

## STRUCTURAL MUTATION AFFECTING INTRACELLULAR TRANSPORT AND CELL SURFACE EXPRESSION OF MURINE CLASS II MOLECULES

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Proteins destined for the cell surface are translocated across the membrane of the endoplasmic reticulum and then vectorially transported through the Golgi complex to the plasma membrane (1). Core glycosylation occurs as the proteins are translocated across the endoplasmic reticulum membrane. Initial processing of the core (high mannose) glycosyl groups occurs as the proteins leave the endoplasmic reticulum; terminal glycosylation and processing occurs in the Golgi compartment.

A number of single and multiple base substitutions have been reported to block the intracellular transport of the Sindbis virus glycoprotein (2), murine class I antigen (3),  $\lambda$  light chain (4), and influenza hemagglutinin (5). Several of these are temperature-sensitive mutants in which defective transport can result from increased aggregation (6), decreased oligomerization (7), or altered chain folding (5) at nonpermissive temperatures. One other mutation involves disruption of an intrachain disulfide bond (3). These studies show that proper protein conformation is essential for normal protein maturation and expression.

In addition, it has been proposed that there are specific signals embedded in the primary protein sequence that are recognized by intracellular transport systems (8). Interaction of a protein with a transport system would explain the differential glycosylation and transport rates of proteins along a common maturational pathway from the endoplasmic reticulum through the Golgi complex and the ultimate sorting of proteins into different pathways. For example, a signal embedded in the NH<sub>2</sub> terminus of the yeast vacuolar glycoprotein, carboxypeptidase Y, appears to be necessary and sufficient to target the carboxy-

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peptidase to the vacuoles (9, 10). These protein-encoded signals are therefore critical for determining a protein's fate in the cell.

Our laboratory has been examining mutations that alter the surface expression of the murine class II Ia molecules. These Ia molecules, which are transmembrane glycoproteins encoded by the H-2 MHC, are expressed primarily on the surface of macrophages and B cells (11). There are two isotypic forms of Ia, I-A and I-E, each of which is a heterodimer composed of noncovalently associated  $\alpha$  and  $\beta$  chains. Each  $\alpha$  and  $\beta$  chain has two extracellular domains, a hydrophobic transmembrane domain, and a short cytoplasmic tail. The most membrane-distal (NH<sub>2</sub>-terminal) domain, is characterized by a high degree of intraspecies polymorphism. This allelic variation is the basis for Ia-restricted antigen recognition by T cells (12, 13).

We demonstrate in this report that a single conservative amino acid substitution in the  $\beta$ 1 domain of the A $_{\beta}$ <sup>d</sup> or E $_{\beta}$ <sup>d</sup> polypeptide can affect surface expression of I-A or I-E molecules, respectively. In contrast, it has been demonstrated by our laboratories (14–16) that the  $\beta$ 1 domain can tolerate even nonconservative changes without altering the level of surface expression for Ia. Therefore, the mutation that we describe may identify a region of the Ia molecule that is critical for intracellular transport and targeting to the cell surface.

### Materials and Methods

*Anti-Ia mAbs.* The mAbs MKD6 (anti-I-A<sup>d</sup>), 17-227, 34-5-3, and 25-9-17 (anti-I-A<sup>b,d</sup>), 14-4-4, 40B, and 13/4 (anti-E $_{\alpha}$ <sup>d</sup>), and 34-1-4 (anti-E $_{\beta}$ <sup>d</sup>) were used in this study. These antibodies were obtained from sources detailed in Glimcher et al. (17).

*FACS Analysis.* Quantitative indirect immunofluorescence analysis was performed as described (18) using an analyzer from Becton Dickinson & Co. (Mountain View, CA). For analysis, 10<sup>6</sup> cells were incubated with 20  $\lambda$  of a previously titrated quantity of the mAb indicated, followed by fluoresceinated goat anti-mouse H and L chain Ig (Cappel Laboratories, Cochranville, PA). Levels of background staining were determined by incubation of cells with the goat anti-mouse reagent in the absence of any anti-Ia mAb.

*Cell Lines and Selection of Variants.* Cells of the M12.4.1 (H-2<sup>d</sup>) B cell lymphoma were  $\gamma$  irradiated with 1,000 rad and then subjected to negative immunoselection by immune lysis with anti-Ia<sup>d</sup> mAbs plus complement. After three rounds of negative immunoselection, cells were surface negative for the particular Ia isotype recognized by the mAbs used. Two surface-negative variants, the M12.A2 (I-A<sup>-</sup>/I-E<sup>+</sup>; described here) and the M12.C3 (I-A<sup>-</sup>/I-E<sup>-</sup>; reference 17), were cloned from original bulk cell populations by limiting dilution. The M12.A2 and M12.C3 cell lines were independently derived from M12.4.1 parental cells negatively selected with a combination of anti-I-A<sup>d</sup> mAbs (34-5-3 and MKD6; M12.A2) or with a combination of anti-I-A<sup>d</sup> plus anti-I-E<sup>d</sup> mAbs (34-5-3, 14-4-4 and 34-1-4; M12.C3).

The panel of T cell hybridomas used in these studies was produced and examined for antigen specificity and Ia restriction as previously described (18). The AODH7.1, AODK1.16.14, and 3DO26.1 cell lines were the kind gift of Dr. P. Marrack (National Jewish Hospital, Denver, CO). T cells were tested for activation in a standard IL-2 release assay described elsewhere (18). Dose-response curves of antigen-presenting cells were performed to ensure maximal stimulation and IL-2 release. The antigens human gamma globulin, beef insulin, pork insulin, OVA (all from Sigma Chemical Co., St. Louis, MO), and keyhole limpet hemocyanin (Calbiochem-Behring Corp., La Jolla, CA) were used in culture as previously described (17, 18).

