REGULATION OF MURINE MHC CLASS II
MOLECULE EXPRESSION

Identification of $\alpha_\beta$ Residues Responsible for Allele-specific
Cell Surface Expression

By JEAN-MARIE BUERSTEDDE, LARRY R. PEASE, ALLAN E. NILSON,
MICHAEL P. BELL, CAROL CHASE, GERTRUD BUERSTEDDE,
AND DAVID J. McKEAN

From the Department of Immunology, Mayo Clinic, Rochester, Minnesota 55905

Class II major histocompatibility molecules are cell recognition structures that,
in combination with processed foreign antigen, form the ligand for CD4$^+$ T
lymphocyte antigen receptors (1). The class II molecules consist of an $\alpha$ and a $\beta$
polypeptide, both of which exhibit a high degree of structural polymorphism (2, 3). Since
T cell antigen recognition is influenced by the allelic origin of both chains, additional
functional diversity is generated by combinatorial association of different $\alpha$ and $\beta$
chain allelic products (4). Biochemical analyses of B lymphoma cell lines (5) and
L cells transfected with different $\alpha$ and $\beta$ alleles (6) have demonstrated that certain
$\alpha_\alpha$ and $\alpha_\beta$ polypeptides do not form heterodimers effectively on the cell surface.
Studies using hybrid gene constructs suggested that amino acid differences in the
NH$_2$-terminal half of the $\beta_1$ domain are responsible for the pairing defects of these
$\alpha/\beta$ chain combinations (7, 8).

In this report we have analyzed a panel of cell lines expressing mutant $\alpha_\beta$ (designated $\alpha_\beta^*$) polypeptides, which were produced by substituting one or more allele
residues in the $\beta_1$ domain (9), to identify $\alpha_\beta$ polymorphic positions responsible for
the preferential association of $\alpha_\beta^*$ polypeptide with the $\alpha_\alpha$ polypeptide rather than
with the $\alpha_\beta$ polypeptide. We have identified polymorphic positions in the NH$_2$-
terminal part of the $\beta_1$ domain that are responsible for this preferential chain
association. In addition, we have also identified mutations in the COOH-terminal
end of the $\beta_1$ domain that either completely inhibit $\alpha_\beta^*$ $\alpha_\beta$ expression or result in
the expression of the $\alpha_\beta^*$ $\alpha_\beta$ molecule primarily in an intracytoplasmic compart-
ment. These results suggest that the substitution at these polymorphic positions in
the NH$_2$- and COOH-terminal ends of the $\beta_1$ domain can alter cell surface expres-
sion of certain $\alpha_\beta$ $\alpha_\alpha$ molecules, resulting in limitation of the potential number of
different class II restriction molecules that can mediate antigen-specific TH cell
activation. In addition, the results from the characterization of one of these allele-specific
expression mutants suggest that the transport of the Ia molecules from the cytoplasm
to the cell surface may be regulated by signals that are determined by the interaction
of polymorphic residues in both the $\beta$ and $\alpha$ polypeptides.
Allele-Specific Ia Expression Variants

Materials and Methods

Cell Lines. The panel of wild-type or mutant Ia-bearing bulk populations and cloned cell lines were prepared by transfecting into M12.C3 cells (generously provided by Dr. L. Glimcher, Harvard Medical School, Boston, MA), plasmids containing the wild-type A\textsuperscript{b} gene or mutant A\textsuperscript{b}* genes, the neomycin resistance gene \textit{pRSVneo} and, in certain cases, with the wild-type A\textsuperscript{d} gene as described previously (9). The transfected cells were cultured in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 \mu g/ml streptomycin, 1 mM L-glutamine, 0.1 mM 2-ME, 10 mM Hepes, and 600 \mu g/ml G418. Cells transfected with the \textit{pSV2-hygro} gene were cultured in the complete RPMI medium containing 600 \mu g/ml hygromycin.

Serologic Phenotypes. Expression of cell surface Ia molecules was quantitated by indirect fluorescence with FITC protein A on a monoclonal AD-reactive antibody, 40F (10), or 39F (10), or 3F12 (12), or anti-A\textsuperscript{d} antibody, H24.199 (13) as previously reported (12).

DNA-mediated Gene Transfer. Mutant Ia-bearing cell lines were supertransfected with plasmids containing the wild-type A\textsuperscript{b} gene and a hygromycin resistance marker. 10\textsuperscript{7} cells were mixed with 70 \mu g of a linearized plasmid containing the wild-type A\textsuperscript{b} gene (9) and 50 \mu g of the linearized \textit{pSV2-hygro} plasmid (14) in 0.6 ml medium containing 140 mM NaCl, 25 mM Hepes, 0.75 mM Na\textsubscript{2}HPO\textsubscript{4}, pH 7.4. Electroporation was carried out as described previously (9). The treated cells were cultured at 37°C for 16 h and then selected in medium containing 600 \mu g/ml hygromycin B (Sigma Chemical Co., St. Louis, MO). Each bulk population used for analysis of A\textsuperscript{b}* A\textsuperscript{d} surface expression originated from at least 24 independent hygromycin-resistant clones. Cell lines were cloned from the hygromycin-resistant bulk populations by limiting dilution.

Northern Blot Analysis. Poly(A)\textsuperscript{+} mRNA (10 \mu g) from each cell line was size selected on a 1.2% agarose formaldehyde gel and transferred to Gene Screen Plus (New England Nuclear, Boston, MA) (15). Hybridization was performed using an A\textsuperscript{b} synthetic oligonucleotide (sequence 269–246) derived from the noncoding strand of the A\textsuperscript{b} gene (16), an A\textsuperscript{b} cDNA probe (Eco RI fragment of pKAK (17)), A\textsuperscript{b} cDNA (Eco RI fragment of pKBK (16)) or actin (p7000 actin; Oncor). Blots were sequentially hybridized as previously described (18).

