Defective Intracellular Transport as a Common Mechanism Limiting Expression of Inappropriately Paired Class II Major Histocompatibility Complex α/β Chains

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Summary

Distinct combinations of class II major histocompatibility complex (MHC) α and β chains show widely varying efficiencies of cell surface expression in transfected cells. Previous studies have analyzed the regions of the class II chains that are critically involved in this phenomenon of variable expression and have shown a predominant effect of the NH2-terminal domains comprising the peptide-binding site. The present experiments attempt to identify the post-translational defects responsible for this variation in surface class II molecule expression for both interisotypic α/β combinations failing to give rise to any detectable cell membrane molecules (e.g., $E\alpha A\beta^k$) and intraisotypic pairs with inefficient surface expression (e.g., $A\alpha^d A\beta^k$). The results of metabolic labeling and immunoprecipitation experiments using L cell transfectants demonstrate that in both of these cases, the α and β chains form substantial amounts of stable intracellular dimers. However, the isotype- and allele-mismatched combinations do not show the typical post-translational increases in molecular weight that accompany maturation of the N-linked glycans of class II MHC molecules. Studies with endoglycosidase H reveal that no or little progression to endoglycosidase H resistance occurs for these mismatched dimers. These data are consistent with active or passive retention of relatively stable and long-lived mismatched dimers in a pre-*medial*-Golgi compartment, possibly in the endoplasmic reticulum itself. This retention accounts for the absent or poor surface expression of these α/β combinations, and suggests that conformational effects of the mismatching in the NH2-terminal domain results in a failure of class II molecules to undergo efficient intracellular transport.

T cell receptor recognition of either self or foreign MHC products is a cell surface event that depends on both the qualitative and quantitative nature of MHC molecules on the presenting cell membrane (1). For MHC class I, each locus encoding a heavy chain can contribute only one new species to a cell's MHC molecule display, albeit with a spectrum of bound peptides. For class II, the situation is more complex, as there is significant polymorphism in both the α and β chains comprising these heterodimeric molecules (2). Because a minimum requirement for class II molecule cell surface expression is assembly of individual α and β chains (2-7), if free intracellular mixing were to occur, the number of possible class II MHC molecules would be the product of the number of allelically and isotypically distinct α and β chains cosynthesized by the cell. However, biochemical analyses of normal cells and transformed cell lines, as well as studies using transfected cells, have shown that not all possible class II α/β pairs are detectable in or expressed on the surface of cells possessing a diversity of α and β chains (2, 3, 7, 8).

Studies carried out over the past several years in this and other laboratories have provided some insight into the basis for these limitations in class II molecule expression. Two different post-translational restrictions have been identified. First, transfection experiments using cells not expressing any endogenous class II gene products have shown that there is a great deal of variability in the potential of distinct α and β chain combinations to reach the surface membrane (9-15). In mice, haplotype-matched (*cis*-encoded) Aα and Aβ chains are efficiently expressed on the cell surface, whereas haplotype-mismatched combinations show variable surface expression efficiency, ranging from almost that of haplotype-matched combinations to no detectable expression at all (9, 10, 13). Interisotypic combinations (e.g., $E\alpha A\beta$) can be expressed on the membrane, but the restrictions on this process are even

more stringent than for isotypically matched combinations (11, 12, 15). Analyses using mutant and recombinant class II genes have established that the sites of incompatibility resulting in poor or absent surface expression in these cases lie predominantly in the region of the class II peptide-binding domain predicted to constitute the interacting pair of β-pleated sheets contributed by the α and β polypeptides, with secondary regions of importance at the ends of the putative binding domain helices (10, 12–14, 16).

A second mechanism limiting class II molecule expression, which operates in cells synthesizing more than one class II α and β combination, involves competition for precursor chains among distinct α/β combinations. Thus, class II heterodimers that are moderately well expressed when only one α and one β chain are present in a cell become undetectable if certain preferred partner α and/or β chains are cosynthesized (8, 17, 18). This feature of class II molecule assembly and expression limits functional surface molecule diversity to a smaller set of α/β combinations than would be expected based on the observed expression of individual α/β pairs by transfectants.

Although these two classes of constraints on class II molecule expression have been identified, less is known about the precise biochemical and cell biological mechanisms underlying these expression phenotypes. Class II MHC dimers are formed rapidly (within a few minutes) of initial chain synthesis (19), and, in normal cells, are coassembled during this time with a third, non-MHC-encoded, nonpolymorphic chain termed the invariant chain (Ii) (7, 20, 21). This complex is transported out of the rough endoplasmic reticulum (RER)[1] and through the Golgi stacks, undergoing post-translational glycosylation during this passage. The class II-Ii complexes then appear to move to an endosome-related compartment, where low pH and acidic proteases contribute to the removal of Ii and the loading of processed peptide antigen into the class II molecule binding site (7, 22–25). The peptide-bearing dimer then transits to the cell surface. Thus, limited or absent membrane expression could reflect either ineffective formation of stable α/β dimers in the RER, impaired egress from the RER or a subsequent intracellular organelle, degradation before attainment of surface expression, or even rapid loss from the cell surface. In the present experiments, we have used biochemical analysis of L cell class II gene transfectants to determine the site(s) of the intracellular defect(s) underlying the poor surface membrane display of several distinct class II MHC α/β combinations.