*Cloning and Sequencing the A $_{\beta}$ <sup>d</sup> and E $_{\beta}$ <sup>d</sup> Genes.* cDNA libraries were constructed from the surface-negative variants M12.A2 and M12.C3 using standard procedures (19). Briefly, RNA was recovered from the variants and then enriched for the poly(A)<sup>+</sup> fraction

by passage over an oligo(dT)-cellulose column (New England Biolabs, Beverly, MA). This material was used as a template for first strand synthesis with an oligo(dT) primer (New England Biolabs) and reverse transcriptase (Molecular Genetics, St. Petersburg, FL). After second-strand synthesis, Eco RI arms (New England Biolabs) were added and the cDNA was ligated into the phage  $\lambda$ gt10 (Vector Cloning Systems, San Diego, CA). Unamplified libraries were screened by Southern analysis with a full-length cDNA  $E_{\beta}^b$  probe (15) that cross-reacts with  $A_{\beta}$ . Phage clones containing full-length copies of the  $E_{\beta}^d$  (M12.C3) and  $A_{\beta}^d$  (M12.A2) were selected from initial pools of phage clones by restriction fragment length polymorphism analysis. The M12.A2  $A_{\beta}^d$  and M12.C3  $E_{\beta}^d$  inserts were then subcloned into the appropriate M13 vectors (Amersham Corp., Arlington Heights, IL) for sequencing by the dideoxy chain termination method (20). Various constructs were made using the restriction sites shown in Fig. 2 and both strands of the  $A_{\beta}^d$  and  $E_{\beta}^d$  cDNAs were sequenced. All restriction enzymes were obtained from New England Biolabs.

*Computer Resources.* Computer resources used to carry out our studies were provided by the National Institutes of Health-sponsored BIONET National Computer Resource for Molecular Biology. This includes data base resources and algorithms from Chou and Fasman (Brandeis University, Waltham, MA), Hopp and Woods (New York Blood Center, NY), and Kyte and Doolittle (University of California at San Diego, La Jolla, CA) for analysis of predicted protein structure and hydrophobicity.

*DNA-mediated Gene Transfer.* A wild-type  $A_{\beta}^b$  gene in pBR327 (21) was cotransfected into the M12.A2 cell with the plasmid pMSV-neo, which contains the neomycin resistance gene (22), using electroporation (23). Neomycin-resistant clones were selected and screened for the expression of I-A<sup>b</sup> on the cell surface, as above.

*Biochemical Analysis of Ia Molecules.* Procedures for the isolation of intrinsically radio-labeled Ia molecules from B lymphoma cell lines have been previously described in detail (24). M12.4.1 and M12.A2 cells were biosynthetically labeled with [<sup>35</sup>S]methionine, solubilized with 0.5% Triton X-100, and immunoprecipitated with the anti-I-A<sup>d</sup> reactive mAb 17-227 covalently coupled to Sepharose. The immunoprecipitates were analyzed with two-dimensional nonequilibrium pH gradient gel electrophoresis as previously described (24).

## Results

*Characterization of the M12.A2 Surface Ia<sup>-</sup> Variant.* The M12.A2 variant was derived from the BALB/c (H-2<sup>d</sup>, I-A<sup>+</sup>/I-E<sup>+</sup>) B cell lymphoma M12.4.1 after  $\gamma$  irradiation and negative immunoselection by immune lysis with a mixture of two anti-I-A<sup>d</sup> mAbs plus complement. The M12.A2 variant, cloned from the bulk population of treated cells by limiting dilution, is surface I-A<sup>-</sup>/I-E<sup>+</sup> by both FACS analysis (Table I) and immunoelectron microscopy (data not shown). Consistent with its surface phenotype, this variant will not present antigen to I-A<sup>d</sup>-restricted T cell hybridomas (Table II). The defect in I-A-restricted antigen presentation appears to be secondary to the loss of cell surface I-A molecules and not to a general loss in ability to process and present antigen since M12.A2 will present antigen normally to I-E<sup>d</sup>-restricted T cells (Table II).

The defect responsible for the surface I-A<sup>-</sup> phenotype of this cell is posttranscriptional since the M12.A2 contains normal levels of messenger RNA for both  $A_{\alpha}$  and  $A_{\beta}$  in Northern analysis (data not shown). I-A molecules can be detected in the cytoplasm of the M12.A2 after permeabilization of the cell membrane with formaldehyde treatment and staining with an anti-I-A<sup>d</sup> mAb (data not shown). Two-dimensional gel analysis of biosynthetically labeled I-A molecules immunoprecipitated with an anti- $A_{\beta}^d$  mAb allows ready identification of the  $\alpha$ ,  $\beta$ , and invariant chains (Fig. 1A). The pattern of oligosaccharide-induced heterogeneity in M12.A2 is indistinguishable from the wild-type pattern (Fig. 1B).

TABLE I  
Surface Phenotype of the M12.4.1 Parent and Mutant M12.A2 and M12.C3 Cell Lines

Antigen-presenting cell line	Anti-I-A <sup>d</sup> mAbs			Anti-I-E <sup>d</sup> mAbs			
	34-5-3	MKD6	25-9-17	14-4-4	34-1-4	40B	13/4
M12.4.1	+	+	+	+	+	+	+
M12.A2	-	-	-	+	+	+	+
M12.C3	-	-	-	-	-	-	-

Schematic representation of a FACS analysis showing binding of the specified mAbs to the surface of the M12.4.1 parent cell line and the M12.A2 and M12.C3 mutants. +, strong staining above background; -, no staining above background.

