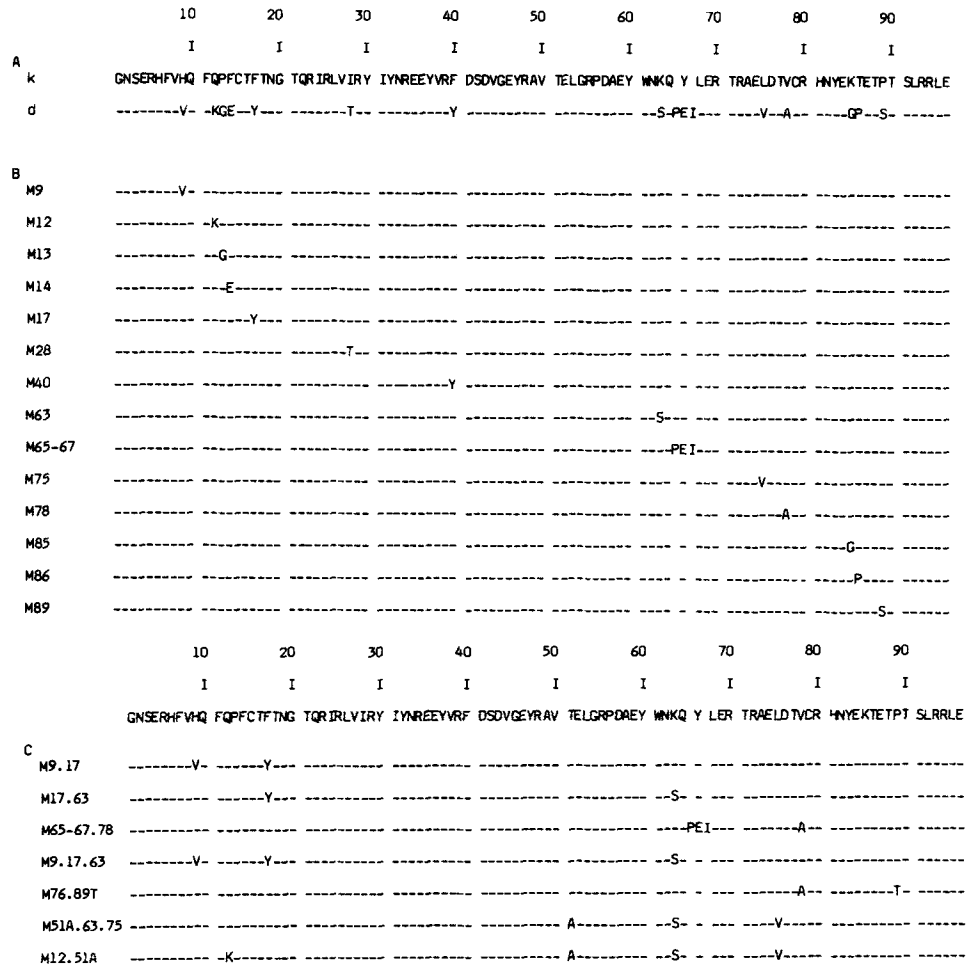


FIGURE 1. Amino acid sequences of the $\beta_1$ domain encoded by the wild-type $A^k_\beta$ and $A^d_\beta$ alleles and mutant $A^k_\beta$ genes (6, 8). (*A*) Sequence of the $\beta_1$ domains of the $A^k_\beta$ and $A^d_\beta$ polypeptides as deduced from the nucleotide sequences of the corresponding genes. (*B*) Sequences of the $\beta_1$ domains of the mutant $A^k_\beta$ polypeptides containing residues of the $A^d_\beta$ polypeptide at single positions. (*C*) Sequences of the $\beta_1$ domains of mutant $A^k_\beta$ polypeptides containing residues of the $A^d_\beta$ polypeptide at multiple positions. The three sequences M76.89T, M51A.63.75, and M12.51A.63.75 contain one additional substitution characteristic of neither the $A^k_\beta$ or $A^d_\beta$ polypeptide. The assignment of amino acid positions was based on the sequence of the $\beta_1$ domain of the $A^d_\beta$ polypeptide, which is two amino acids longer than the $\beta_1$ domain of the $A^k_\beta$ polypeptide.

is present. After transfection, neomycin (G-418)–resistant bulk cell populations were cloned by limiting dilution. Individual clones were tested by cytofluorometric analysis to obtain cell lines that expressed similar levels of the mutant $A^k_\beta$ polypeptide with the $A^k_\alpha$ polypeptide on the cell surface. Since the $A^k_\alpha$ and $A^k_\beta$ polypeptides preferentially associate with each other transfected M12.C3 cells expressing the $A^k_\beta$ polypeptide and high levels of $A^k_\alpha$ polypeptide express relatively small amounts of the $A^d_\alpha$ $A^k_\beta$ hybrid molecules. In contrast, cells transfected with