Materials and Methods

Transfected Cells Synthesizing Various Class II α and β Chains. Drug-resistant, stable gene transfectants of the L cell subline DAP.3 were prepared as previously described using the calcium phosphate coprecipitation method (26). The specific transfectants used in the present experiments and the respective introduced class II genes are as follows: AT5.2 (Eα/Aβ^k [12]), AT7.1G9 (Eα/A$\beta1^k$Eβ [12]),

[1] *Abbreviations used in this paper:* CT, COOH terminal; PAS, protein A-Sepharose; RER, rough endoplasmic reticulum.

RT10.3 (Eα/Eβ^d [11]), RT8.17H3A5 (Aα^d/Aβ^k [10]), and RT7.3HB4.5 (Aα^k/Aα^k [27]). For those α/β combinations yielding cell surface molecules detected by mAbs, populations of cells expressing levels of surface class II dimers similar to murine B cell lymphomas and hybridomas were selected by preparative flow microfluorimetry (9) and/or magnetic bead sorting. For those combinations not giving surface-expressed class II molecules, clones of drug-resistant colonies were screened and those with the highest levels of specific class II mRNA were chosen for study. All transfected cells were maintained in culture in DMEM (high glucose) with 10% heat-inactivated FCS in the presence of the appropriate selecting drug.

Monoclonal and Anti-COOH-terminal Peptide Antibodies. mAbs were used as culture supernatants, both for immunofluorescent staining and for immunoprecipitation. For class II molecules containing the $\beta1$ domain of Aβ^k, the antibody 10-2.16 was used (28); for cells synthesizing Eα, the antibody 14-4-4S was used (29). Polyclonal rabbit antisera reactive with each murine class II chain were generated by immunization with peptides corresponding to the COOH-terminal 13–15 residues of each chain together with an NH$_2$-terminal cysteine. These peptides were conjugated to KLH using m-maleimidebenzoic acid N-hydroxysuccinimide ester (MBS). The sequences of the peptides used were: Aβ, CQK-GPRGPPPAGLLQ; Eβ, CNQKGQSGLQPTGLLS; Aα, CRSG-GTSRHPGPL; and Eα, CKGIKKRNVVERRQGAL. The specificity of each of the antisera pools used in this study is documented in Fig. 1.

Flow Cytometry. Cells were analyzed for cell surface expression of class II dimers as previously described (12), using mAbs as a first-step reagent, followed by FITC-conjugated goat anti–mouse Ig (Cappel Laboratories, West Chester, PA). Stained cells were analyzed using a FACScan® flow cytometer (Becton Dickinson & Co., Mountain View, CA).

Metabolic Labeling, Immunoprecipitation, and SDS-PAGE. SDS-PAGE analysis of metabolically labeled and immunoprecipitated proteins from transfected cells was carried out by slight modifications of previously described techniques (30, 31). Briefly, transfected cells were harvested using trypsin-EDTA, washed three times in PBS with 2.5% dialyzed FCS, and precultured for 1–2 h at 37°C in RPMI or DMEM lacking leucine, but supplemented with 5% dialyzed FCS, 2 mM glutamine, 10 mM Hepes, and gentamicin. These precultured cells were pelleted and resuspended in the same medium containing 200–300 μCi/ml [^{3}H]leucine as [^{3}H]leucine in water, with 1/10 volume of 10× HBSS added to maintain isotonicity. After incubation for the indicated time, cells were either washed into label-free complete medium and reincubated for the chase times indicated, or directly pelleted and lysed in 4 mM CHAPS/0.05 M Tris/0.15 M NaCl for 45 min at 4°C. Nuclei and insoluble debris were removed by centrifugation, and the supernatant was used for immunoprecipitation. Lysates were pre-cleared using normal rabbit serum and *Staphylococcus aureus*, Cowan strain 1 (PanSorbin; Gibco Laboratories, Grand Island, NY), followed by protein A–Sepharose (PAS). Pre-cleared lysates were then mixed with antibody overnight, and antigen–antibody complexes were isolated with PAS. The isolated immunoprecipitates were either directly analyzed on SDS-10% polyacrylamide gels under reducing conditions or dissociated in SDS sample buffer and reprecipitated before gel electrophoresis.

For dissociation and reprecipitation, immune complexes adsorbed to PAS were suspended in 0.0625 M Tris, pH 6.8, containing 1% SDS, incubated at room temperature for 15 min, boiled for 3 min, and centrifuged to remove PAS. Supernatants were adjusted to 0.2% SDS by dilution in cell lysis buffer containing 0.5% NP-40. Samples were then incubated for 3–4 h at 4°C, and any free, renatured anti-

body was removed by incubation with PAS. Supernatants were then incubated overnight at 4°C with rabbit antisera, immune complexes isolated with PAS, and the eluted complexes run on SDS-PAGE.

All SDS gels were treated with En³Hance (New England Nuclear, Boston, MA), dried, and autoradiographs prepared at −70°C.

Endoglycosidase-H Treatment. The N-linked glycans of immunoprecipitated proteins were analyzed for sensitivity to endoglycosidase H (Endo H) (Boehringer Mannheim Biochemicals, Indianapolis, IN) digestion by suspending immune complexes adsorbed to PAS in 30 μl of 50 mM sodium citrate, pH 5.5, containing 0.1% SDS and 500 mIU/ml of Endo H (32). Mock or enzyme-treated samples were incubated at 37°C overnight under toluene vapor. 2× concentrated Laemmli sample buffer was added to adjust samples to 2% SDS, 0.0625 M Tris, pH 6.8, with 10% glycerol. The samples were boiled for 3 min and the dissociated proteins analyzed by SDS-PAGE.