TABLE II  
Loss of Ia-restricted Antigen Presentation by M12-derived Mutant Cell Lines

T cell hybridoma	Specificity	Antigen-presenting cells		
		M12 (wild-type)	M12.C3	M12.A2
B3.C6	Auto:I-A <sup>d</sup>	+	-	-
B8/C3/X	Pork insulin:I-A <sup>d</sup>	+	-	-
BCF1A20	Beef insulin:I-A <sup>d</sup>	+	-	-
LC26	Allo:I-E <sup>d</sup>	+	-	+
LC50	Allo:I-E <sup>d</sup>	+	-	+
LC62	Allo:I-E <sup>d</sup>	+	-	+
LC70	Allo:I-E <sup>d</sup>	+	-	+
LC76	Allo:I-E <sup>d</sup>	+	-	+
AODH7.1	HGG:I-E <sup>d</sup>	+	-	+
AODK1.16.14	KLH:I-E <sup>d</sup>	+	-	+
3D026.1	OVA:I-E <sup>d</sup>	+	-	+

The M12.4.1 (I-A<sup>+</sup>/I-E<sup>+</sup>) wild-type parent and M12.C3 (I-A<sup>-</sup>/I-E<sup>-</sup>) and M12.A2 (I-A<sup>-</sup>/I-E<sup>+</sup>) variants were tested for their ability to present antigen to a panel of autoreactive, alloreactive, and antigen-specific Ia<sup>d</sup>-restricted T cell hybridomas. The results, from a standard IL-2 release assay (18), are presented schematically. The antigen specificity and Ia-restricting element are shown for each T cell hybridoma. +, wild-type response; -, no IL-2 release above background.

Three points can be made from this analysis. First, coprecipitation of the  $\alpha$  and  $\beta$  chains with the anti-A $\beta$ <sup>d</sup> mAb shows that the A $\beta$ <sup>d</sup>:A $\alpha$ <sup>d</sup> heterodimer is formed in the M12.A2 cell and, therefore, that chain pairing has not been disrupted. Second, detection of invariant chain indicates that the A $\beta$ :A $\alpha$  heterodimer forms a complex with the invariant chain that is precipitable by the anti-A $\beta$ <sup>d</sup> mAb. Although it has been shown that the presence of the invariant chain is not an absolute requirement for surface expression of Ia (25), it has been postulated that formation of the  $\alpha$ : $\beta$ :invariant chain complex facilitates intracellular transport (26) and may assure proper glycosylation of Ia (27). Third, the normal pattern of glycosylation for both the  $\alpha$  and  $\beta$  chains indicates that the A $\beta$ <sup>d</sup>:A $\alpha$ <sup>d</sup> heterodimer has been transported from the endoplasmic reticulum to the Golgi compartment; most of the induced complexity results from the processing of the

