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Association with BiP and aggregation of class II MHC molecules synthesized in the absence of invariant chain

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Class II molecules of the major histocompatibility complex (MHC) are composed of two polymorphic glycoprotein chains (α and β), that associate in the ER with a third, non-polymorphic glycoprotein known as the invariant chain (Ii). We have examined the relationship between the intracellular transport and physico-chemical characteristics of various combinations of murine α, β and Ii chains. Biochemical and morphological analyses of transfected fibroblasts expressing class II MHC chains show that both unassembled α and β chains, as well as a large fraction of $\alpha + \beta$ complexes synthesized in the absence of Ii chain, are retained in the ER in association with the immunoglobulin heavy chain binding protein, BiP. Analyses by sedimentation velocity on sucrose gradients show that most incompletely assembled class II MHC species exist as high molecular weight aggregates in both transfected fibroblasts and spleen cells from mice carrying a disruption of the Ii chain gene. This is in contrast to the sedimentation properties of $\alpha\beta$Ii complexes from normal mice, which migrate as discrete, stoichiometric complexes of M_r ~200 000-300 000. These observations suggest that assembly with the Ii chain prevents accumulation of aggregated α and β chains in the ER, which might relate to the known ability of the Ii chain to promote exit of class II MHC molecules from the ER.

Introduction

Many proteins expressed at the surface of eukaryotic cells have been shown to be part of oligomeric complexes (reviewed by Hurtley and Helenius, 1989). In the majority of cases, assembly of such oligomeric complexes occurs shortly after synthesis of the individual subunits in the endoplasmic reticulum (ER). Failure to assemble complete complexes has often been associated with retention of subunits or incomplete complexes within the ER (reviewed by Rose and Doms, 1988; Hurtley and Helenius, 1989). Although ER retention of incompletely assembled complexes has been extensively documented in the literature, its causes remain poorly understood. It is also unclear whether the underlying mechanisms that function to retain incompletely assembled complexes in the ER are related to those involved in retaining other structurally abnormal proteins, such as mutant or aberrantly processed proteins. Two potential mechanisms have been invoked to explain the retention of these abnormal proteins: (i) stable interaction with ER resident proteins and (ii) formation of transport-incompetent aggregates (Rose and Doms, 1988; Hurtley and Helenius, 1989). Like many abnormal proteins, some unassembled subunits and incompletely assembled complexes have been shown to associate with both soluble (Blount and Merlie, 1991; Forsayeth *et al.*, 1992; Melnick *et al.*, 1992) and transmembrane (Degen and Williams, 1991; Hochstenbach *et al.*, 1992; David *et al.*, 1993) ER resident proteins. However, despite ample evidence for the aggregation of mutant proteins in the ER, it is currently not known whether such aggregation is also common to unassembled chains of multiprotein complexes. In the present study, we have addressed this issue by examining the relationship between the intracellular transport and physical characteristics of various combinations of class II molecules of the major histocompatibility complex (MHC) and the associated invariant (Ii) chain.

Surface class II MHC molecules are non-covalent heterodimers, composed of two polymorphic integral membrane glycoprotein subunits, known as α and β (reviewed by Kaufman *et al.*, 1984). The α and β chains assemble in the ER, where they associate with homotrimers of a third integral membrane glycoprotein, Ii (Jones *et al.*, 1979; Kvist *et al.*, 1982; Machamer and Cresswell, 1982; Marks *et al.*, 1990) to form a nine subunit $(\alpha\beta Ii)_3$ complex (Roche *et al.*, 1991; Lamb and Cresswell, 1992). Once assembly is completed, the complexes are transported out of the ER and through the Golgi system. Eventually, the complexes are delivered into a prelysosomal compartment (Neefjes *et al.*, 1990; Peters *et al.*, 1991; Romagnoli *et al.*, 1993) where the Ii chain is removed by proteolytic degradation (Blum and Cresswell, 1988; Nguyen and Humphreys, 1989). Upon removal of the Ii chain, $\alpha\beta$ dimers become competent for binding peptides generated by proteolysis of internalized antigens (Roche and Cresswell, 1990; Teyton *et al.*, 1990; Germain and Hendrix, 1991). The mature class II molecules are subsequently delivered to the cell surface, where they function to present peptides to $CD4^+$ T cells.