Results

Stable α/β Dimers Form in Transfectants Showing No Surface Class II MHC Molecule Expression

We first asked whether combinations of α and β chains showing no detectable cell surface expression assemble into stable heterodimers by performing immunochemical studies on L cell transfectants cotransfected with plasmid DNA encoding the chains comprising such combinations. For these experiments, it was desirable to use antibodies that are not conformation sensitive. Many monoclonal anti–class II antibodies do not bind free α or β chains and are sensitive to the altered conformation of dimers composed of atypical (e.g., allele-mismatched) combinations of α and β (10). To increase the probability of detecting all forms of the class II chains within the L cells, rabbit antisera raised against synthetic peptides corresponding to the COOH-terminal (CT) segments of α or β were utilized for immunoprecipitation. These reagents react with free α and β chains, and also with SDS-denatured proteins (J.E. Coligan, and W.L. Maloy, unpublished observations), making it likely that they would detect all conformational forms of the class II chains, regardless of secondary structure.

To ascertain the specificity of these reagents, their reactivity with the class II MHC α and β polypeptides synthesized by normal hematopoietic cells was tested. BALB/c spleen cells were biosynthetically labeled and aliquots of the detergent lysates of the labeled cells were reacted with anti-CT Aβ or anti-CT Eβ antisera. Antigen-antibody complexes were isolated and denatured in SDS. The SDS concentration was reduced to 0.2% by dilution in an NP-40-containing buffer, and aliquots of the immunoprecipitated material were tested for reactivity with Aα-, Aβ-, Eα-, or Eβ-specific antisera. Gel analyses of the immunoprecipitated material (Fig. 1) demonstrate, first, that each of the antisera reacts only with the chain corresponding to the immunizing peptide, and second, that only isotype-matched α chains are recovered after initial precipitation with the β-specific antibodies (e.g., Aα with Aβ and Eα with Eβ).

Using these antisera, we examined L cells synthesizing an isotype-matched, well-expressed α/β combination (EαEβ)
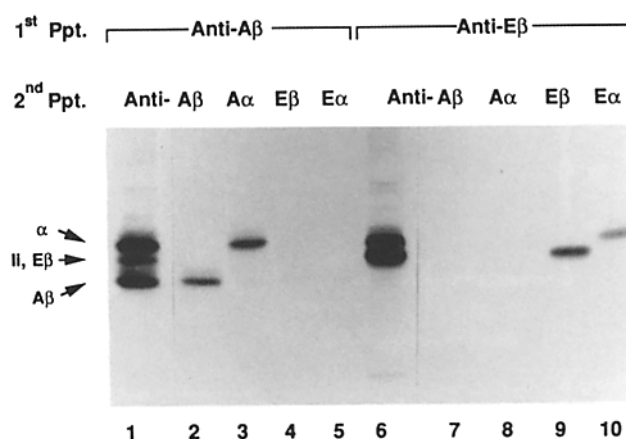

Figure 1. Reactivity of anti-CT antisera with murine class II polypeptides. BALB/c spleen cells were labeled with [³H]leucine for 6 h, then solubilized in 0.5% NP-40/0.05 M Tris/0.15 M NaCl. Precleared lysates were divided in half and incubated with either anti-CT Aβ (lanes 1–5) or anti-CT Eβ (lanes 6–10) antisera. Antigen-antibody complexes were isolated with PAS, then eluted by boiling in 1% SDS, as described in Materials and Methods. SDS was reduced to 0.2% by dilution, and aliquots of the samples were incubated with antisera raised against synthetic peptides corresponding to CT Aβ (lanes 2 and 7), Aα (lanes 3 and 8), Eβ (lanes 4 and 9), or Eα (lanes 5 and 10), as indicated. Immunoprecipitates were analyzed by SDS-10% PAGE. 5% of the Aβ and Eβ primary immunoprecipitates were analyzed directly, and are shown in lanes 1 and 6, respectively. The bands are identified to the left of the gel.