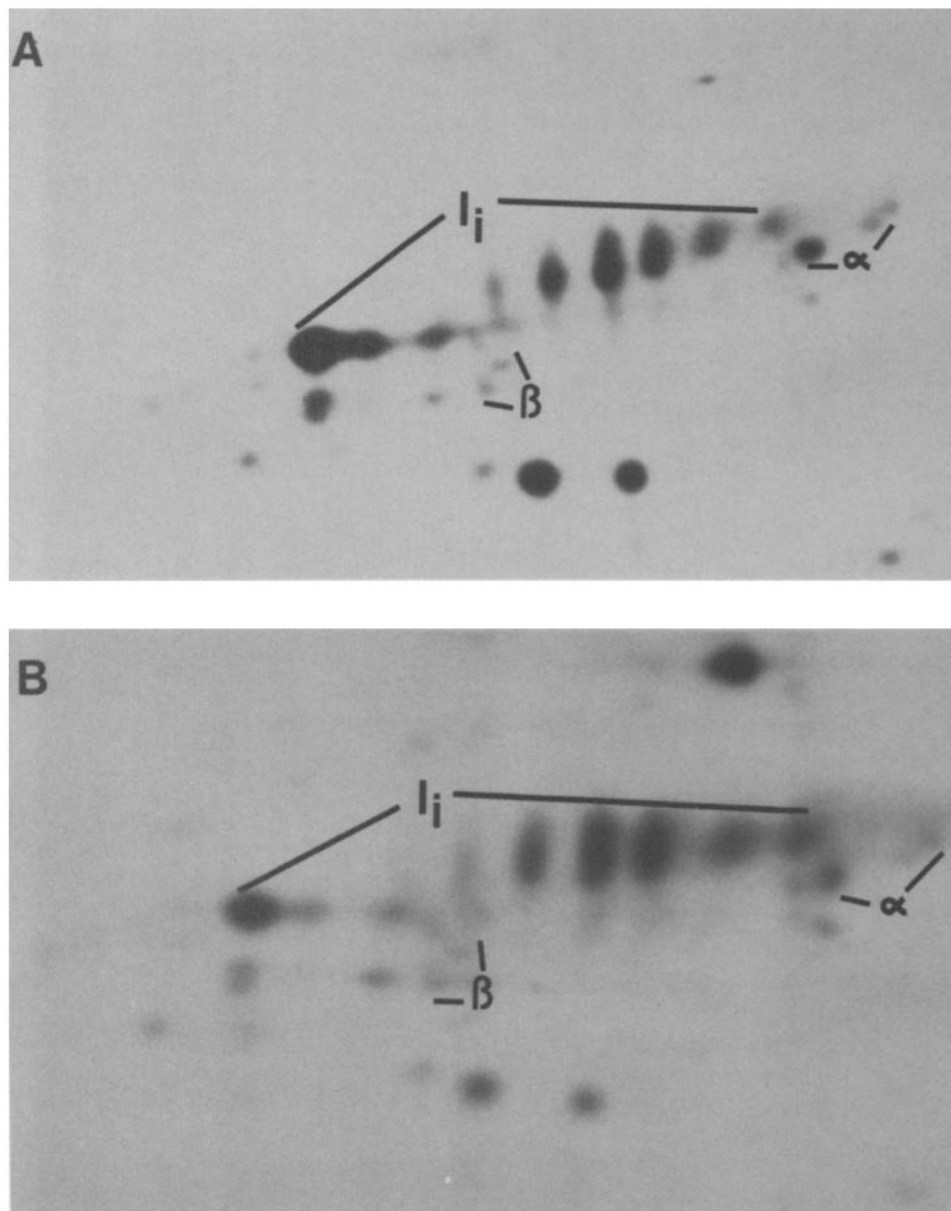


FIGURE 1. Two-dimensional nonequilibrium pH gradient gel electrophoresis of 17-227 (anti-I-A<sup>d</sup>) immunoprecipitates from (A) [<sup>35</sup>S]methionine-labeled M12.A2 detergent extract and (B) [<sup>35</sup>S]methionine-labeled M12.4.1 detergent extract. The A<sub>α</sub><sup>d</sup> and A<sub>β</sub><sup>d</sup> polypeptides are labeled as α and β, respectively, and the invariant chain is labeled as I<sub>i</sub>. The polypeptides were first separated by two-dimensional nonequilibrium pH gradient gel electrophoresis (horizontal dimension: basic, *left*; acidic, *right*) and then separated by SDS-PAGE in the vertical dimension (highest mol wt, *top*).

core (high mannose) *N*-linked glycosyl groups that has been shown to occur in the medial and *trans* faces of the Golgi apparatus (28).

Transfection of M12.A2 with a wild-type A<sub>β</sub><sup>b</sup> gene results in surface expression

of the  $A_{\beta}^b:A_{\alpha}^d$  heterodimer (data not shown). This does not rule out the possibility that the  $A_{\alpha}^d$  polypeptide also contains a mutation, but the successful expression of I-A after DNA-mediated gene transfer of a wild-type  $A_{\beta}^b$  gene shows that a hypothetical mutation in the  $A_{\alpha}^d$  polypeptide is not sufficient to block intracellular transport and cell surface expression of I-A molecules. These data suggest that the defect in this variant is due to a structural mutation in the endogenous  $A_{\beta}^d$  polypeptide.

*Characterization of the M12.C3 Surface Ia<sup>-</sup> Variant.* The M12.C3 cell line was cloned by limiting dilution from irradiated, negatively selected M12.4.1 cells as previously described (17). It had been negatively selected with a mixture of anti-I-A<sup>d</sup> and I-E<sup>d</sup> mAbs and is surface I-A<sup>-</sup>/I-E<sup>-</sup> by FACS analysis (Table I) and immunoelectron microscopy (data not shown). M12.C3 will not present antigen to any cloned antigen-specific, I-A<sup>d</sup>- or I-E<sup>d</sup>-restricted T cell hybridoma examined (Table II), but can participate in a class I-restricted response (29). The M12.C3 cell line contains normal levels of mRNA for  $A_{\alpha}$ ,  $E_{\beta}$ , and  $E_{\alpha}$  by Northern analysis (17).

Previous analysis of I-E molecules immunoprecipitated from the M12.C3 variant showed reduced heterogeneity of both the  $E_{\alpha}$  and  $E_{\beta}$  chains, with the  $E_{\beta}$  chain having only the core-glycosylated form (17). After permeabilization, it is possible to detect I-E<sup>d</sup> polypeptides in the cytoplasm (data not shown). The previously observed decrease in glycosylation-induced complexity of both  $E_{\alpha}^d$  and  $E_{\beta}^d$  would be consistent with an accumulation of I-E in the endoplasmic reticulum.

Transfection of the M12.C3 variant with an  $E_{\beta}^b$  gene resulted in surface expression of the  $E_{\beta}^b:E_{\alpha}^d$  heterodimer on the cell surface (15). This DNA-mediated gene transfer study shows that any hypothetical  $E_{\alpha}^d$  mutations are not sufficient to block intracellular transport or cell surface expression of I-E molecules. The surface expression of the  $E_{\beta}^b:E_{\alpha}^d$  heterodimer therefore suggests that the defect in the M12.C3 variant is the result of a structural mutation in the endogenous  $E_{\beta}^d$  polypeptide.

*Sequence Analysis of the Mutant  $A_{\beta}^d$  and  $E_{\beta}^d$  Genes.* cDNA libraries were prepared from both variants and the  $A_{\beta}^d$  and  $E_{\beta}^d$  genes were cloned from the M12.A2 and M12.C3 libraries, respectively. The coding regions of these genes were fully sequenced in both orientations using the strategies shown in Fig. 2. Nucleotide sequence data and predicted amino acid sequences, presented in Fig. 3, show that both genes contain identical single base A → G transitions that result in a predicted Asn → Ser substitution at residue 82 of the  $A_{\beta 1}$  domain (M12.A2) and at residue 83 of the  $E_{\beta 1}$  domain (M12.C3). In addition, the  $A_{\beta}$  gene contains a single-base difference from the published *d* haplotype sequence in the leader domain. This base change, A → G in the codon for residue -4, matches a wild-type *d* haplotype sequence obtained in our laboratory. There are also two single-base differences in the transmembrane and cytoplasmic regions that are silent (changing the codons for amino acids 222 and 227 from CAC → CAT and GGA → GGC, respectively). The  $A_{\beta}^d$  gene contains no mutations in the first 60 bases of the 3' untranslated (UT)<sup>1</sup> region; the last 210 bases of the 3' UT region have not been sequenced since the  $A_{\beta}^d$  3' UT region contains an internal Eco RI site

<sup>1</sup> Abbreviation used in this paper: UT, untranslated.