TABLE I

*Properties of $A^{k}_{\beta}$-reactive mAbs*

| mAb | Crossreactivity | Strain combination | Reference |
|---|---|---|---|
| 39B | I-$A^{f,r,u}$ (Ia.1) | A.TH anti-A.TL | 33 |
| 39E | I-$A^{f,r,u}$ (Ia.1) | A.TH anti-A.TL | 33 |
| 40M | I-$A^{f,r,u}$ (Ia.1) | A.TH anti-A.TL | 33 |
| 10-2.16 | I-$A^{f,r,s}$ (Ia.17) | CWB anti-C3H | 34 |
| 11-3.25 | I-$A^{f,r,s}$ (Ia.17) | Balb/c anti-CKB | 34 |
| 4-2.1 | I-$A^{f,r,s,j}$ (Ia.17) | B10.P anti-B10.M | 35 |
| 4-2.3 | I-$A^{f,s,j}$ (Ia.18) | B10.P anti-B10.M | 35 |
| 40F | I-$A^{f,u}$ | A.TH anti-A.TL | 33 |

Chain specificity was determined previously (15, 19).

TABLE II

*Cytofluorometric Analysis of M12.C3 Cell Lines Transfected with Mutant $A^{k}_{\beta}$ Genes that Encode Residues of the $A^{d}_{\beta}$ Polypeptide at Single Positions in the $\beta_1$ domain*

| mAb | Cell lines | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | T. Neo* | T.$A^{k}$‡ | T9 | T12 | T13 | T14 | T17 | T28 | T40 | T63 | T65–67 | T75 | T78 | T85 | T86 | T89 |
| 39B | – | +++ | +++ | +++ | +++ | ++ | +++ | +++ | +++ | +++ | – | +++ | +++ | ++ | ++ | +++ |
| 39E | – | +++ | +++ | +++ | +++ | ++ | ++ | +++ | +++ | +++ | – | +++ | +++ | +++ | +++ | +++ |
| 40M | – | +++ | +++ | +++ | +++ | ++ | +++ | +++ | +++ | +++ | – | +++ | +++ | +++ | +++ | +++ |
| 10.2-16 | – | +++ | +++ | +++ | +++ | ++ | ++ | +++ | ++ | +++ | – | +++ | +++ | +++ | ++ | +++ |
| 11-3.25 | – | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | – | +++ | +++ | +++ | +++ | +++ |
| 4-2.1 | – | +++ | +++ | +++ | +++ | ++ | ++ | +++ | ++ | +++ | – | +++ | +++ | +++ | +++ | +++ |
| 4-2.3 | – | +++ | +++ | +++ | ++ | ++ | +++ | +++ | +++ | +++ | – | N.D. | +++ | ++ | +++ | +++ |
| 40F | – | +++ | +++ | +++ | +++ | ++ | +++ | +++ | +++ | – | +++ | +++ | +++ | +++ | +++ | +++ |
| 39J§ | – | +++ | +++ | ++ | + | ++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| K24-199‖ | – | + | – | ++ | + | – | – | – | – | + | – | – | – | – | – | – |

The transfected cell lines were divided into four categories based upon levels of antibody binding relative to the wild-type TA3 cell line (20). The peak fluorescence channel difference between cells stained with the negative antibody and cells stained with the antibody in question was divided through the peak channel difference obtained for the TA3 cell line with the same antibodies in the same experiment and then multiplied by 100. (–) Values <5%; (+) values 5–25%; (++) values 26–75%; (+++) values >75%.

* Cell line transfected only with the neomycin resistance gene.

‡ Cell line transfected with the wild-type $A^{k}_{\alpha}$ and $A^{k}_{\beta}$ gene.

§ 39J recognizes the $A^{k}_{\alpha}$ polypeptide (19).

‖ K24-199 recognizes the $A^{d}_{\alpha}$ polypeptide.

the $A^{k}_{\beta}$ gene alone express high levels of the $A^{d}_{\alpha}$ $A^{k}_{\beta}$ hybrid molecules (data not shown).