and others producing non–cell surface–expressed α/β combinations (Eα plus Aβ^k or Eα plus a hybrid β chain containing the β1 NH₂-terminal domain from Aβ^k and the β2, transmembrane, and cytoplasmic segments of Eβ [Aβ1^{k}Eβ] [12]). Typical FACS® profiles of these transfectants after indirect immunofluorescence staining using monoclonal anti–class II primary antibodies are shown in Fig. 2. They demonstrate the dramatic effect of the NH₂-terminal domain on cell surface coexpression of α and β, as previously reported (10, 12). Precleared lysates of biosynthetically labeled cells were incubated with antisera reactive with either the CT of the α or the β chain expressed by the transfectant. SDS-PAGE analysis of these immunoprecipitates (Fig. 3 A) indicated that in each case, incubation of detergent lysates with Eα-specific reagents led to coisolation of the β chain, regardless of whether or not an α/β dimer would ultimately be expressed at the cell surface (lanes 4, 5, and 6). The Eα/Aβ^k transfectant showed a lower amount of coprecipitated β chain, consistent with anti-β precipitations showing a substantially lower level of total β chain in these cells (data not shown). Screening of additional clones from this and other transfectants did not identify a cell with an amount of β chain similar to the other two cells used in this analysis. Therefore, the remainder of the experiments used the Eα/Aβ1^{k}Eβ transfectant as a representative mixed isotype combination failing to show cell surface expression. Fig. 3 B shows the reciprocal coprecipitation from lysates of this latter cell of α and β chains by each anti-CT antibody. Fig. 3 C demonstrates that α chain coprecipitation is observed using the Aβ1^k-specific mAb 10-2.16 (10) (lane 2). The capacity of the 10-2.16 mAb to precipi-
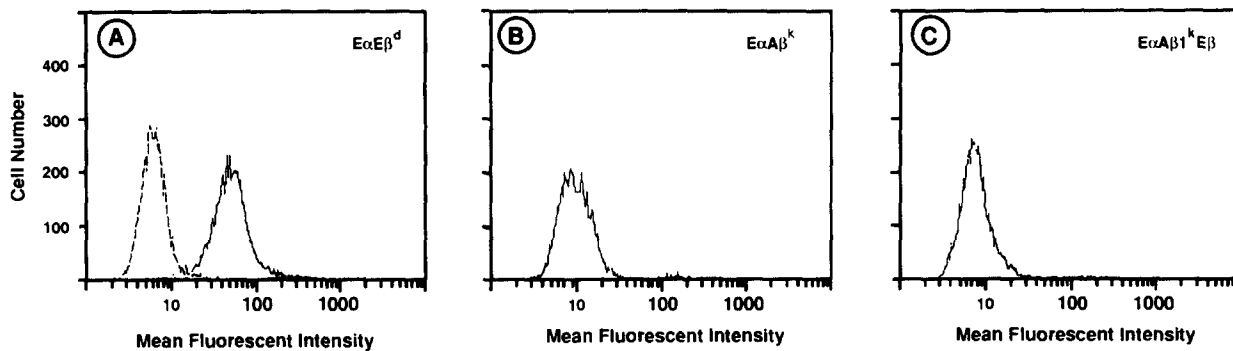
Figure 2. Cell surface expression of isotype-matched and isotype-mismatched α/β combinations. L cells transfected with plasmid DNA encoding the isotype-matched $E\alpha/E\beta^d$ combination (A), the isotype-mismatched $E\alpha/A\beta^k$ combination (B), or the isotype-mismatched combination $E\alpha/A\beta1^kE^b$ (C), all of which express high levels of α and β mRNA, were stained for cell surface expression of class II, using the $E\alpha$-specific antibody 14-4-4s (A, solid line) or the $A\beta$-specific antibody 10-2.16 (B and C). Also shown is background staining obtained with the FITC-goat anti–mouse Ig (GAM) developing reagent (A, dashed line). In B and C, all staining curves, including the background control, were identical.

tate an α/β dimer from the $E\alpha/A\beta1^kE\beta$ cotransfected cells also establishes that the failure to detect surface class II dimers on these cells with this antibody (see Fig. 2) is not a result of using the inappropriate reagent, but is a true reflection of the inability of such α/β dimers to reach the plasma membrane.

Poorly or Nonexpressed Dimers Show Defective Post-translational Modification

Nonexpressed α/β Combinations. Pulse-chase studies were performed to determine the intracellular fate and post-translational modifications of various assembled α/β dimers. L cell transfectants synthesizing α/β combinations giving either high or undetectable levels of surface molecules were pulse labeled and then chased in nonradioactive media for 90 min or 3 h. Immunoprecipitates were prepared from these cells and analyzed by SDS-PAGE. The results of one such experiment, shown in Fig. 4, suggest that for the non–cell surface–expressed combination of $E\alpha$ with $A\beta1^kE\beta$, although the α and β chains assemble into a dimer, they do not undergo the same types of post-translational modifications as do the dimers of the well-expressed, wild-type $E\alpha E\beta$ combination. Thus, in contrast to the chains of the latter dimer, which undergo a time-dependent increase in both molecular weight and microheterogeneity (lanes 2, 4, and 6), both the α and the β chain of the $E\alpha A\beta1^kE\beta$ dimer remain homogeneous in molecular weight (lanes 1, 3, and 5). Interestingly, with time, the α and β chains of the dimers that are not transported to the cell surface undergo a slight decrease in apparent molecular weight (compare lane 1 to lanes 3 and 5). This analysis indicates that the nonexpressed but assembled dimers persist for relatively long periods of time within the cell, in a modified form that is biochemically distinct from α/β dimers that are ultimately expressed at the cell surface.

Poorly Expressed α/β Combinations. Class II α and β chains that are isotype and allele matched (e.g., $A\alpha^k A\beta^k$) are expressed at the cell surface with high efficiency. In contrast,

some isotype-matched (e.g., $A\alpha A\beta$) but allele-mismatched (e.g., $A\alpha^d A\beta^k$) combinations are very poorly expressed at the cell surface (9, 10, 14, 16). To identify the intracellular events that lead to these differences in cell surface expression, and compare them to the intracellular handling of the nonexpressed interisotypic dimers described above, we examined
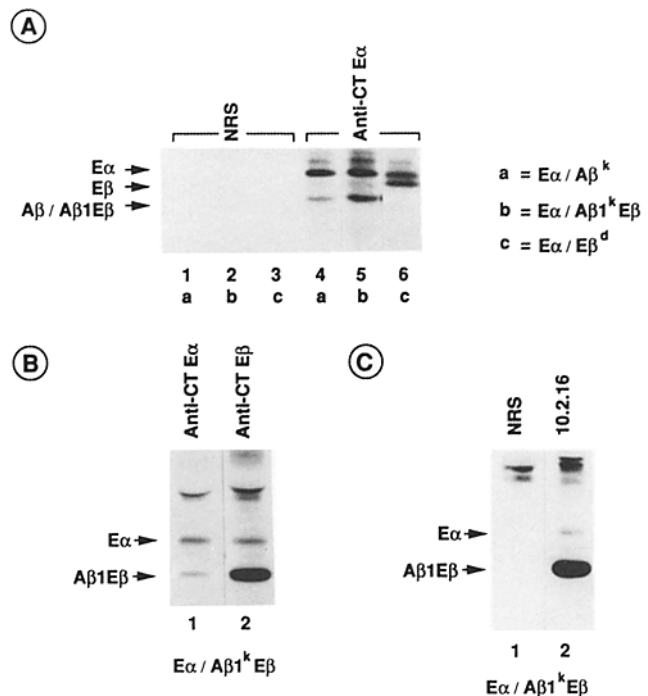