Biochemical Analysis of Ia Molecules. Procedures for the isolation of intrinsically radiolabeled Ia molecules from murine cell lines have been described in detail in a previous report (5). Briefly, the transfected M12.C3 cell lines were biosynthetically radiolabeled with [\textsuperscript{35}S]methionine, solubilized with a 0.5% Triton X-100–containing lysis buffer and the Ia molecules were immunoprecipitated from the cell lysate with either an A\textsuperscript{b}-reactive mAb (10-2.16 [19], 40 M [10]) or an isotype-matched control (CB101) bound to protein A-Sepharose. The immunoprecipitates were analyzed by two-dimensional nonequilibrium pH gradient electrophoresis as described previously (5).

Results

To investigate systematically the role of polymorphic residues in the A\textsuperscript{b} polypeptide, we have constructed by site-directed mutagenesis a series of A\textsuperscript{b}* genes encoding single or multiple residues characteristic of the A\textsuperscript{b} allele in the B\textsubscript{1} domain (9) (Fig. 1). Each A\textsuperscript{b}* gene was transfected together with a wild-type A\textsuperscript{b} and/or a neomycin-resistance gene into the A\textsuperscript{d} expression-negative B lymphoma line M12.C3 (9). Since this cell line expresses functional A\textsuperscript{b} mRNA but not A\textsuperscript{a} mRNA, M12.C3 cells transfected with different combinations of A\textsuperscript{b} and/or A\textsuperscript{b}* genes together with a neomycin-resistance marker can be used to test the ability of A\textsuperscript{b} polypeptides to associate with A\textsuperscript{b} or A\textsuperscript{b} polypeptides. M12.C3 cells transfected with the wild-type A\textsuperscript{b} gene express A\textsuperscript{b} A\textsuperscript{d} molecules on their surface, while M12.C3 cells transfected with both wild-type A\textsuperscript{b} and A\textsuperscript{b}* genes express primarily A\textsuperscript{b} A\textsuperscript{d} molecules on their surface (9).

Cell Surface Expression of M.78 and M.86 A\textsuperscript{b}* Mutants. Ia cell surface expression
in the Ia gene–transfected, neomycin-resistant bulk populations was evaluated through quantitative immunofluorescence by staining with Aα-, Aβ-, or Aβ-reactive mAbs. Most of the 14 cell populations resulting from the transfection of Aβ* genes encoding single d allele residues at different positions express Aβ* Aα molecules on the cell surface at a level comparable to that obtained from cells transfected with the wild-type Aβ gene (9, data not shown). M12.C3 cells transfected with a wild-type Aβ gene and a mutant Aβ* gene encoding a polypeptide containing the substitution of a single d allele residue at positions 78 or 86 (mutant Aβ* genes are designated M.78 or M.86, respectively) express normal levels of cell surface Aβ* Aα molecules. However, M12.C3 cells transfected with only the M.78 or M.86 Aβ* genes (transfected cells are designated T.78 or T.86, respectively) do not express detectable levels of Aβ* Aα molecules on the cell surface (Fig. 2 a). Additional experiments indicated that the alteration of Aβ* Aα

Figure 1. Amino acid sequences of the Aβ and Aβ β1 domains (32). Mutant Aβ* genes encoding d allele residues at each of the 14 polymorphic positions, at multiple positions, or in polymorphic regions of the β1 domain have been constructed by site-directed mutagenesis (9). The boundaries and designation chosen for these polymorphic regions are indicated below the sequences. A detailed description of the methods used to construct the Aβ* genes and to transfect the genes into M12.C3 cells is given elsewhere (9).
cell surface expression in these cells was not due to ineffective transfection of the
M.78 or M.86 Aβ* gene. Supertransfection of the Aβ gene and a hygromycin resis-
tance marker into T.78 β and T.86 β which expressed Aβ* and Aδ mRNA, resulted
in high levels of Aβ* Aδ cell surface expression as determined by staining with Aβ-
or Aδ-reactive antibodies (our unpublished results). When T.86 β bulk cultures were
cloned, cells were found by flow cytometric analysis that expressed very low levels
of T.86 Aβ* Aδ molecules on the cell surface (<5% of the level expressed by wild-
type T.A δ β cells based on mean peak channel fluorescence values; data not shown).
In contrast, no cell surface la molecules were detected on cloned T.78 β cells. These
results demonstrate that the substitution of a single d allele residue at position 78
or 86 in the Aβ β domain either inhibits completely or nearly completely the cell
surface expression of the Aβ* Aδ molecule but does not impair surface expression
of the Aβ* Aδ molecule.

Since wild-type Aβ and Aδ polypeptides pair efficiently with each other (5), we
anticipated that additional d allele substitutions in the Aβ* β1 domain might correct
the surface expression defect caused by single d allele substitutions at positions 78
or 86. Evidence for such intragenic complementation was observed when an Aβ*
gene containing d allele substitutions at positions 65-67 and 78 was transfected into
M12.C3 cells (T.65-67, 78 β). Quantitative immunofluorescence analysis of the
resulting bulk population demonstrated that the T.65-67, 78 Aβ* polypeptide is ex-
pressed, albeit inefficiently, with the Aδ polypeptide (Fig. 2, a and b). To rule out
the possibility that the relatively low level of T.65-67, 78 Aβ* Aδ cell surface expres-
sion is due to inefficient transfection, we compared levels of Aβ* Aδ surface expres-
sion with Aβ mRNA levels in the cloned lines T65-67, 78 β and T.A δ β expressing
the M.65-67, 78 Aβ* Aδ molecule or the wild-type Aβ Aδ molecules, respectively.
Although the T.65-67, 78 β cells express lower amounts of Aβ* Aδ molecules on the
surface than the wild-type T.A δ β cells, the T.65-67, 78 β cells express two to three
times higher Aβ* mRNA levels (Fig. 3).