*Loss of $A^{k}_{\beta}$* Epitopes. A panel of mAbs raised in different strain combinations or showing different patterns of crossreactivity (Table I) was selected for this analysis. The diverse origin of the antibodies was intended to maximize the number of potentially different antibody binding sites that could be investigated. As shown in Table II, the $A^{k}_{\beta}$-reactive antibodies did not bind to cells transfected with only the neomycin resistance gene (T.Neo), but did bind to cells transfected with the wild-type $A^{k}_{\beta}$ gene together with the $A^{k}_{\alpha}$ gene (T.$A^{k}$). Among the 14 cell lines that were transfected with mutant $A_{\beta}$ chain genes, only those two that expressed $A^{k}_{\beta}$ polypeptides containing amino acids of the $A^{d}_{\beta}$ polypeptide at positions 63 or 65–67 showed complete loss of antibody binding. The substitution of serine for lysine at position 63 in the $A^{k}_{\beta}$ polypeptide eliminated binding of

TABLE III

*Cytofluorometric Analysis of M12.C3 Cell Lines Transfected with Mutant $A^{k}_{\beta}$ Genes that Encode Residues of the $A^{d}_{\beta}$ Polypeptide at Multiple Positions in the $\beta_1$ Domain*

| mAbs | Cell lines | | | | | | |
|---|---|---|---|---|---|---|---|
| | T9.17 | T17.63 | T65–67.78 | T9.17.63 | T78.87.T | T51A.63.75 | T12.51A.63.75 |
| 39B | +++ | ++ | – | +++ | +++ | +++ | +++ |
| 39E | +++ | ++ | – | ND | +++ | +++ | ND |
| 40M | +++ | +++ | – | +++ | +++ | +++ | +++ |
| 10.2–16 | +++ | ++ | – | +++ | +++ | +++ | +++ |
| 11-3.25 | +++ | +++ | – | +++ | +++ | +++ | +++ |
| 4-2.1 | +++ | +++ | – | +++ | +++ | +++ | +++ |
| 4-2.3 | +++ | ++ | – | +++ | +++ | +++ | +++ |
| 40F | +++ | – | ++ | – | +++ | – | – |
| 39J* | +++ | +++ | +++ | +++ | +++ | +++ | +++ |

The TA3 cell line was used as a standard (see under Fig. 1).

* 39J recognizes the $A^{x}_{\alpha}$ polypeptide.

antibody 4OF, whereas the substitution of the three amino acids proline, glutamic acid, and isoleucine for a single tyrosine at position 66 in the $A^{k}_{\beta}$ polypeptide eliminated the binding of those antibodies that define the allospecificities Ia.1, Ia.17, and Ia.18 (mAbs 39B, 39E, 40M, 10-2.16, 11-3.25, 4-2.1, 4-2.3). These cytofluorometric analyses, which used saturating amounts of anti-Ia antibodies, were designed to evaluate relative expression levels of cell surface Ia molecules. Additional analyses will be needed to determine if alterations in antibody binding affinities have been introduced by these mutations in the $\beta_1$ domain. One of the mutant clones (T14) expresses lower levels of $A^{k}$ molecules on the cell surface than the TA3 cell line (Table II). Cytofluorometric analysis of the T14 bulk cell population indicated this mutation did not result in significant qualitative alteration of the $A^{k}$ molecule and the $A^{k}$ expression level on the clone is not representative of most of the cells in the bulk population.

*Multiple Amino Acid Alterations in the $\beta_1$ Domain of the $A^{k}_{\beta}$* Polypeptide. The technique of site-directed mutagenesis used in this study allowed us to simultaneously obtain mutations at several positions within the second exon of the $A^{k}_{\beta}$ gene by annealing multiple oligonucleotides in the same experiment. The $\beta_1$ domain amino acid sequences encoded by these mutant genes are shown in Fig. 1*c*. Three genes in this series (*M12.51A.63.78*, *M51A.63.78*, and *M78.89T*) contained unintended nucleotide substitutions that changed the codons at positions 51 and 89 to condons found in neither the d nor the k allele. Cell lines expressing the genes that encode changes at multiple positions in the $\beta_1$ domain were stained with $A^{k}_{\beta}$-reactive mAbs (Table III). Only those cell lines that expressed $A_{\beta}$ polypeptides containing amino acids characteristic of the $A^{d}_{\beta}$ polypeptide at position 63 or 65–67 exhibited loss of binding with $A^{k}_{\beta}$-reactive antibodies. These results support the previous finding that among the residues that differ between the $A^{k}_{\beta}$ and $A^{d}_{\beta}$ polypeptide only the residues at positions 63 and 65–67 determine the binding sites of the tested $A^{k}_{\beta}$-reactive antibodies.