Figure 3. Stable assembly of α and β chains in transfected L cells. L cells transfected with plasmids encoding the class II chains indicated in each panel were biosynthetically labeled with [^{3}H]leucine for 3–4 h, solubilized in 6 mM CHAPS, 0.05 M Tris, 0.15 M NaCl, and pretreated with NRS and PAS. Aliquots of the pretreated detergent lysates were incubated with the antisera or mAb listed above each lane. Antigen-antibody complexes were isolated with PAS and immunoprecipitates were analyzed by SDS-10% PAGE. Shown at the left of each panel is the migration position of the α and β chains.
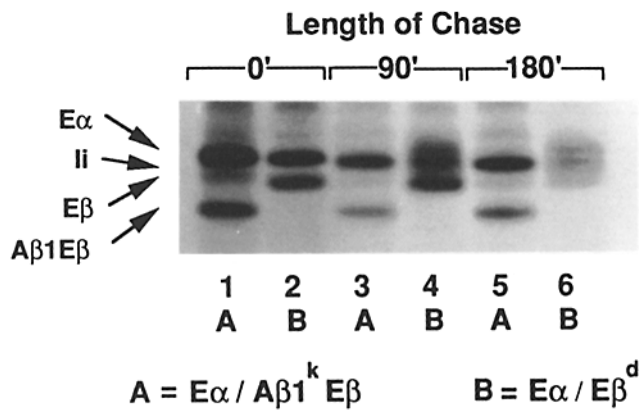
Length of Chase

┌──0'──┬──90'──┬──180'──┐

Eα
Ii
Eβ
Aβ1Eβ

1　2　3　4　5　6
A　B　A　B　A　B

$A = E\alpha / A\beta1^k E\beta$　　　$B = E\alpha / E\beta^d$

Figure 4. Intracellular fate of efficiently expressed isotype- and allele-matched α/β dimers vs. inefficiently expressed isotype-mismatched α/β dimers. L cells synthesizing the surface-nonexpressed interisotypic α/β combination $E\alpha A\beta1^k E\beta$ (A) or the efficiently expressed intraisotypic combination $E\alpha E\beta^d$ (B) were biosynthetically labeled with [³H]leucine for 20 min (0), or pulsed and then chased in nonradioactive medium for 90 or 180 min (90 or 180, respectively). Detergent lysates were prepared from the labeled cells and immunoprecipitated with antisera specific for Eα. Immunoprecipitates were analyzed by SDS-10% PAGE. The position of each of the class II chains and of Ii is indicated on the left of the figure.

Length of Chase

0 h　3 h　8 h　0 h　3 h　8 h

α →
Ii →
β →

1　2　3　4　5　6

$\underline{\text{A}\alpha^k / \text{A}\beta^k}$　$\underline{\text{A}\alpha^d / \text{A}\beta^k}$

Figure 5. Comparison of the post-translational processing of allele-matched and allele-mismatched intraisotypic α/β chain combinations. L cell transfectants producing the efficiently expressed allele-matched chains $A\alpha^k A\beta^k$ or the inefficiently expressed allele-mismatched chains $A\alpha^d A\beta^k$ were biosynthetically labeled with [³H]leucine for 30 min and either harvested immediately from culture ($T = 0$), or chased for 3 or 8 h in nonradioactive medium. Detergent lysates were immunoprecipitated with antisera specific for Aα, and immunoprecipitates were analyzed by SDS-10% PAGE. The positions of the α, β, and Ii chains are indicated on the left of the figure.

L cell transfectants that synthesized the same Aβ chain (Aβ^k) but that differed in the allelic origin of the α chain. Aβ^k is well expressed with its normal cis-encoded partner chain (Aα^k), but is poorly expressed with the d allelic variant of Aα (9). L cells producing Aβ^k and either Aα^k or Aα^d were pulse labeled for 30 min and chased in nonradioactive medium for 3 or 8 h. Class II chains were isolated using Aα or Aβ CT-specific antisera and the immunoprecipitated material analyzed by SDS-PAGE.