![Figure 3](image_url)  
**Figure 3.** Quantitation of Aβ-specific mRNA from cloned cell lines
expressing the Aβ* Aδ molecule. Poly(A)* mRNA from each cell line
was hybridized sequentially with synthetic oligonucleotides derived
from the noncoding strand of the Aβ gene, the Aδ gene, or actin. Lane
1, M12.C3; lane 2, T.A δ β; lane 3, T.65-67, 78 β; lane 4, T13 β.
To examine this question further, $A_{b*}$ genes containing multiple $d$ allele regional substitutions in the $\beta_1$ domain (see Fig. 1 for boundaries of regions) were transfected into M12.C3 cells. The construction and serologic characterization of these $A_{b*}$ genes was described previously (9). Whereas cells transfected with $A_{b*}$ genes containing $d$ allele residues in regions C or CD do not express $A_{b*} A_d$ molecules on the cell surface, M12.C3 cells transfected with $A_{b*}$ genes containing $d$ allele residues in D, ABC, ACD, BCD, or AC express high levels of $A_{b*} A_d$ complexes on the cell surface (Table 1). The cell surface expression of $A_{b*} A_d$ molecules by cells transfected with the $A_{b*}$ gene containing the region D substitutions (T.D $\beta$ cells) indicates that the addition of $d$ allele residues at positions 83 and/or 84 correct the cell surface expression defect observed in the T.86 $\beta$ mutant. Additional substitutions in the B region are also corrective (compare T.BCD$\beta$ with T.CD$\beta$). Thus, while the substitution of $d$ allele residues at positions 78 or 86 results in either undetectable or very low levels of $A_{b*} A_d$ cell surface expression, the $A_{b*} A_d$ molecule can be expressed at varying degrees of efficiency if the $A_{b*}$ polypeptide contains additional $d$ allele residues in other regions of the $\beta_1$ domain.

Analysis of T.78 $\beta$ and T.86 $\beta$ Cells for Expression of $A_{b*} A_d$ Molecules Intracytoplasmically. The observation that T.78 $\beta$ and T.86 $\beta$ cells express cell surface $A_{b*} A_d$ molecules after they are supertransfected with the $A_d$ gene suggested that T.78$\beta$ and T.86$\beta$ cells might express the $A_{b*}$ polypeptides that do not associate stably with $A_d$ molecules intracytoplasmically. To evaluate this possibility, detergent lysates of [$^{35}$S]methionine T.78 $\beta$ and T.86 $\beta$ cloned cells were immunoprecipitated with an anti-$A_b$ mAb and the immune complexes were analyzed by two-dimensional gel electrophoresis. Although spots comigrating with $A_{b*}$, $A_{b*}$, and invariant (Ii) polypeptides were isolated from the T.86 $\beta$ anti-$A_b$ immunoprecipitates, the migration pattern of the polypeptides isolated from the T.86 $\beta$ Ia preparation was different from the pattern observed with the wild-type Ia preparation (Fig. 4). In the wild-type anti-$A_b$ immunoprecipitate, the $A_{b*}$ polypeptide migrates as two major spots: a lower molecular weight precursor form and a higher molecular weight, more anionic spot that contains additional complex sugars and a terminal sialic acid residue. The T.86 $A_{b*}$ polypeptide migrates predominantly in the precursor form, with a small proportion of the molecules migrating as the higher molecular weight, mature form. Similarly, the T.86 $\beta$ Ia preparation contains an $A_d$ polypeptide that migrates predominantly as the core-glycosylated form, with a relatively small proportion of the molecules in the higher glycosylated, more anionic forms. The T.86 Ia immunoprecipitate also contains relatively large amounts of highly sialylated Ii polypeptides as compared with the Ia polypeptides isolated from the wild-type T.$A^k$ $\beta$ cells. This result is consistent with previous observations that intracytoplasmic precursor Ia molecule preparations contain more Ii polypeptide than Ia molecules isolated from the plasma membrane (20, 21). When comparisons are made of the numbers of T.86 $\beta$ cells and T.$A^k$ $\beta$ cells used in these experiments, the autoradiographic exposure times, and the intensity of the $\alpha$ and $\beta$ spots from the two preparations, the results suggest that the T.86 $\beta$ cells and the T.$A^k$ $\beta$ cells contain comparable amounts of $A_{b*} A_d$ polypeptides. Although quantitative immunofluorescence results suggest that these T.86 $\beta$ cells express <5% of the amounts of cell surface Ia as compared with the T.$A^k$ $\beta$ cells, anti-$A_{b*}$ immunoprecipitates from the T.86 $\beta$ lysates contain much more than 5% of the T.86 $A_{b*}$ or $A_d$ molecules in the highly glycosylated,
Figure 4. Autoradiographs of Ia polypeptides analyzed on two-dimensional NEPHGE gels. Anti-A\(\beta\) immunoprecipitates were isolated from the following \(^{[35}S\)lactoalbumin cell lysates: (A) 40 M or (B) control (CBI01) immunoprecipitates isolated from wild-type T.A\(\beta\) cell lysates; (C) 10-2.16 or (D) control immunoprecipitates isolated from T.86\(\beta\) cell lysates; (E) 10-2.16 or (F) control immunoprecipitates isolated from T.76\(\beta\) cell lysates. Spots corresponding to A\(\beta\), A\(\beta\), and I\(\alpha\) are indicated on the appropriate autoradiographs. In F, the expected location of the A\(\beta\) polypeptide is marked. Separation of proteins on the horizontal axis was by NEPHGE and in the vertical direction by SDS-PAGE.
mature forms. Together these results suggest that T.86 A\(b^*\) A\(d\) molecules are not being transported efficiently to the cell surface and that these Ia molecules are accumulating within the cytoplasm.