*Regional Changes in the $\beta_1$ Domain of the $A^{k}_{\beta}$* Polypeptide. The polymorphic residues within the $\beta_1$ domain of the $A^{d}_{\beta}$ and $A^{k}_{\beta}$ polypeptide cluster in stretches

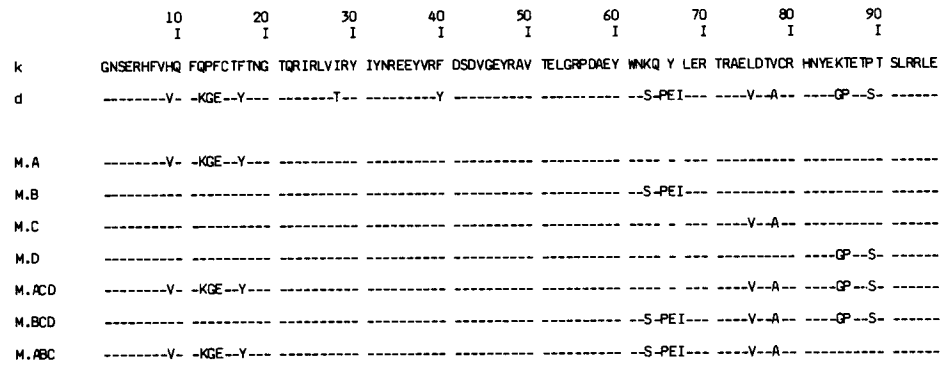


FIGURE 2. Sequences of the $\beta_1$ domain of the $A^k_\beta$ and $A^d_\beta$ and of mutant $A^k_\beta$ polypeptides containing changes of polymorphic regions.

of the primary sequence (Fig. 2*a*). Based on this pattern, we divided the $\beta_1$ domain into four regions of pronounced interallelic variability. The regions (Fig. 2*a*) encompass residues at position 9–17 (region ··A··), 63–67 (region ··B··), 75–78 (region ··C··), and 85–89 (region ··D··). Sequences encoding the amino acids of the $A^d_\beta$ polypeptide in these four regions were introduced into the $A^k_\beta$ gene by site-directed mutagenesis. Oligonucleotides corresponding to each of the four regions were used simultaneously in a single mutagenesis reaction and allowed the isolation of clones containing single as well as multiple mutations (Fig. 2*b*).

Transfection of the wild-type $A^k_\alpha$ gene together with mutant $A^k_\beta$ genes that encode region ··A·· of the $A^d_\beta$ polypeptide did not result in cell surface expression of Ia molecules containing the $A^k_\alpha$ polypeptide as determined by quantitative immunofluorescence analysis with $A^k_\alpha$-reactive mAbs (data not shown). Staining of the transfected cells with an $A^d_\alpha$-reactive mAb (K24-199) revealed that the mutant $A^k_\beta$ polypeptides containing substitutions characteristic of the d allele in region ··A·· were expressed on the cell surface in association with the endogenous $A^d_\alpha$ polypeptide of the M12.C3 cell line (Table IV). This finding is in agreement with previous reports that the $A^d_\beta$ polypeptide does not associate with the $A^k_\alpha$ polypeptide (36, 37) and that a region controlling $\alpha/\beta$ chain association maps to the $NH_2$-terminal half of the $\beta_1$ domain (10, 38).

Cell lines expressing the mutant $A^k_\beta$ polypeptides with regional changes in combination with either the $A^k_\alpha$ polypeptide or, in the case of a substitution of region A, in combination with the $A^d_\alpha$ polypeptide, were stained with the antibodies reactive to $A^k_\beta$ (Table IV). All cell lines expressing $A^k_\beta$ genes that encode region B of the $A^d_\beta$ polypeptide did not react with any $A^k_\beta$-reactive antibodies. Also of particular interest is the finding that the cell line expressing the gene that encodes regions ACD of the $A^d_\beta$ polypeptide retained binding of all $A^k_\beta$-reactive antibodies. Substitutions of amino acids of the $A^d_\beta$ polypeptide at positions 28 and 40 also do not influence binding of the $A^k_\beta$ reactive mAbs (Table II). Together these findings clearly demonstrate immunodominance of the region B of the $A^k_\beta$ polypeptide, showing the $A^k_\beta$ antibody binding sites are maintained despite substitutions of all $A^d_\beta$ characteristic residues in the $\beta_1$ domain outside of region B.

TABLE IV

*Cytofluorometric Analysis of M12.C3 Cell Lines Transfected with Mutant $A^{x}_{\beta}$ Genes that Encode Single or Multiple Regions of the $A^{d}_{\beta}$ Polypeptide in the $\beta_1$ Domain*

| mAbs | Cell lines | | | | | | |
|---|---|---|---|---|---|---|---|
| | T.A. | T.B. | T.C. | T.D. | T.ABC | T.ACD | T.BCD |
| 39B | +++ | − | +++ | +++ | − | +++ | − |
| 39E | +++ | − | +++ | +++ | − | +++ | − |
| 40M | +++ | − | +++ | +++ | − | +++ | − |
| 10.2-16 | +++ | − | +++ | +++ | − | +++ | − |
| 11-3.25 | +++ | − | +++ | +++ | − | +++ | − |
| 4-2.1 | +++ | − | +++ | +++ | − | +++ | − |
| 4-2.3 | +++ | − | +++ | +++ | − | +++ | − |
| 40F | +++ | − | +++ | +++ | − | +++ | − |
| 39J* | − | +++ | +++ | +++ | − | − | +++ |
| K24-199‡ | +++ | + | − | − | +++ | +++ | − |

The TA3 cell line was used as a standard (see under Fig. 1).
* 39J recognizes the $A^{x}_{\alpha}$ polypeptide.
‡ K24-199 recognizes the $A^{d}_{\alpha}$ polypeptide.