As can be seen in Fig. 5, coprecipitation of α with β-specific antibodies in the pulse-labeled samples (lanes 1 and 4) demonstrates that the assembly of the α and β chains is similar in both cell lines. This establishes that, as for the interisotypic dimers, the defect in cell surface expression is not α/β coassembly. Rather, as was seen in the L cells producing the surface-nonexpressed interisotypic $E\alpha A\beta1^k E\beta$ dimer, the subsequent intracellular processing of the interallelic Aα^dAβ^k dimer appears to be defective. In the cell line generating the efficiently expressed Aα^kAβ^k dimer, the individual α and β chains undergo an increase in molecular weight and microheterogeneity between the pulse and 3-h chase period (lanes 1 and 2), and these forms persist at 8 h (lane 3). In contrast, in the cell line producing the allele-mismatched Aα^dAβ^k combination, there are minimal detectable increases in molecular weight in the α or β chains at the 3-h chase period (compared lanes 4 and 5). In fact, at 3 h, there is a significant decrease in the amount of labeled class II MHC molecules recovered in the immunoprecipitates, and a significant fraction of the α chains that are precipitated display a lower apparent molecular weight compared with the α chains from pulse-labeled cells, as was also seen with the isotype-mismatched $E\alpha A\beta1^k E\beta$ combination.
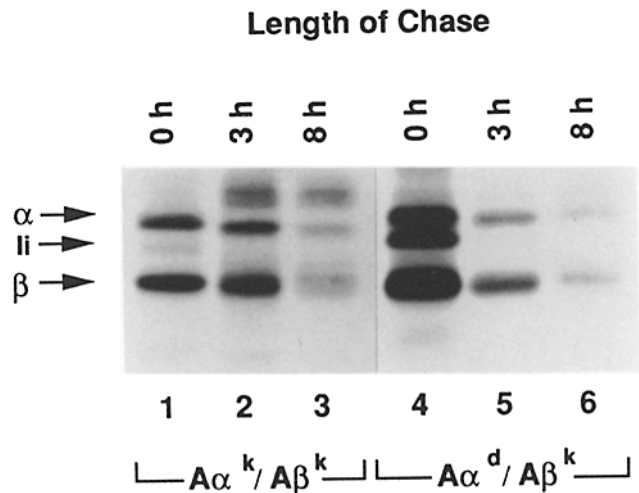
Biochemical Evidence for RER or Early Cis-Golgi Retention of α/β Dimers Showing Defective Cell Surface Expression

The preceding analysis of nonexpressed and poorly expressed class II MHC α and β combinations indicates a major defect in post-dimerization transport that is reflected in an absence of the usual post-translational modifications revealed by SDS-PAGE. To determine where inside the cell the processing events were interrupted, immunoprecipitated proteins were subjected to degradation by Endo H, which primarily cleaves immature, high-mannose N-linked oligosaccharides that are characteristic of glycoproteins that have not yet entered the medial-Golgi (33). In the experiment shown (Fig. 6), three types of class II dimers were examined; the isotype-matched pair $E\alpha E\beta^d$, which is efficiently expressed at the cell surface, the isotype-mismatched pair $E\alpha A\beta1^k E\beta$, which is not detectably expressed at the cell surface, and the haplotype-mismatched pair $A\alpha^d A\beta^k$, which is inefficiently expressed at the cell surface. Cells were pulse labeled for 45 min (P) and either harvested from culture or chased for an additional 4 h (C). Immunoprecipitates, prepared using anti-α antibodies, were denatured in SDS, and divided into two aliquots. One aliquot was treated with Endo H (EH), and the other was mock digested (UT). After such treatment, samples were incubated with either anti-α or anti-β CT antibodies, and individual chains were reprecipitated, then analyzed by SDS-PAGE (Fig. 6).

In the L cells synthesizing the efficiently expressed $E\alpha E\beta^d$ dimer, the α chains (compare Fig. 6 A, lanes 1 vs. 3) and β chains (Fig. 6 A, lanes 5 vs. 7) are sensitive (S) to Endo
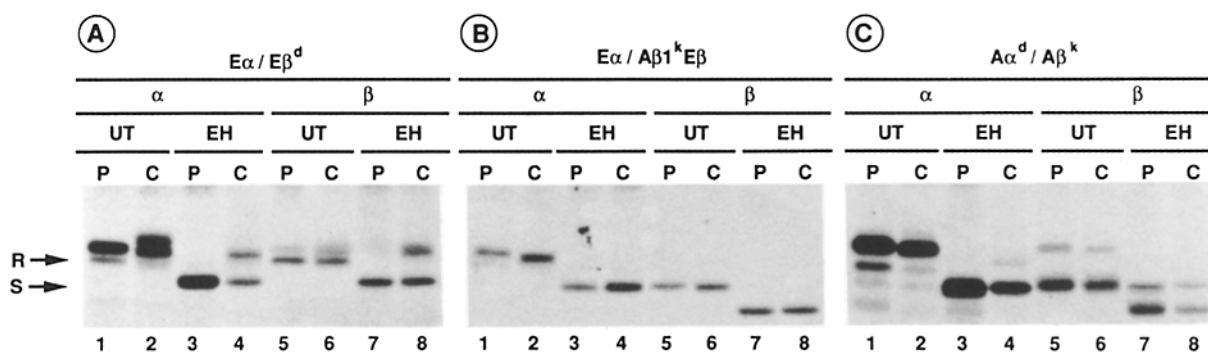
(A) Eα / Eβ^d   (B) Eα / Aβ1^k Eβ   (C) Aα^d / Aβ^k

α				β				α				β				α				β			
UT		EH		UT		EH		UT		EH		UT		EH		UT		EH		UT		EH	
P	C	P	C	P	C	P	C	P	C	P	C	P	C	P	C	P	C	P	C	P	C	P	C

R →   S →

1 2 3 4 5 6 7 8   1 2 3 4 5 6 7 8   1 2 3 4 5 6 7 8

Figure 6. Analysis of intracellular processing of efficiently expressed, inefficiently expressed, and nonexpressed class II MHC dimers. L cell transfectants synthesizing the indicated class II chains were pulse labeled with [³H]leucine for 15 min and either harvested directly from culture (Pulse, P) or chased in nonradioactive medium for 90 min (Chase, C). Detergent lysates were made from the labeled cells and α/β immunoprecipitates were prepared using anti-CT α-specific rabbit antisera. Immunoprecipitates were either digested with Endoglycosidase H (EH) or mock digested (UT), as described in Materials and Methods. Samples were then denatured in SDS to separate α and β chains and renatured with NP-40 buffer. α and β chains were reisolated using CT-α or -β specific antisera as indicated. Immunoprecipitates were analyzed by SDS-10% PAGE. R and S to the left of the figure refer to the positions of Endo H–resistant and Endo H–sensitive class II chains, respectively.