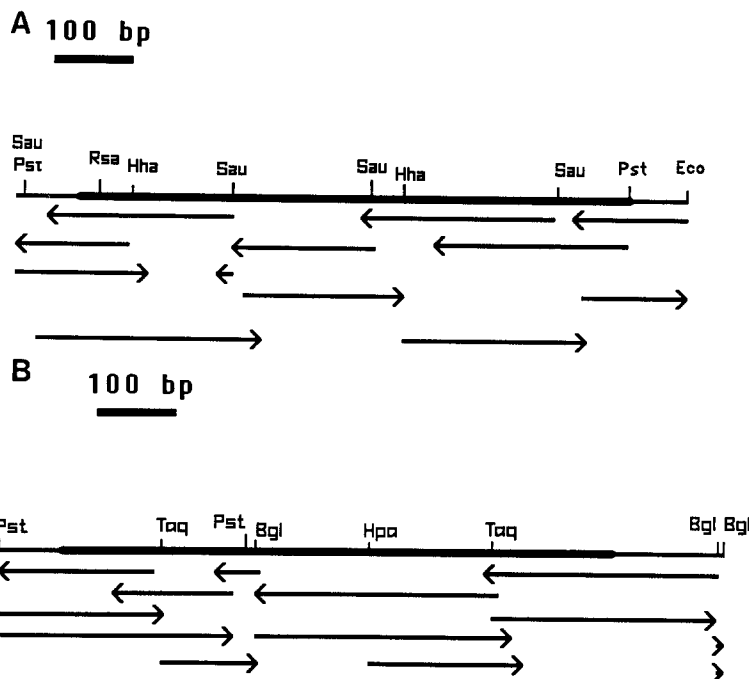


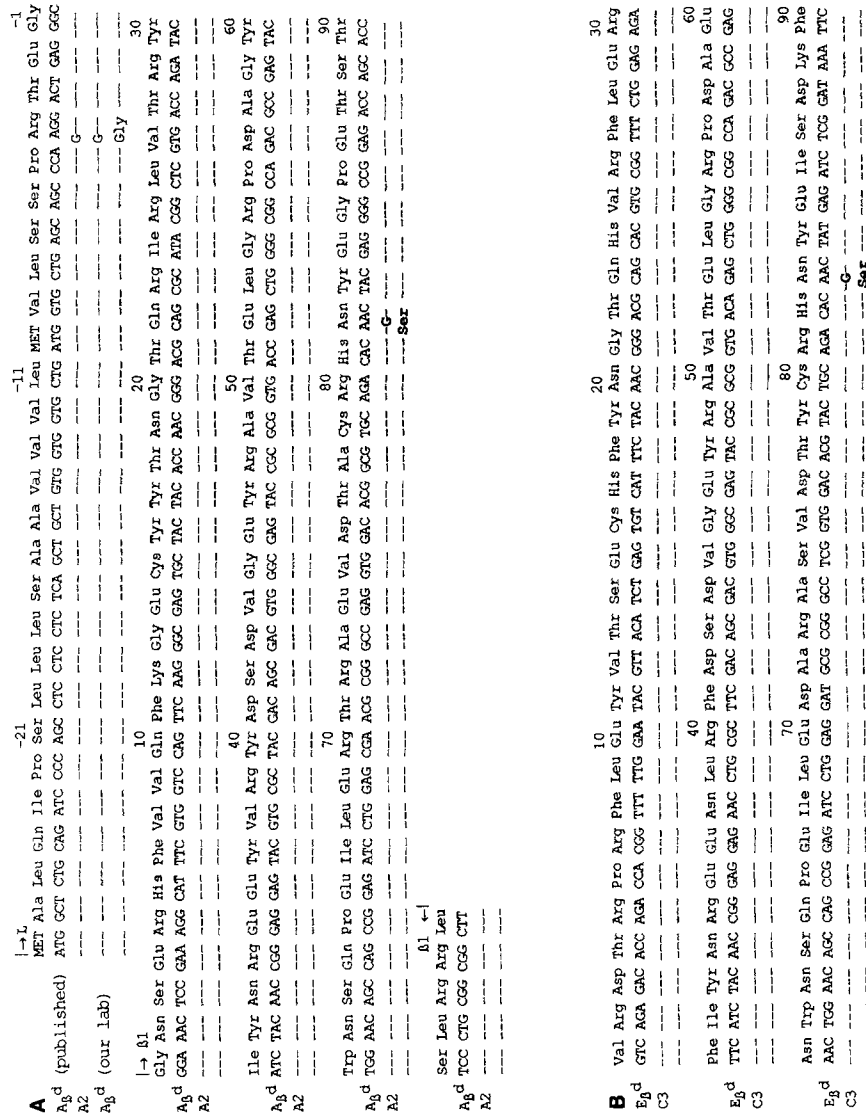
FIGURE 2. Partial restriction maps and sequence strategies for the  $A_{\beta}^d$  and  $E_{\beta}^d$  genes. The  $A_{\beta}^d$  (A) and  $E_{\beta}^d$  (B) inserts were subcloned into the appropriate M13 vectors for sequencing by the dideoxy chain termination method (20). Various constructs were made using the restriction sites shown and both strands of the  $A_{\beta}^d$  and  $E_{\beta}^d$  cDNAs were fully sequenced. The coding sequences are depicted by thick lines, whereas 5' leader and partial 3' untranslated sequences are depicted by thinner lines. *Bgl*, *Bgl* II; *Eco*, *Eco* RI; *Hha*, *Hha* I; *Hind*, *Hind* III; *Hpa*, *Hpa* II; *Pst*, *Pst* I; *Rsa*, *Rsa* I; *Sau*, *Sau* 3A1; *Taq*, *Taq* I.

and these bases were lost during cloning. The M12.C3  $E_{\beta}^d$  gene, which is wild type in the leader and remaining coding domains, contains two base differences from the published *d* haplotype sequence in the first 210 bases of the 3' UT region. The last 100 bases of the 3' UT region were not sequenced.