*Gain of $A^{d}_{\beta}$* Epitopes. To investigate the possibility that antibody binding sites characteristic of the d allele have been introduced by the substitution of $A^{d}_{\beta}$ residues into the $A^{k}_{\beta}$ polypeptide, eight different $A^{d}_{\beta}$-reactive antibodies were tested for binding to the panel of transfected cell lines. Antibodies derived from different strain combinations or showing different patterns of crossreactivity were included (Table V). None of the $A^{d}_{\beta}$-reactive antibodies stained M12.C3 cells expressing the $A^{k}_{\beta}$ polypeptide in combination with either the $A^{k}_{\alpha}$ or $A^{d}_{\alpha}$ polypeptide (designated T.$A^{k}$ and T.$A^{k}_{\beta}$, respectively, Table VI). In contrast, all antibodies bound to transfected M12.C3 cells that expressed an $A^{k/d}_{\beta}$ hybrid gene construct in which the second exon of the $A^{k}_{\beta}$ gene was replaced by the second exon of the $A_{\beta}$ gene (designated T.$A^{k/d}$, Table VI). This binding pattern demonstrates that the binding sites of all of the $A^{d}_{\beta}$-reactive antibodies are determined by polymorphic residues in the $\beta_1$ domain of the $A^{d}_{\beta}$ polypeptide.

The $A^{d}_{\beta}$-reactive antibodies were first tested against the cell lines expressing $A^{k}_{\beta}$ genes that encoded regional changes. Antibodies 25-9-17S, 34-5-3S, Y-212, Y-237, and Y-276 bound to all the cell lines expressing the mutant $A^{k}_{\beta}$ polypeptide containing region ··B·· residues of the $A^{d}_{\beta}$ polypeptide. These antibodies could be further divided into three groups on the basis of the reactivity with the two cell lines that express $A^{k}_{\beta}$ polypeptides with changes at either position 63 or positions 65–67. Antibody Y-212 stained the cell line expressing the mutant $A^{k}_{\beta}$ polypeptide containing serine at position 63 (T63), antibodies 25-9-17S, 34-5-3S, and Y-237 stained the cell line expressing the mutant $A^{k}_{\beta}$ polypeptide that contained proline, glutamic acid, and isoleucine at position 65–67 (T65–67) and antibody Y-276 did not bind to either T63 or T65–67 cells. These results, which were the reciprocal of those observed with the $A^{k}_{\beta}$-reactive mAbs, demonstrate that the presence of amino acids characteristic of the $A^{d}_{\beta}$ polypeptide at positions 63 and 65–67 of the $A^{k}_{\beta}$ polypeptide results in binding of most $A^{d}_{\beta}$-reactive antibodies tested.

Four exceptional cases were noted. Antibody MKD6 bound only to the cell

TABLE V
*Properties of $A^d_\beta$-reactive mAbs*

| mAb | Crossreactivity | Strain combination | Reference |
|---|---|---|---|
| 25-9-17S | I-$A^{b,p,q}$ | C3H anti-C3H.SW | 39 |
| 34-5-3S | I-$A^{b,p,q}$ | C3H anti-BDF1 | 39 |
| Y-212 | I-$A^{b,p,q,r,v}$ | A/J anti-B10.A(5R) | 23 |
| Y-219 | I-$A^{b,r,v}$ | A/J anti-B10.A(5R) | 23 |
| Y-237 | I-$A^{b,p,q,v}$ | A/J anti-B10.A(5R) | 23 |
| Y-270 | I-$A^{b,r,v}$ | A/J anti-B10.A(5R) | 23 |
| Y-276 | I-$A^{b,p,q,v}$ | A/J anti-B10.A(5R) | 23 |
| MKD6 | I-$A^{p,q}$ | (B6 × AJ) anti-B10.D2 | 40 |