Two-dimensional gel analysis of anti-A\(b\) immunoprecipitates from \[^{35}\text{S}\]methionine T.78 \(\beta\) cell lysates demonstrated the presence of a complex comprising the core-glycosylated T.78 A\(b^*\) polypeptide and the core-glycosylated I\(i\) polypeptide (Fig. 4). No A\(d\) polypeptide was detected in the T.78 \(\beta\) preparation. Thus, although the T.78 A\(b^*\) polypeptide does not associate stably with the endogenous A\(d\) polypeptide, it does associate with the I\(i\) polypeptide. Since the T.78 A\(b^*\) and I\(i\) polypeptides are found exclusively in their precursor forms, it is likely that this A\(b^*\) I\(i\) complex is located within the endoplasmic reticulum. Although little is known about the requirements for I\(i\) polypeptide association with the Ia \(\alpha/\beta\) complex, these results demonstrate that the I\(i\) polypeptide is capable of associating with the A\(b\) polypeptide in the absence of the A\(a\) polypeptide.

Altered Cell Surface Expression of T.13 A\(b^*\) A\(a\) Molecules. Transfection of the M.13 A\(b^*\) gene resulted in low levels of cell surface expression of the T.13 A\(b^*\) A\(a\) molecule on the T.13 \(\beta\) bulk cell population (Fig. 2 a). This low level of detectable cell surface expression of the T.13 A\(b^*\) A\(a\) molecule could be due to a mutation-induced alteration of the serologic epitope resulting in a change of affinity of the A\(b^*\) or A\(a^*\) reactive mAbs for the T.13 A\(b^*\) A\(a\) molecules rather than a quantitative alteration in the level of expression of the T.13 A\(b^*\) A\(a\) molecules. As shown previously by Braunstein and Germain (8), the relative affinity of an anti-Ia antibody for different A\(a\) A\(b\) molecules can be evaluated by quantitative immunofluorescence analysis using increasing dilutions of the mAb. When the antibody is limiting, the fluorescence intensity of an Ia-bearing cell line is dependent primarily on the affinity of the antibody for a specific epitope. When such an analysis is performed with increasing dilutions of 40F mAb (anti-A\(a^*\)), the T.13 \(\beta\), wild-type T.A\(k^*\) \(\beta\) and wild-type TA3 cells stain equivalently (Fig. 5 a). These results suggest that the low level of T.13 A\(b^*\) A\(a\) cell surface expression is not due to a mutation-induced alteration in antibody affinity. Northern blot analysis of a cell line cloned from this bulk population (T.13BA) demonstrated that the low level of T.13 A\(b^*\) A\(a\) expression is not due to inefficient transcription of the M.13 A\(b^*\) gene. As shown in Fig. 3, the T.13BA cell line expresses more A\(a^*\) mRNA than the wild-type transfected T.A\(k^*\) \(\beta\) cell line, although surface A\(b^*\) A\(a\) expression is lower in the T.13 \(\beta\) cell line.

NH\(_2\)-terminal A\(b\) Residues Regulate A\(b^*\) A\(a^*\) Cell Surface Expression. To identify the polymorphic residues in the A\(b\) \(\beta_1\) domain that are important for A\(b^*\) A\(a^*\) surface expression, A\(b^*\) genes encoding one or more d allele residues in the \(\beta_1\) domain were cotransfected together with the wild-type A\(a\) gene into M12.C3 cells. Quantitative immunofluorescence analyses with A\(b^*\), A\(b^{10}\), or A\(b^d\)-reactive mAbs demonstrated that, with the exception of the cases noted below, expression of A\(b^*\) A\(a\) molecules in the bulk populations transfected with the A\(b^*\) and A\(a^*\) gene is comparable to expression of A\(b^*\) A\(a\) molecules in the bulk population transfected with the wild-type A\(b\) and A\(a\) genes (9 and our unpublished results). In addition, cell lines generated from these bulk populations express high levels of A\(b^*\) A\(a^*\) molecules, but low levels of A\(b^*\) A\(a^d\) molecules, as determined by surface staining with A\(a^d\) or A\(a^d\)-reactive mAbs (9). Transfection of the A\(b\) gene together with the A\(b^*\) gene encoding d allele residues in region A (T.A.) (see Fig. 1) did not result in cell surface expression of
the T.A A\^a\_1\_\* A\^a\_2 molecule (Fig. 6, Table I). However, M12.C3 cells transfected with only the M.A A\^a\_1\_\* gene do express high levels of T.A A\^a\_1\_\* A\^a\_2 molecules on their cell surface (9). These results confirm previous studies that localized the positions responsible for preferential \(\alpha/\beta\) chain association to the NH\(_2\)-terminal half of the \(\beta_1\) domain (7) and further localized these residues to positions 9–17.

Surface expression of the A\^a\_1\_\* A\^a\_2 molecule in bulk populations and cell lines transfected with A\^a\_2 genes encoding a polypeptide substituted with a single \(\delta\) allele residue at either position 12 or 13 and wild-type A\^a\_2 genes has been shown previously to be impaired (9). Flow immunocytometric analysis was used to determine if the substitutions at position 12 or 13 altered the relative affinity of the anti-A\^a\_1\_\* mAb 39J for T.12 or T.13 A\^a\_1\_\* A\^a\_2 molecules (Fig. 5 b). Although the T.13 cells express relatively small amounts of cell surface Ia, the fluorescence curve obtained with cloned T.13.