*Conformational and Hydrophobicity Analyses of the Mutant Polypeptides.* The predicted amino acid substitution of Ser for Asn at residue 82 of the  $A_{\beta}^d$  and residue 83 of the  $E_{\beta}^d$  polypeptides is conservative since both Asn and Ser are uncharged polar residues. Preliminary analysis of the  $A_{\beta}^d$  gene from the M12.A2 variant with the Chou and Fasman algorithm suggests that the Asn  $\rightarrow$  Ser substitution causes no detectable change in the secondary structure of the  $A_{\beta}^d$  polypeptide (data not shown). Similarly, analysis with the Hoop and Doolittle, and Wood and Kyte algorithms indicate that this mutation slightly decreases the protein's predicted hydrophobicity in the region of the mutation. Analysis of the  $E_{\beta}^d$  gene from the M12.C3 variant suggests that the Asn  $\rightarrow$  Ser substitution may disrupt the end of a  $\beta$ -pleated sheet and reinforce a possible turn sequence in the folded  $E_{\beta}^d$  polypeptide. The hydrophobicity of this protein is also decreased slightly by the substitution.

### Discussion

The M12.A2 and M12.C3 variants were derived from the M12.4.1 B cell lymphoma. Although these variants lack surface expression of I-A (M12.A2) or



**FIGURE 3.** (A) Comparison of the nucleotide sequence and predicted amino acid sequence for the wild-type and mutant A<sub>2</sub> genes in the leader and β1 domains. Only the sequence for the wild-type A<sub>2</sub> gene is given in full; the other sequences are given as dashed lines, except where they differ from the published A<sub>2</sub> sequence. The predicted amino acid sequences are given immediately above and below the nucleotide sequences. The published A<sub>2</sub> sequences are from reference 30. A wild-type A<sub>2</sub> sequence obtained in our laboratory is provided in the leader domain only. L, leader; β1, β1 domain. (B) Comparison of the nucleotide and predicted amino acid sequences for the wild-type and mutant E<sub>2</sub> genes in the β1 domain. Only the sequence for the wild-type E<sub>2</sub> gene is given in full. The mutant sequence is given as dashed lines, except where it differs from the E<sub>2</sub> gene. The predicted amino acid sequences are given immediately above and below the nucleotide sequences. The E<sub>2</sub> sequences are from reference 31.



I-E (M12.C3) molecules, accumulation of the appropriate Ia molecule can be detected in the cytoplasm. This accumulation appears to be the result of mutations in the  $A_{\beta}^d$  and  $E_{\beta}^d$  genes. Sequence analysis showed that both genes contain an identical, shared mutation at amino acid 82/83 of the  $\text{NH}_2$ -terminal  $\beta 1$  domain that causes a change from Asn (wild-type) to Ser (mutant). In addition, both genes contain unique mutations that do not alter the sequence of the mature polypeptide. Possible explanations for the effect these mutations have on transport of the Ia molecule are discussed below.

*Failure to Transport the I-A and I-E Polypeptides Is Likely Due to the Single-shared Amino Acid Substitution.* The shared mutation in the  $\beta 1$  domain that results in a change from Asn (wild-type) to Ser (mutant) is the likely cause for the altered surface expression of Ia in the M12.A2 and M12.C3 cells. The M12.C3  $E_{\beta}^d$  gene is wild-type in the leader and coding sequences except for the mutation in the  $\beta 1$  domain that is shared with the M12.A2  $A_{\beta}^d$  gene. In addition to the shared mutation, the M12.A2  $A_{\beta}^d$  gene contains silent mutations in the transmembrane and cytoplasmic regions of the  $A_{\beta}$  polypeptide. The M12.A2 gene also contains a single mismatch from the published  $A_{\beta}^d$  sequence in the leader domain. This latter mismatch is apparently due to an error in the published  $A_{\beta}^d$  sequence since the M12.A2 sequence is identical to a wild-type  $A_{\beta}^d$  sequence obtained in our laboratory (Fig. 3A). Therefore, the shared mutation is the only mutation that alters the amino acid sequence in the mature  $A_{\beta}^d$  and  $E_{\beta}^d$  polypeptides.

*The Shared Amino Acid Substitution Does Not Apparently Alter Glycosylation of the Ia Molecules.* It has previously been shown that glycosylation can affect protein folding and, ultimately, transport (6). It seems unlikely in this instance that altered glycosylation is responsible for the observed defect in Ia expression, however, since the wild-type Asn residue is not part of the N---Asn-X-Ser/Thr---C recognition sequence required for N-linked glycosylation (1). Further, Ia can be expressed on the cell surface after treatment of cells with tunicamycin (32), which blocks N-linked glycosylation (33), showing that N-linked glycosylation is not critical for Ia expression.

It is also unlikely that an altered pattern of O-linked glycosylation at the mutant Ser residue has blocked surface expression of Ia in the M12.A2 or M12.C3 cell. The results presented in Fig. 1 show that the glycosylation pattern of the mutant  $A_{\beta}^d$  (M12.A2) and wild-type  $A_{\beta}^d$  (M12.4.1) polypeptides are indistinguishable. This indicates that additional O-linked glycosylation of the mutant Ser has not occurred and argues against a hypothetical alteration in glycosylation being responsible for the defect in Ia expression observed in the M12.A2 and M12.C3 cells.