TABLE VI
*Cytofluorometric Analysis of Mutant $A^k_\beta$-bearing Cell Lines with Antibodies Reactive to $A^d_\beta$*

| mAbs | T.$A^{k}$* | T.$A^{k}_{\beta}$‡ | T.$A^{d/k}$§ | T28 | T40 | T40$\beta$§ | T63 | T65 | T.A | T.B | T.C | T.D | T.ABC | T.ACD | T.BCD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 25-9-17S | – | – | +++ | – | – | – | – | ++ | – | +++ | – | – | +++ | – | +++ |
| 34-5-3S | – | – | +++ | – | – | – | – | ++ | – | +++ | – | – | +++ | – | +++ |
| Y-212 | – | – | +++ | – | + | ++ | +++ | – | – | ++ | – | – | + | – | + |
| Y-219 | – | – | +++ | – | + | ++ | – | – | – | – | – | – | – | – | – |
| Y-237 | – | – | +++ | – | – | – | – | + | – | ++ | – | – | ++ | – | + |
| Y-270 | – | – | +++ | – | + | +++ | – | – | – | – | – | – | – | – | – |
| Y-276 | – | – | +++ | – | – | – | – | – | – | +++ | – | – | +++ | – | +++ |
| MKD6 | – | – | +++ | – | – | – | – | – | – | – | – | – | ++ | – | ++ |
| 39J¶ | +++ | – | – | +++ | +++ | – | +++ | +++ | – | +++ | +++ | +++ | – | – | +++ |
| K24-199** | + | ++ | +++ | – | – | +++ | + | – | – | + | – | – | +++ | +++ | – |

The TA3 cell line was used as a standard (see under Fig. 1).
* Cell line transfected with the wild-type $A^k_\alpha$ and $A^k_\beta$ gene.
‡ Cell line transfected only with the wild-type $A^k_\beta$ gene.
§ Cell line transfected with an $A^k_\beta$ gene construct in which the second exon was derived from the $A^d_\beta$ gene.
‖ Cell line transfected only with the M40 $A^k_\beta$ gene.
¶ 39J recognizes the $A^k_\alpha$ polypeptide.
** K24-199 recognizes $A^d_\alpha$ polypeptide.

lines expressing the mutant $A^k_\beta$ polypeptides containing regions ··ABC·· or regions ··BCD of the $A^d_\beta$ polypeptide (Table VI). Therefore, the epitope recognized by this antibody is either determined by variable residues of regions ··B·· and ··C·· or determined in a more complex fashion. A recent analysis (38) of L cells transfected with $A_\alpha$ genes and various combinations of half-exon–shuffled $A_\beta$ genes is consistent with these observations. Their results indicated that although the binding of the MKD6 antibody to these exon-shuffled Ia molecules was dependent upon the presence of d allele residues in the carboxyl half of the $\beta_1$ domain, polymorphic residues in both the $NH_2$-terminal half of the $\beta_1$ domain and in the $A_\alpha$ polypeptide influenced the binding affinity of the MKD6 antibody. Antibodies Y-219 and Y-270, which did not stain any of the cell lines expressing $A^k_\beta$ polypeptides with regional changes, did react with the cell line expressing the mutant $A^k_\beta$ polypeptide containing tyrosine characteristic of the $A^d_\beta$ polypeptide at position 40 (Table VI). Binding was stronger to the cell line expressing this mutant $A^k_\beta$ polypeptide in association with the $A^d_\alpha$ polypeptide (T40$\beta$) than to the cell line expressing the mutant $A^k_\beta$ polypeptide predominantly in association with the $A^k_\alpha$ polypeptide (T40). This result suggests that the binding sites of the antibodies Y-219 and Y-270 may be influenced by polymorphic residues on both the $\alpha$ and $\beta$ chain polypeptides. The reactivity pattern of

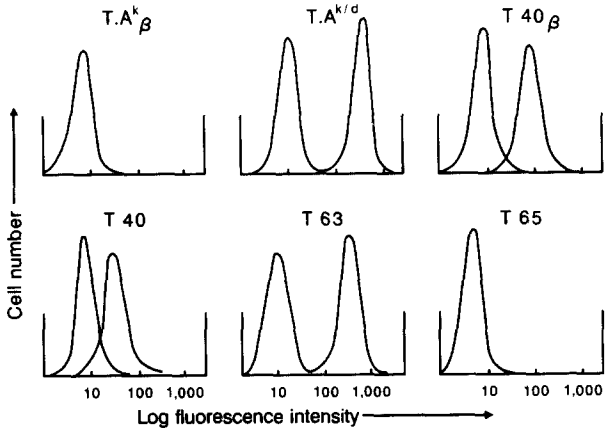


FIGURE 3. Quantitative immunofluorescence profiles using monoclonal antibody Y-212 illustrating representative levels of cell surface Ia expression obtained on cell lines transfected with different $A^k_\alpha$ and/or $A^k_\beta$ genes. Categories of expression levels used in Tables III, IV, V, and VII are represented by: (−) T.$A^k_\beta$ and T65; (+) T40; (++) T40$\beta$; (+++) T.$A^{k/d}$ and T63.