**Figure 5.** Comparative analysis of the relative affinities of (top) 40F or (bottom) 39J mAbs for wild-type. (Top) Anti-A\^a\_2-reactive mAb 40F on wild-type T.A A\^a\_2 (A\^a\_1\_\* A\^a\_2) cells, T.12 (A\^a\_1\_\* A\^a\_2) cells, and T.13 (A\^a\_1\_\* A\^a\_2) cells. Each point represents the mean fluorescence level obtained from the transfected cells stained with a comparable dilution of an inappropriate mAb from the mean fluorescence level obtained from the transfected cells stained with 39J or 40F. (Bottom) Anti-A\^a\_1\_\* reactive mAb 39J on wild-type T.A A\^a\_1\_\* cells, T.12 (A\^a\_1\_\* A\^a\_2) cells, and T.13 (A\^a\_1\_\* A\^a\_2) cells.

**Figure 6.** Quantitative immunofluorescence profiles of hygromycin-resistant bulk populations after surface staining with the A\^a\_1\_\* reactive mAb 39J. The wild-type A\^a\_2 gene and a hygromycin-resistance marker were transfected into T.12B cells (–), T.13B cells (– –), wild-type T.A A\^a\_2 cells (– – –), or T.A \(\beta\) cells (-----). The profile obtained with the isotype-matched control antibody was superimposable with the profile obtained with 39J on the T.A \(\beta\) cells.
TABLE I
Surface Expression of Aβ* Aβ and Aβ* Aβ Molecules After Transfection of M12.C3 Cells with Wild-Type Aβ Genes and/or Aβ* Genes Encoding d Allele Regional Changes

| Cell lines | T.A | T.ABC | T.AC | T.ACD | T.BCD | T.CD | T.C | T.D |
|------------|-----|-------|------|-------|-------|------|-----|-----|
| Aβ Aβ+1   | +   | +     | +    | +     | +     | -    | -   | +   |
| Aβ Aβ+5   | -   | -     | -    | -     | +     | +    | +   | +   |

Results provided in this table represent a summary of previously reported quantitative immunofluorescence analysis on transfected bulk populations using a panel of Aβ-, Aβ-, and Aβ-reactive mAbs (9). Expression level designation: (-) <5% of the mean peak channel fluorescence obtained with the M12.C3 cells transfected with wild-type Aβ and Aβ genes (T.A-K cells); (+) mean peak channel fluorescence comparable (± 20%) to the profile obtained with wild-type T.A-K cells.

1 Cells resulting from transfection of a mutant Aβ* gene.
2 Cells resulting from transfection of a Aβ* gene plus wild-type Aβ gene.

Aβ* Aβ cells does not differ significantly from the curve obtained from cloned cells expressing either T.12 Aβ* Aβ or the wild-type T.A-K molecules. This analysis was repeated three different times with comparable results. These results indicate that the single d allele substitution at either position 12 or 13 does not alter our ability to detect the T.12 or T.13 Aβ* Aβ molecules.

To determine if these altered levels of Aβ* Aβ surface expression are caused by variation in the amount of Aβ* mRNA expression, we identified cloned cell lines expressing the Aβ gene and similar levels of either the M.12, M.13 or wild-type Aβ mRNA (T.12B, T.13B and T.Aβ β [2] [clone 2], respectively) for further analysis. These cell lines were supertransfected with the wild-type Aβ gene together with a hygromycin resistance marker (14). Immunofluorescence profiles of the three hygromycin-resistant bulk populations demonstrated that Aβ* Aβ surface expression is lower in the cells transfected with the M.12 Aβ or M.13 Aβ gene than the Aβ Aβ expression in cells transfected with the wild type Aβ and Aβ genes (Fig. 6). Northern blot analysis of cell lines cloned from these bulk populations demonstrated that the low level of Aβ* Aβ expression is not due to inefficient transcription of the Aβ gene. As shown in Fig. 7, the cell lines expressing the T.12 Aβ* Aβ (lane 3) or T.13 Aβ* Aβ molecules (lane 4) contain more Aβ specific mRNA (relative to levels of actin mRNA) than the cell line expressing the wild-type Aβ Aβ molecule (lane 2), although Aβ* Aβ surface expression is lower in the former cell lines. These results suggest that the absence of cell surface expression of the T.A Aβ* Aβ molecule may be due primarily to the additive effects of substituting d allele residues at positions 12 and 13. In contrast to what was observed with the T.78 or T.86 Aβ mutants, no additional d allele substitutions in the Aβ polypeptide are corrective for the observed defects in T.A Aβ* Aβ cell surface expression (Table 1).

Discussion
A panel of Aβ* mutants, which contain one or more d allele residues in the β1 domain, have been used to identify β1 domain polymorphic positions that are respon-
Figure 7. Quantitation of $A_k^\delta$-specific mRNA from cells cloned from the hygromycin-resistant bulk populations. Poly(A)$^+$ mRNA from each cell line was hybridized sequentially with synthetic oligonucleotides derived from the noncoding strand of the $A_k^\delta$ gene, the $A_k^\delta$ gene or actin. Lane 1, M12.C3; lane 2, wild-type $A_k^\delta$ cells transfected with the $A_k^\delta$ gene; lane 3, T120 cells transfected with the $A_k^\delta$ gene; and lane 4, T13β cells transfected with the $A_k^\delta$ gene.