H after pulse labeling, as expected for glycoproteins that express high mannose oligosaccharides, but by 4 h, a significant fraction of the α chains (Fig. 6 A, lanes 3 vs. 4) and β chains (Fig. 6 A, lanes 7 vs. 8) have Endo H–resistant (R) N-linked glycans. This resistance presumably reflects the transport of the dimer from the RER to the Golgi complex, where processing of the N-linked oligosaccharides occurs (34). In contrast, in the cell lines that assemble dimers that are poorly expressed at the cell surface, the α and β chains remain Endo H–sensitive (S) throughout the chase (Fig. 6, B and C, lanes 3 vs. 4, and 7 vs. 8, respectively). This implies that they have not yet entered the subcellular compartment in which the glycosyltransferases responsible for conversion of oligosaccharides from the high mannose to complex type reside. It thus appears likely that the poorly expressed α/β dimers remain in the RER or the cis-Golgi, where they are degraded or from which they directly exit to a degradative compartment. Interestingly, the time-dependent change in apparent molecular weight of the transport-defective α and β chains is no longer apparent in the endo H–treated samples (compare Fig. 6, B and C, lanes 1 vs. 2 to lanes 3 vs. 4, and lanes 5 vs. 6 to lanes 7 vs. 8), indicating that the decrease in apparent molecular weight of such claims seen in Figs. 4 and 5 is due to modifications of the N-linked oligosaccharides.

Discussion

A large body of prior work has documented that in cells cosynthesizing a variety of allelic and isotypic forms of class II MHC α and β chains, only a limited subset of all possible α/β dimer combinations is assembled and transported to the surface membrane (2, 35). In part, this limitation reflects preferences in stable α/β pairing during initial dimer assembly so that favored combinations outcompete less-favored pairs for available free chains (17, 18). However, even in the absence of competing chains, some combinations of α and β give little or no detectable cell surface expression (9–15). In

the experiments described in this report, we have analyzed the intracellular fate of combinations of α and β chains showing poor or absent cell surface expression under noncompetitive conditions, which maximizes our ability to visualize the formation and post-translational processing of any dimers these combinations might form.

The fate of cosynthesized α and β chains has been examined biochemically in L cell transfectants representing three distinct expression phenotypes: (a) two different cell lines producing isotype- and/or allele-matched α/β combinations showing efficient cell surface expression (Eα/Eβ^d, Aα^k/Aβ^k); (b) a cell line making an inefficiently expressed allele-mismatched combination (Aα^d/Aβ^k); and (c) a cell line producing an isotype-mismatched α/β combination showing no detectable surface expression (Eα/Aβ1^kEβ). Biosynthetic labeling studies revealed that despite very marked differences in the efficiency with which each pair gave rise to surface molecules, these α/β combinations showed similar initial levels of assembled heterodimers stable to detergent solubilization and immunoprecipitation. These results establish that the defect in surface expression in the latter two cell lines is not primarily due to deficient assembly per se, although our experimental system does not permit us to exclude some contribution of less efficient assembly to the overall phenotype. After initial assembly, however, the post-translational processing of the various dimers appears to be distinct. The allele- and/or isotype-matched combinations undergo readily detectable post-translational modification of their N-linked carbohydrate chains, whereas the α/β pairs showing poor cell membrane expression do not. These data indicate that both locus-specific and allelically polymorphic residues in the NH₂-terminal domains of class II molecules control conformational features of the assembled dimers that regulate post-translational processing and intracellular transport.

Our results differ from those recently reported by Karp et al. (15), who did not observe stable intracellular dimers of human class II α and β combinations that failed to give

detectable surface molecules. There are several possible explanations for the differences seen in these two experimental systems. The first is that the control of expression of human and mouse class II gene products is distinct in the two species. It is conceivable that the greater number of class II gene products produced in individual human cells (2) has led to a need for stricter regulation of assembly, so that competition for component chains does not deplete intracellular chain levels required for adequate expression of each isotype. The importance of such intracellular competition in determining patterns of cell surface class II molecule expression has been demonstrated in both our transfection model (18) and more recently in $A\beta$ transgenic mice (36). A second possibility is that there may be substantially more diversity in the capacity of various α/β combinations to stably assemble than revealed in the set of molecules we have examined in this report. The pair tested by Karp et al. (15) might then represent one of the more unfavored combinations. It is also likely that detection of assembled dimers depends on the experimental conditions used to immunoprecipitate the chains, with instability of weakly associated dimers in certain buffer/detergent combinations. The importance of the particular conditions of cell lysis and immunoprecipitation in preservation of TCR-CD3 associations is well known and perhaps relevant in this regard (37). This same explanation may underlie the failure to coprecipitate $A\beta^f$ with $E\alpha$ from cells of A.TFR5 mice (38), which show weak but significant expression of $E\alpha$ epitopes on the cell surface, as our own studies indicate that such expression requires dimer formation and is not due to $E\alpha$ transport to the membrane alone (A.J. Sant, C. Layet, and R.N. Germain, unpublished observations).