antibody Y-212 differs from that observed with antibodies Y-219 and Y-270 in that antibody Y-212 reacts with cells expressing d allele residues at either position 63 (T.63) or position 40 (T.40). Cells expressing the M.40 $A^k_\beta$ polypeptide associated with the $A^d_\alpha$ polypeptide (T.40$\beta$) react more strongly with Y-212 than cells expressing the M.40 $A^k_\beta$ polypeptide associated with $A^k_\alpha$ (T.40) (Fig. 3). However, antibody Y-212 did not bind stronger to cells expressing M.63 $A^k_\beta$ polypeptide associated with the $A^k_\alpha$ polypeptide than to cells expressing M.63 $A^k_\beta$ polypeptide associated with the $A^k_\alpha$ polypeptide (data not shown). The observation that the antibody binding site recognized by mAb Y-212 is determined by the polymorphic residues at both positions 40 and 63 suggests that these two residues may be adjacent to each other in the three-dimensional conformation of the $A^d$ molecule.

## Discussion

The extensive allelic structural diversity of Ia polypeptides determines the unique components of allospecific antibody binding sites. Although allelic amino acid variation in the $\beta_1$ domain, and presumably the $\alpha_1$ domain determine T cell recognition as well as allospecific antibody binding (9, 10, 23), it has not been possible to determine the significance of particular residues or regions within the $\beta_1$ domain due to the high number of polymorphic residues encoded by the naturally existing alleles. We have begun to address this question by producing a panel of cell lines expressing mutant $A^k_\beta$ polypeptides that contain amino acid substitutions characteristic of the d allele at single or multiple positions within the $\beta_1$ domain.

Analysis of this panel of cell lines with eight different $A^k_\beta$-reactive and eight different $A^d_\beta$-reactive mAbs demonstrated that a limited number of polymorphic residues within the $\beta_1$ domain determine the binding of these allospecific antibodies. Amino acid substitutions characteristic of the $A^d_\beta$ polypeptide at positions 63 and 65–67 in the $A^k_\beta$ polypeptide resulted in the loss of binding of all antibodies reactive to $A^k_\beta$. The introduction of amino acids of the $A^d_\beta$ polypeptide at positions 40, 63, and/or 65–67 resulted in the binding of most $A^d_\beta$-reactive antibodies.

These results suggest that the binding sites of these $A^k_\beta$- and $A^d_\beta$-reactive antibodies are determined by the polymorphic amino acids at positions 40, 63, and 65–67.

Results from previous studies have indicated that certain amino acid substitutions in the polymorphic region around positions 63 and 65–67 result in the loss of antibody binding sites on the $A_\beta$ chain. Substitution of three amino acids at positions 67, 70, and 71 in the $A^b_\beta$ polypeptide resulted in the loss of multiple allospecificities in the mutant mouse strain bm12 (26, 41). Similarly, five immunoselected mutant Ia-bearing cell lines exhibiting defects in many of the $A^k_\beta$ antibody binding sites, contained single base substitutions in their $A^k_\beta$ genes which led to amino acid changes at positions 59, 64, or 70 (21, 42). Analysis of three cell lines transfected with mutant $A^b_\beta$ genes encoding $A^k_\beta$ characteristic residues at positions 9, 13, or 65–67 revealed that only the change at position 65–67 resulted in loss of antibody binding sites characteristic of the $A^b_\beta$ polypeptide (24). These studies were limited, however, because only the loss of antibody binding was observed and only the effects resulting from alteration of a small portion of the $\beta_1$ domain was evaluated.