The substitution of $d$ allele residues in the NH$_2$-terminal end of the $A_k^\delta$ β1 domain (region A; polymorphic residues 9, 12, 13, 14 and 17) results in the complete absence of detectable $A_k^\delta$ $A_k^\delta$ complexes within the transfected M12.C3 cells. Although the substitution of $d$ allele residue at any one of these five positions does not result in the complete absence of $A_k^\delta$ $A_k^\delta$ cell surface expression, substitution at either positions 12 or 13 impairs significantly the expression of $A_k^\delta$ $A_k^\delta$ molecules. The effects of the substitutions at these two positions may be additive and together be responsible for the complete loss of expression observed with the T.A $A_k^\delta$ $A_k^\delta$ mutant. The expression defect resulting from the substitution of $d$ allele residues in region A cannot be overcome by the addition of other $d$ allele residues at other positions in the β1 domain (9). Although we have analyzed these mutants with anti-$A_k^\delta$ mAbs that are reactive to two different $A_k^\delta$ serologic epitopes, we cannot completely rule out the possibility that our inability to detect this $A_k^\delta$ $A_k^\delta$ molecule is because the β1 domain substitutions alter the $A_k^\delta$ serologic epitopes. These results are consistent with a previous report demonstrating that the polymorphic residues in the NH$_2$-terminal half of the β1 domain were responsible for inhibiting the expression of the $A_k^\delta$ $A_k^\delta$ molecule (6, 7). Our results demonstrate that there are a minimum of two and a maximum of five NH$_2$-terminal polymorphic residues that may alter the conformation of the $A_k^\delta$ polypeptide, such that it will not interact effectively with the $A_k^\delta$ molecule to produce the hybrid $A_k^\delta$ $A_k^\delta$ molecule.

The substitution of a single $d$ allele residue at position 86 in the $A_k^\delta$ polypeptide results in the expression of very low levels of the mutant T.86 $A_k^\delta$ $A_k^\delta$ molecules on the cell surface. Analysis of the T.86 β Ia complexes on two-dimensional gels demonstrated that although the highly glycosylated, mature forms of both the T.86 $A_k^\delta$ and $A_k^\delta$ polypeptides are present, a large proportion of these two polypeptides migrate in positions corresponding to the less glycosylated precursor forms. However, the two-dimensional gel analyses also indicate that both T.86 β cells and wild-type cells express comparable levels of α/β complexes. Together these results suggest that the T.86 $A_k^\delta$ $A_k^\delta$ molecules are not being transported properly to the cell surface and are accumulating in an intracellular compartment. Ii polypeptide, which has
been hypothesized to be involved in the intracellular transport of Ia molecules (20, 21), is associated with the T.86 A₈* A₄ complex and is not likely responsible for the altered Ia expression. It is possible that the T.86 substitution produces a subtle alteration in α-β or α-β-Ii association, which might result in abnormal rates of degradation of the A₈* A₄ molecules intracellularly. Studies are in progress to evaluate this possibility.

The presence of sialic acid-mediated charge heterogeneity on the T.86 A₈*, A₄, and Ii polypeptides indicates that at least a portion of the T.86 Ia molecules are being transported intracellularly to the trans cisternae of the Golgi apparatus (22). Previous reports have indicated that the routing of proteins to the lysosomes, secretory storage vesicles, or to the plasma membrane appears to be regulated within the Golgi stack (23). Results from several model systems have suggested that proteins destined for the plasma membrane are unelectively transported from the Golgi apparatus, while routing to the secretory vesicles or lysosomes requires some sort of signal, presumably encoded within the protein's sequence (24-27). The altered expression observed with T.86 β mutants could result if the d allele substitution at position 86 either creates a signal that causes the A₈* A₄ molecules to be retained in the Golgi apparatus (or routed to the lysosomes) or alters a signal that is responsible for transporting the A₈* A₄ molecule to the cell surface. At this time we do not have sufficient experimental data to distinguish this signal-mediated regulation of class II expression from a potential α/β chain pairing defect in these mutants.

Griffith et al. (28) have reported two other in vitro immunoselected Ia mutants that exhibit a mutation and pattern of expression similar to what is observed with T.86 β. M12.C3 and M12.A2 cell lines express E₄ and A₄ molecules, respectively, that are expressed intracytoplasmically but not on the plasma membrane and contain sialic acid-mediated charge heterogeneity on their α, Ii, and/or β polypeptides. The altered phenotypes are due to point mutations (Asn → Ser) at position 83 in the M12.C3 E₄ molecules or position 82 in the M12.A2 A₄ molecules. It was speculated that the M12.C3 and M12.A2 β chain mutations may have altered a positive transport signal that is needed to direct the Ia molecule to the cell surface (28). This transport signal was predicted to comprise five amino acids between residues 79 and 83, which are conserved in almost every known class II β polypeptide. If the mutation in the T.86 β Ia molecules alters the same transport regulatory mechanism that is responsible for the altered transport of the M12.C3 and M12.A2 Ia molecules, then additional constraints need to be made on the putative transport signal hypothesis proposed by Griffith et al. (28). The 86 substitution occurs outside the previously designated boundaries of the hypothesized signal sequence and, most importantly, functions in an allele-specific manner (i.e., the T.86 A₈* polypeptide is expressed normally with the A₄ polypeptide but not with the A₄ polypeptide). Thus, if a positive (or negative) transport signal is located in this region of the β1 domain, then the signal must either be altered by or comprise, in part, polymorphic residues in the A₄ polypeptide. Since the substitution of additional d allele residues in the β1 domain corrects the expression defect, it is likely that a putative signal would be conformationally determined. The substitution of d allele residues at the polymorphic position 86 or the apparent random substitution at the nonpolymorphic positions 82 or 83 in the A₈ or E₄ sequences, respectively, may be responsible for creating a similar expression phenotype. It therefore seems most likely that the mutations
may have altered the conformation of the Ia molecule (resulting in rapid degradation of the molecule) rather than altered a signal responsible for the transport of the Ia molecules from the Golgi apparatus.