The class II molecules that are retained within the transfectants we have studied do not have any detectable conformational features that distinguish them from efficiently transported dimers. We have tested their reactivity with a panel of mAbs, and have not observed the absence of any serological epitopes predicted to be present from studies on conventional dimers containing these chains (A.J. Sant, L.R. Hendrix, and R.N. Germain, unpublished observations). This contrasts with results obtained studying expression-defective mutants of various viral envelope proteins (39, 40), but perhaps is more related to the limited number of epitopic sites seen by murine anti-MHC mAbs than to an absence of conformational differences among the well- and poorly expressed class II MHC α/β dimers.

In addition, both well- and poorly or non-expressed dimers appear to coassemble with invariant chain (see Fig. 4, lane 1). Although we do not know whether the sites or affinity of interaction are equivalent in the two cases, recent work in this laboratory has revealed that the cell surface expression that is observed for such complexes as $A\alpha^d A\beta^k$ is due in large measure to the ability of the invariant chain to "rescue" these dimers from retention in a late ER/early Golgi compartment (41). These recent results showing that Ii can contribute in a positive sense to cell surface expression of otherwise poorly expressed dimer combinations make it unlikely that inappropriate α/β dimer interaction with invariant chain

is responsible for the transport defect(s) we have observed. Because it is not required for and does not markedly augment the transport and surface expression of haplotype- and isotype-matched dimers (41–43), it remains possible, however, that a limitation in availability of Ii in the L cells contributes disproportionately to the poor expression observed for allele- and isotype-mismatched α/β combinations in these cells.

The structural features of proteins that regulate their intracellular movement are poorly defined at the present time. One major area of uncertainty is whether successful transport between organelles is a consequence of expression of an appropriate positive signal, which allows recognition by a receptor protein responsible for transport, or if successful transport is due to the lack of expression of retention signals. Evidence in favor of each of these mechanisms exists in different model systems (44–49). A variety of reports provide strong support for the view that some proteins are retained in the endoplasmic reticulum as a result of their association with other proteins containing specific retrieval signals that mediate their recycling from an intermediate compartment interposed between the endoplasmic reticulum and cis-Golgi compartment (50–52). One such retrieval (retention) protein is BiP, one of the KDEL-containing lumenal endoplasmic reticulum proteins believed to play an important role in polypeptide folding (53). Rothman (54) has speculated that BiP and related polypeptide chain binding proteins interact with varying affinity with discrete peptide patches on proteins during their import into the endoplasmic reticulum (54). When appropriate folding occurs, these sites become unavailable for continued BiP binding, and coretrieval of the protein with BiP from the salvage pathway ceases. This allows egress to the later Golgi and secretory/transport compartments. Inappropriately folded molecules would not lose these interaction patches and be subject to continuous retrieval by association with a KDEL-containing molecule.

This model of transport blockade may be applicable to both our present results and the earlier work of Griffith et al. (55), who studied B lymphomas with defects in surface class II molecule expression. An identical mutation at a conserved residue in the $A\beta^d$ or $E\beta^d$ chains led to assembly of immunoprecipitable dimers without full transport to the membrane. For the $A\alpha^d A\beta^d$ molecule, fully modified N-linked glycans were observed, suggesting arrest in a post-medial Golgi compartment, whereas for $E\alpha^d E\beta^d$, the dimers exhibited only core glycosylation, consistent with the results reported here (56). These dimers again were serologically indistinguishable from wild-type, despite their arrested intracellular transport. It would thus appear that rather subtle structural variation from fully wild-type molecules is sufficient to interfere with normal class II molecule movement within the cell.

We have not yet precisely localized the site of retention of the aberrantly assembled class II molecules by ultrastructural methods. The time-dependent loss in molecular weight that the nonexpressed dimers undergo appears to be due to the activity of glycosidases rather than proteases, based on the finding that the difference in molecular weight disappears

when the N-linked oligosaccharides are removed by Endo H. Trimming of N-linked oligosaccharides chains occurs in both the RER and cis-Golgi, through the activity of glucosidases and mannosidases (34). Based on the magnitude of decrease in molecular weight that α and β undergo (1,500), it is likely that they have been completely trimmed, suggesting that they have reached the cis-Golgi compartment. Glycoproteins bearing such trimmed carbohydrate groups remain sensitive to Endo H. Addition of N-acetyl glucosamine, through the activity of GlcNAc transferase I and II, which occurs in the medial-Golgi compartments, renders molecules resistant to Endo H (33). Given the complete sensitivity of the surface nonexpressed but assembled dimers to Endo H, our data suggest that the block in intracellular transport is

before arrival in the medial-Golgi compartment. The newly described intermediate or salvage compartment between the RER and Golgi compartment (52) may actually constitute the site of carbohydrate trimming referred to above, consistent with a retention mechanism involving retrograde transport back to the RER in association with BiP-like molecules. However, preliminary studies examining the relative association of the transportable vs. retained dimers with such endoplasmic reticulum–resident polypeptides have not shown any striking differences. Additional studies of the transport-defective molecules reported here will be necessary to define the mechanism of this block, and should provide useful information about intracellular protein trafficking in general.

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