There remains the possibility that the antibody binding sites on the $A^k_\beta$ and $A^d_\beta$ polypeptides are not located at the polymorphic residues at positions 40, 63, and 65–67. The amino acid substitutions at these positions could change the conformation of the binding sites located in different parts of the $A^k$ or $A^d$ molecule by allosteric effects. However, we do not favor this interpretation. The regions around the residues at positions 40, 63, and 65–67 contain multiple charged residues and are predicted (data not shown) to be markedly hydrophilic according to a previously described algorithm (43). Consequently, these regions are expected to be exposed on the surface of the molecule. In addition, single or multiple substitutions of amino acids at other polymorphic positions in the $\beta_1$ domain of the $A^k_\beta$ polypeptide do not measurably alter antibody binding sites. Preliminary results indicate that substitutions of $A^d_\beta$ characteristic residues in either the $NH_2$- or COOH-terminal part of the $A^k$ $\beta_1$ domain impair the ability of the mutant $A^k_\beta$ polypeptide to be expressed with either the $A^k_\alpha$ or $A^d_\beta$ polypeptide (our unpublished results). Thus, the polymorphic residues in the $NH_2$- and COOH-terminal part of the $A_\beta$ chain may be located near polymorphic residues of the $A_\alpha$ chain and may not be sufficiently exposed at the surface of the molecule to provoke a strong antibody response. A similar hypothesis was formulated by Braunstein and Germain (38) from analyses of L cells expressing $A_\beta$ polypeptides encoded by half-exon–shuffled genes. Their results suggested that the polymorphic residues in the $NH_2$-terminal half of the $\beta_1$ domain determines the allele-specific $\alpha/\beta$ chain pairing, whereas the polymorphic residues in the COOH-terminal half of the $\beta_1$ domain determine the antigenic determinants recognized by most $A_\beta$-reactive antibodies. Finally, the observed gain of binding sites for the $A^d_\beta$ antibodies by substitution of d allele residues at positions 40, 63, or 65–67 parallels the observed crossreactivity patterns on known Ia alleles. For example, antibodies Y-219 and Y-270, which react with T.40 cells, crossreact on the alleles b, d, r, and v, but not on alleles, f, k, q, s, u, and p. Alleles b and d both have a tyr at position 40 while alleles f, k, q, s, and, u have a phe at position 40 (alleles r, v, and p have not been sequenced). Similar comparisons are observed for antibodies 29-9-175, 34-535, Y-237, and Y-276. These results suggest that each

of these antibody binding sites is determined primarily by one polymorphic residue or a limited number of polymorphic residues on each crossreacting allele.

The analysis of allospecific antibody binding sites presented here is limited to those residues that differ between the $A^k_\beta$ and $A^d_\beta$ polypeptide. It has previously been shown that other residues in addition to the polymorphic residues that we have identified influence the binding of certain $A^k_\beta$-reactive mAbs. For example, sequence analysis of genes encoding five different mutant $A^k_\beta$ polypeptides, selected with $A^k_\beta$-reactive mAbs for altered antibody binding properties, revealed that substitutions of nonpolymorphic residues adjacent to the polymorphic region around positions 63 and 65–67 can also affect the conformation of the binding sites recognized by these $A^k_\beta$-reactive antibodies (21, 42). Moreover, polymorphic residues other than those located at positions 40, 63, and 65–67 may influence the binding of some of the tested mAbs. For example, mAbs 39B, 39E, 40M, and 40F, whose binding to the $A^k_\beta$ polypeptide is lost after substitution of the residues at positions 63 or 65–67, do not bind to the $A^s_\beta$ polypeptide, although this allele shares amino acids from position 62–69 and at position 40 with the $A^k_\beta$ polypeptide (8, 33). This example demonstrates that in comparisons of alleles exhibiting multiple amino acid differences it is difficult to identify which amino acids determine antibody binding sites solely on the basis of crossreactivity patterns and sequence data.

The physiologic significance of Ia antigens stems from their role as restriction elements for helper T cells. Recent studies indicate that T cell recognition of Ia molecules is influenced by polymorphic residues on both $\alpha$ and $\beta$ polypeptides (10) and by residues in different regions of the $\beta_1$ domain (24, 44). In contrast, most allospecific antibody binding sites on Ia molecules are determined by either the $\alpha$ or $\beta$ polypeptides, and as we have shown here, only a limited number of $\beta_1$ polymorphic residues are involved in determining these sites. However, the results of the assays used to identify T cell recognition sites are affected by at least two potentially different regions on the Ia molecule, regions that bind Ia antigen and regions that bind the T cell receptor. Analysis of this panel of mutant $A^k$-expressing cell lines with $A^k$- or $A^d$-restricted T lymphocytes that exhibit specificity for defined peptide antigens together with binding analyses with labeled peptides to isolated mutant $A^k$ molecules should facilitate the characterization of the complex molecular interactions that occur between Ia, processed antigen, and the TCR.

## Summary

To identify which polymorphic residues determine the allospecific antibody binding sites on $A_\beta$ polypeptides, mutant $A^k_\beta$ genes were constructed encoding single or multiple amino acids of the d allele at 14 polymorphic positions in the $\beta_1$ domain. Cell lines expressing these genes were analyzed by quantitative immunofluorescence using 16 mAbs reactive to $A^k_\beta$ or $A^d_\beta$. Substitution of d allele residues at positions 63 and 65–67 in the $A^k_\beta$ polypeptide resulted in the loss of binding of all $A^k_\beta$-reactive antibodies and the gain of binding of most $A^d_\beta$-reactive antibodies. Two $A^d_\beta$-reactive mAbs bound to the mutant $A^k_\beta$ polypeptide containing d allele–characteristic residue at position 40. In contrast, substitution of the

other polymorphic residues in the $NH_2$-terminal and COOH-terminal regions of the $\beta_1$ domain did not alter antibody binding.

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