Although the T.86 Aβ* Aα molecules are expressed at very low levels on the cell surface, the Aβ* polypeptides containing d allele residues at positions 85, 86, and 89 (region D) are expressed normally with either Aα or Aβ polypeptides. Thus, interallelic conversion of certain stretches of amino acids, rather than single substitutions, might be selected evolutionarily because of the resulting functional consequences of the altered a/β surface expression. The fact that the altered expression resulting from at least one other chemically conservative substitution (e.g., val → ala at position 78) is more difficult to correct by the addition of more d allele residues (TCβ and T.CDβ) are not expressed with Aα, while T.AC, T.ACD and T.BCD are) may be because position 78 is critical for maintaining Aβ* Aα or Aβ* Aβ Ii chain association. Sequence analyses of murine and human β chain genes have shown that the region around residue 78 is conserved evolutionarily. Our results with the T.78 β cells suggest that there may be significant constraints on the potential mutations that can occur in this region of the β1 domain.

Bjorkman et al. (29, 30) have reported the crystal structure of a human class I molecule. Residues in the α1 and α2 domains form a platform comprising an eight-stranded β-plated sheet topped by two α helices, which together form a groove that is believed to be the recognition site for processed antigen. Brown et al. (31) have recently used the class I crystal structure to model the three-dimensional conformation of a class II α1-β1 domain. In this model, region A of the β1 domain is predicted to be located in the bottom of the groove between the two α helices. Since region A is predicted to be located in the middle of a β strand that is adjacent to a β strand that comprised the NH2 terminus of the α chain, the introduction of d allele residues into region A of the Aβ polypeptide could disrupt the interaction of these two β strands, resulting in destabilization of the a/β complex. Residue 78 is predicted to be located on one of the two α helices at a point just above a β strand, which comprises the NH2-terminal region of the β1 domain. The side chain of residue 78 is predicted to point inwards, toward the groove that contains the hypothetical antigen binding site. In this orientation, it would not be adjacent to the α polypeptide or be readily exposed on the exterior of the molecule in a site that could interact with an exogenous transport molecule. Residue 86 is predicted to be near the end of the same α helix, which contains residue 78, in a region whose conformation cannot be predicted from the class I molecule structure. Thus, although this hypothetical model of the class II structure supports the idea that the substitution of d allele residues in region A disrupts a/β chain association, it does not help us to determine why the substitution of d allele residues at positions 78 and 86 alter the cell surface expression of the Aβ* Aα molecule.

In a previous report from this laboratory we demonstrated that the majority of the known Aβ* and Aβ- characteristic serologic epitopes are not affected by substituions of polymorphic residues in the NH2- or COOH-terminal regions but can be localized to the polymorphic positions 40, 63, and 65–67 (9). These results suggested that these three serologically immunodominant positions are exposed on the exterior of the molecule and may be spatially adjacent. The results reported here further define the model of the I-A molecule by identifying Aβ* and Aα polymorphic
residues in both the NH2- and COOH-terminal regions of the β1 that may interact with λα polymorphic residues and/or other potential intracellular transport molecules to determine α/β chain cell surface expression.

Summary

A panel of mutant class II genes have been constructed using site-directed mutagenesis and DNA-mediated gene transfer. Using this technique, λβ polypeptides have been altered by substituting one or more λβ-specific residues at polymorphic positions in the β1 domain. Transfection of M12.C3 B lymphoma cells with most mutant λβ genes results in the expression of λβ molecules on the cell surface. However, the substitution of a single d allele residue at position 78 or 86 in the λβ polypeptide results in either the complete absence or very low levels, respectively, of cell surface expression of the λβ* λδ molecule, but does not alter λβ* λδ expression. The T.86 λβ* λδ is expressed primarily in an intracellular compartment while the T.78 λβ* λδ molecule does not appear to be produced. The core-glycosylated T.78 λβ* polypeptide does, however, form a complex intracellularly with the core-glycosylated Ii polypeptide. Substitution of the combination of d allele residues at λβ polymorphic positions 9, 12, 13, 14, and 17 results in the absence of λβ* λδ cell surface expression but does not alter the expression of this mutant λβ* polypeptide with the λδ polypeptide. These allele-specific expression mutants demonstrate that substitution at certain β1 domain positions may result in the alteration of Ia cell surface expression and that the transport of Ia molecules from the Golgi apparatus to the cell surface may be regulated by signals that are determined by the interaction of polymorphic residues in both the α and β polypeptides.

Received for publication 4 April 1988.

References

1. Schwartz, R. H. 1985. T lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. Annu. Rev. Immunol. 3:237.
2. Mengle-Gaw, L., and H. O. McDevitt. 1985. Genetics and expression of murine Ia antigens. Annu. Rev. Immunol. 3:367.
3. Giles, R. C., and J. D. Capra. 1985. Structure, function and genetics of human class II molecules. Adv. Immunol. 37:1.
4. Kimoto, M., and C. G. Fathman. 1980. Antigen reactive T cell clones. I. Transcomplementing hybrid I-A-region gene products function effectively in antigen presentation. J. Exp. Med. 152:759.
5. Schlauder, G. G., M. Bell, B. N. Beck, A. Nilson, and D. J. McKeon. 1985. The structure-function relationship of I-A molecules. A biochemical analysis of I-A polypeptides from mutant antigen-presenting cells and evidence of preferential association of allelic forms. J. Immunol. 135:1945.
6. Germain, R. N., D. M. Bentley, and H. Quill. 1985. Influence of allelic polymorphism on assembly and surface expression of class II MHC (Ia) molecules. Cell. 43:233.
7. Lechler, R. I., F. Ronchese, N. S. Braunstein, and R. N. Germain. 1986. Analysis of the roles of Aα and Aδ using DNA-mediated gene transfer. J. Exp. Med. 163:678.
8. Braunstein, N. S., and R. N. Germain. 1987. Allele-specific control of Ia molecule surface expression and conformation: implications for a general model of Ia structure-function relationships. Proc. Natl. Acad. Sci. USA. 84:2921.
9. Buerstedde, J.-M., L. R. Pease, M. P. Bell, A. E. Nilson, G. Buerstedde, D. Murphy, and D. J. McKean. 1988. Identification of an immunodominant region on the I-A β-chain using site-directed mutagenesis and DNA-mediated gene transfer. J. Exp. Med. 167:473.