*The Amino Acid Substitution in the  $\beta 1$  Domain May Alter Protein Conformation and Create a Negative Transport Signal.* Analysis of mutant Ia molecules that contain single amino acid alterations at nonpolymorphic (15, 16, 34) or polymorphic (14, 35, 36) residues shows that an Ia molecule can tolerate even nonconservative changes in the first domain without alteration of membrane expression. This makes the profound effect (nonexpression) of the conservative Asn  $\rightarrow$  Ser amino acid alteration we have described even more striking. One possible explanation for the defect in Ia expression is that Ser at residue 82/83 creates a negative, "stop transport" signal.

As discussed above, this negative signal is not likely to be due to an altered glycosylation of Ia. Rather, such a signal could be generated as the result of an

alteration in protein conformation. Any purported effect of the shared mutation must allow, however, for the apparently normal interchain pairing, association with the invariant chain, and creation of mAb binding sites (which are dependent upon chain association) that we have observed in the I-A<sup>d</sup> and I-E<sup>d</sup> molecules of the M12.A2 and M12.C3 mutants.

These considerations appear to eliminate the possibility that the Ser disrupts the formation of the intrachain disulfide bond that occurs at Cys residue 79/80. Disruption of a disulfide bond, which has been shown to block intracellular transport of a murine class I antigen (3), would have a substantial effect on protein conformation. The binding of the MKD6 mAb to I-A<sup>d</sup> molecules in the cytoplasm of the M12.A2 variant (data not shown) further argues against disruption of this disulfide bond since binding of the MKD6 mAb is highly sensitive to the protein conformation of the  $\beta$ 1 domain (D. J. McKean, unpublished observation).

Similarly, the shared mutation appears to differ from temperature-sensitive mutations that block protein transport (2, 5). These temperature-sensitive mutations may cause aggregation (6), decrease oligomerization (7), or disrupt chain folding at nonpermissive temperatures (5). Growth of the M12.A2 at a range of temperatures (30–40°C) did not permit surface expression of the I-A<sup>d</sup> molecule (data not shown).

The Ser substitution might have a more subtle effect on tertiary protein folding, intrachain, and/or interchain pairing. The physical blockade of transport could be a secondary effect of this disruption; e.g., through a reduced solubility of the protein in the endoplasmic reticulum or Golgi complex. Our preliminary analysis with the Chou and Fasman algorithm suggests that the Asn → Ser substitution either does not alter ( $A_{\beta}^d$ ) or minimally affects ( $E_{\beta}^d$ , reinforced turn) these polypeptides. These differences may help account for the differential processing of the respective mutant Ia molecules in the cytoplasm (I-A<sup>d</sup>, wild-type glycosylation pattern; I-E<sup>d</sup>, only core glycosylation). Alternatively, the differential localization may be secondary to the known slower metabolic turnover of the I-E molecule (37) or other intrinsic properties of I-A and I-E that have not yet been described. Any definitive statement about the effect of the single-shared amino acid substitution on Ia structure must await the resolution of the crystallographic structure of Ia.

*The Shared Substitution May Delete a Positive Transport Signal.* The independent derivation of the identical mutation (resulting in the Ser substitution) in both Ia isotypes emphasizes the importance of this region in determining the structure and function of Ia molecules. One intriguing possibility is that the single-base substitution eliminates a positive transport signal. That is, the Asn at residue 82 ( $A_{\beta}^d$ ) or 83 ( $E_{\beta}^d$ ) may form part of a recognition sequence for an intracellular system necessary for transport and targeting of Ia to the cell surface. Such a recognition signal, which has been postulated to explain both organelle-specific targeting and differential rate of expression for a number of proteins, has not yet been clearly identified for a glycoprotein expressed on the cell surface.

A likely region to contain such a recognition signal in Ia is the 5-mer Cys-Arg-His-Asn-Tyr, which immediately spans the affected Asn in M12.A2 and M12.C3. This 5-mer is conserved in almost every class II  $\beta$ -chain polypeptide whose gene has been sequenced (Fig. 4), suggesting the evolutionary importance of this

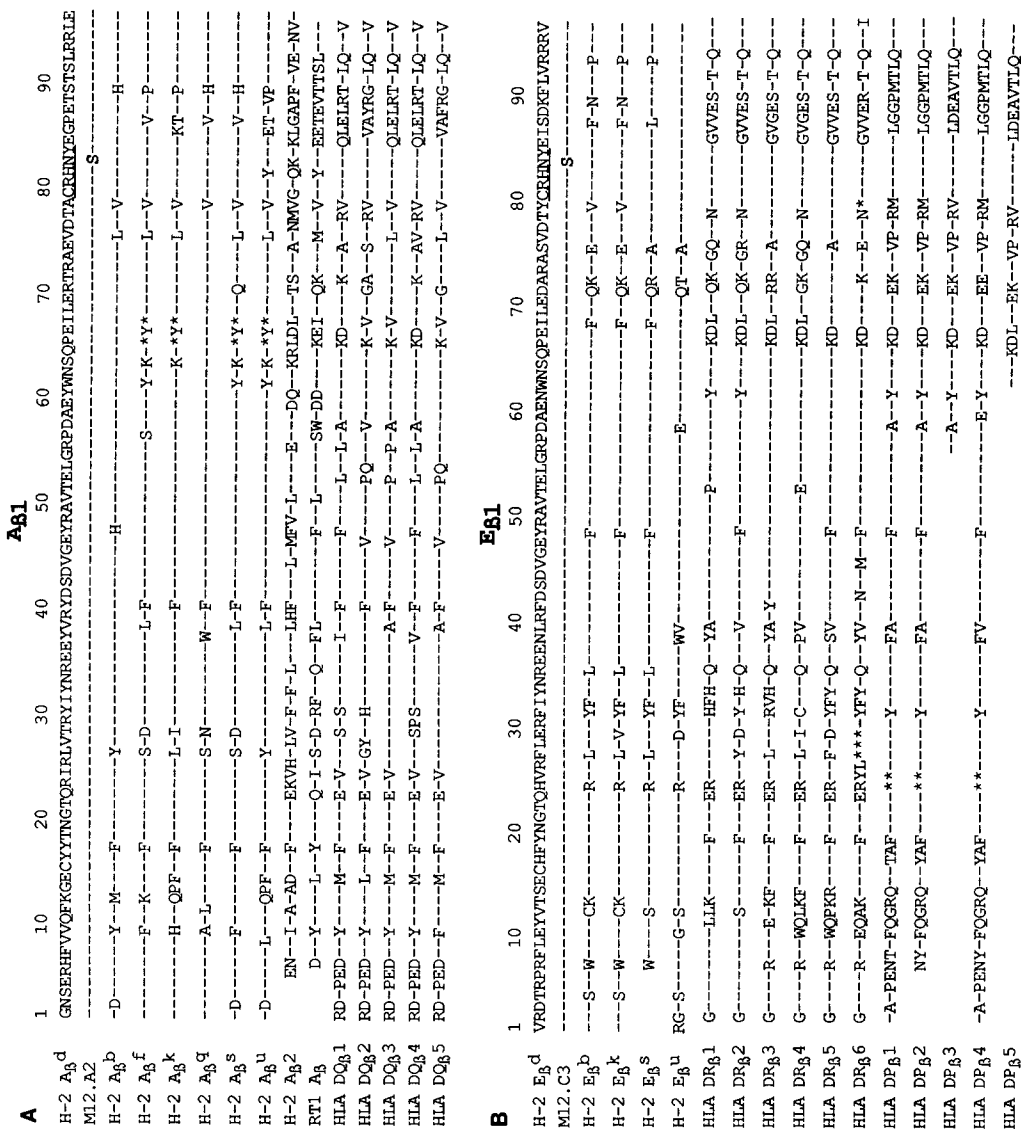


FIGURE 4. Comparison of the predicted M12.A2 A<sub>β</sub><sup>d</sup> and M12.C3 E<sub>β</sub> amino acid sequences in the β1 domain with published human, murine, and rat sequences. (A) Comparison of A<sub>β</sub> sequences. Only the murine A<sub>β</sub> sequence is given in full; all other sequences are given as dashed lines, except where they differ from A<sub>β</sub><sup>d</sup>. The murine A<sub>β</sub> sequence is from reference 30. The murine sequences for A<sub>β</sub><sup>f,q,k,s</sup> and <sup>u</sup> are from reference 38. The sequence for murine Aβ2 is from reference 39. The rat RT1 sequence is from reference 40. Other sequences are provided in reference 41. \* represents a break in the sequence inserted for maximal alignments. (B) Comparison of E<sub>β</sub> sequences. Only the murine E<sub>β</sub> sequence is given in full; all other sequences are given as dashed lines, except where they differ from E<sub>β</sub><sup>d</sup>. The E<sub>β</sub><sup>d</sup> sequence is from reference 31. The 5' E<sub>β</sub><sup>k</sup> sequence is from reference 15. All other sequences are provided in reference 41. \* represents a break in the sequence inserted for maximal alignments.

region in class II  $\beta$ -chain gene structure and function. Small linear sequences have previously been shown to be capable of providing a specific signal that affects intracellular transport. For example, the 4-mer sequence Lys-Asp-Glu-Leu (K-D-E-L) has been reported to provide a "stop transport" signal for proteins destined to remain in the endoplasmic reticulum (42). The  $\beta$ -chain 5-mer could therefore provide the signal necessary to target and transport Ia to the cell membrane.

There are three  $\beta$ -chain genes, the murine  $A_{\beta}^u$  (38),  $A_{\beta}2$  (39), and rat RT1  $A_{\beta}$  (40), that encode altered sequences in this 5-mer region. The encoded  $A_{\beta}^u$  polypeptide, which contains a single His<sub>81</sub>  $\rightarrow$  Tyr<sub>81</sub> amino acid substitution, is expressed on the cell surface. As far as has been determined, the murine  $A_{\beta}2$  gene does not encode a protein that is expressed on the cell surface. Expression of the sequenced rat gene has not been examined. The successful cell surface expression of the  $A_{\beta}^u$  polypeptide suggests that if the 5-mer is a recognition sequence, then some amino acid changes (e.g., His<sub>81</sub>  $\rightarrow$  Tyr<sub>81</sub>) can be tolerated but others (e.g., Asn<sub>82</sub>  $\rightarrow$  Ser<sub>82</sub>) can not.

Additional, independently derived, surface Ia-negative variants are currently being analyzed to see if they contain similar or identical mutations to that described in this report. The frequency and type of mutations that can be defined should provide additional insight into whether negative transport signals are being created or positive signals are being eliminated. The class II glycoproteins provide a well-defined model system to examine these issues.

### Summary

We have selected Ia variants from the Ia<sup>+</sup> (H-2<sup>d</sup>) M12.4.1 B cell lymphoma that are negative on the cell surface for one or both Ia isotypes. The molecular analysis of two such independently selected cell lines, M12.A2 and M12.C3, is reported here. This analysis revealed that the genes encoding  $A_{\beta}^d$  (M12.A2) and  $E_{\beta}^d$  (M12.C3) contained identical single-nucleotide transitions that resulted in the substitution of Ser (mutant) for Asn (wild-type) at residue 82/83 of the extracellular NH<sub>2</sub>-terminal (membrane distal)  $\beta$ 1 domain. This conservative substitution caused a cytoplasmic accumulation of I-A or I-E molecules in the respective cell line although predicted secondary-structure analysis suggests a minimal effect on protein conformation. Thus, the mutation appears to have either created a negative signal that stops transport or eliminated a positive signal that is required for transport and targeting to the cell surface.

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