10. Pierres, M., M. Devaux, M. Dosseto, and S. Marchetto. 1981. Clonal analysis of the B- and T-cell responses to 1a antigens. I. Topology of epitope regions of I-A^k and I-E^k molecules analyzed with 35 monoclonal alloantibodies. Immunogenetics. 14:481.

11. Ozato, K., and D. H. Sachs. 1981. Monoclonal antibodies to mouse MHC antigens. III. Hybridoma antibodies reacting to antigens of the H-2b haplotype reveal genetic control of isotype expression. J. Immunol. 126:317.

12. Beck, B. N., L. H. Glimcher, A. E. Nilson, M. Pierres, and D. J. McKean. 1984. The structure-function relationship of I-A molecules. Correlation of serologic and functional phenotypes of four I-A^k mutant cell lines. J. Immunol. 133:3177.

13. Koch, N., G. J. Hammerling, N. Tada, S. Kimura, and U. Hammerling. 1982. Cross-blocking studies with monoclonal antibodies against I-A molecules of haplotypes b, d and k. Eur. J. Immunol. 12:909.

14. McPhaul, M., and P. Berg. 1987. Formation of functional asialoglycoprotein receptor after transfection with cDNAs encoding the receptor proteins. Proc. Natl. Acad. Sci. USA. 83:8863.

15. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.

16. Benoist, C. O., D. J. Mathis, M. R. Kanter, and V. E. Williams. 1983. Regions of allelic hypervariability in the murine Aα immune response gene. Cell. 34:169.

17. Landis, D., B. N. Beck, J.-M. Buerstedde, S. deGaw, D. Klein, N. Koch, D. Murphy, M. Pierres, T. Tada, K. Yamamoto, C. Benoist, and D. Mathis. 1986. Chain specificities of anti-Ia monoclonal antibodies. J. Immunol. 137:3002.

18. Geliebter, J., R. A. Zeff, D. H. Schulze, L. R. Pease, E. H. Weiss, A. L. Mellor, R. A. Flavell, and S. G. Nathenson. 1986. Interaction between K^b and Q4 gene sequences generates the K^b> mutation. Mol. Cell. Biol. 6:645.

19. Oi, V. P., P. P. Jones, J. W. Goding, L. A. Herzenberg, and L. A. Herzenberg. 1978. Properties of monoclonal antibodies to mouse Ig allotypes. Curr. Top. Microbiol. Immunol. 81:115.

20. Moosic, J. P., E. Sung, A. Nilson, P. P. Jones, and D. J. McKean. 1982. The selective solubilization of different murine splenocyte membrane fractions with Lubrol WX and Triton X100 distinguishes two forms of 1a antigens. J. Biol. Chem. 257:7684.

21. Sung, E., and P. P. Jones. 1981. The invariant chain of murine Ia antigens: its glycosylation, abundance and subcellular localization. Mol. Immunol. 18:899.

22. Roth, J., D. J. Taatjes, J. M. Lucocq, J. E. Weinstein, and J. C. Paulson. 1985. Demonstration of an extensive trans-tubular network continuous with the Golgi apparatus stack that may function in glycosylation. Cell. 43:287.

23. Rothman, J. E. 1987. Protein sorting by selective retention in the endoplasmic reticulum and Golgi stack. Cell. 50:521.

24. Wieland, F. T., M. L. Gleason, T. A. Serafini, and J. E. Rothman. 1987. The rate of bulk flow from the endoplasmic reticulum to the cell surface. Cell. 50:289.

25. Orci, L., B. S. Glick, and J. E. Rothman. 1986. A new type of coated vesicle carrier that appears not to contain clathrin: its possible role in protein transport within the Golgi stack. Cell. 46:171.

26. Von Figura, K., and A. Hasilik. 1986. Lysosomal enzymes and their receptors. Annu. Rev. Biochem. 55:167.

27. Kelly, R. B. 1985. Pathways of protein secretion in eukaryotes. Science (Wash. DC). 230:25.

28. Griffith, I. J., N. Nabari, Z. Ghogawala, C. G. Chase, M. Rodriguez, D. J. McKean,
and L. H. Glimcher. 1988. Structural mutation affecting intracellular transport and cell surface expression of murine class II molecules. J. Exp. Med. 167:541.

29. Bjorkman, P. J., M. A. Saper, B. Samraoui, W. S. Bennett, J. L. Strominger, and D. C. Wiley. 1987. Structure of the class I histocompatibility antigen, HLA-A2. Nature (Lond.). 329:512.

30. Bjorkman, P. J., M. A. Saper, B. Samraoui, W. S. Bennett, J. L. Strominger, and D. C. Wiley. 1987. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. Nature (Lond.). 329:512.

31. Brown, J. H., T. Jardetzky, M. A. Saper, B. Samraoui, P. J. Bjorkman, and D. C. Wiley. 1988. A hypothetical model of the foreign antigen binding site of class II histocompatibility antigens. Nature (Lond.). 332:845.

32. Choi, E., K. McIntyre, R. N. Germain, and J. G. Seidman. 1983. Murine I-A\(_g\) chain polymorphism: nucleotide sequences of three allelic I-A \(\beta\) genes. Science (Wash. DC). 221:283.