As with other oligomeric complexes, synthesis of both the α and β chains is required for expression of class II MHC molecules at the cell surface (Murphy *et al.*, 1980; Miller and Germain, 1986). Recently, the Ii chain has been shown to play several roles in the intracellular transport and function of class II molecules. While early studies indicated that Ii chain synthesis is not strictly essential for attaining significant levels of class II MHC expression at the surface of transfected fibroblasts (Miller and Germain, 1986; Sekaly

et al., 1986), more recent studies have demonstrated that the Ii chain enhances the efficiency of class II transport from the ER (Layet and Germain, 1991; Anderson and Miller, 1992; Bikoff *et al.*, 1993; Viville *et al.*, 1993). In addition, the Ii chain has been shown to prevent access of peptides to the peptide binding groove in the class II molecules, until its removal in the prelysosomal compartment (Roche and Cresswell, 1990; Teyton *et al.*, 1990; Roche *et al.*, 1992). The Ii chain has also been shown to direct class II molecules to the prelysosomal compartment by virtue of a targeting signal in its cytoplasmic domain (Bakke and Dobberstein, 1990; Lotteau *et al.*, 1990; Lamb *et al.*, 1991; Romagnoli *et al.*, 1993). Through these roles the Ii chain contributes to both the efficiency and the fidelity of antigen presentation.

The present study was undertaken to examine the intracellular fate and physico-chemical properties of unassembled class II MHC chains and of class II MHC $\alpha + \beta$ complexes synthesized in the absence of Ii chain. The results of our experiments reveal that the free α and β chains, and to a large extent $\alpha + \beta$ complexes, are retained within the ER of transfected fibroblasts in association with the immunoglobulin heavy chain binding protein, BiP. In addition, sedimentation velocity analyses show that the class II MHC chains retained in the ER exist as heterogeneous, high molecular weight aggregates in both transfected fibroblasts and splenic cells from mice carrying a disruption of the Ii chain gene. This is in contrast with the assembly of newly synthesized class II MHC chains with the Ii chain in splenic cells from normal mice, in which discrete stoichiometric complexes are formed that are competent for transport out of the ER. The aggregation of incompletely assembled subunits and the association of the aggregates with ER resident proteins such as BiP may be responsible for the retention of such species within the ER.

Results

Fate of class II MHC α and β chains synthesized in the absence of Ii chain in transfected fibroblasts

The fate of newly synthesized class II MHC chains in the absence of the Ii chain was first examined in the African green monkey fibroblast cell line, COS-1. These cells have been previously shown to lack any detectable expression of Ii chain (Miller and Germain, 1986; Sekaly *et al.*, 1986). COS-1 cells were analyzed following transient transfection with plasmids carrying the mouse $A\alpha^k$ (Benoist *et al.*, 1983) and $A\beta^k$ (Estess *et al.*, 1986) cDNAs. COS-1 cells express the SV40 large T antigen, which promotes replication of plasmids carrying the SV40 origin (Gluzman, 1981), like those used in our studies. This leads to high levels of expression of the transfected genes. When expressed separately in COS-1 cells, both $A\alpha^k$ and $A\beta^k$ chains remained sensitive to endoglycosaminidase H (endo H) for long periods after synthesis (Figure 1). Only a small amount of $A\beta^k$ (<5%) developed endo H resistance at 6 h after synthesis (Figure 1, lane 18). Immunofluorescence microscopy of cells transfected with either $A\alpha^k$ (Figure 2a) or $A\beta^k$ (Figure 2b) showed prominent staining of a cytoplasmic network characteristic of the ER and of the nuclear envelope. The network was identified as the ER by colocalization with ER resident proteins recognized by a specific polyclonal antiserum (Louvard *et al.*, 1982; data not

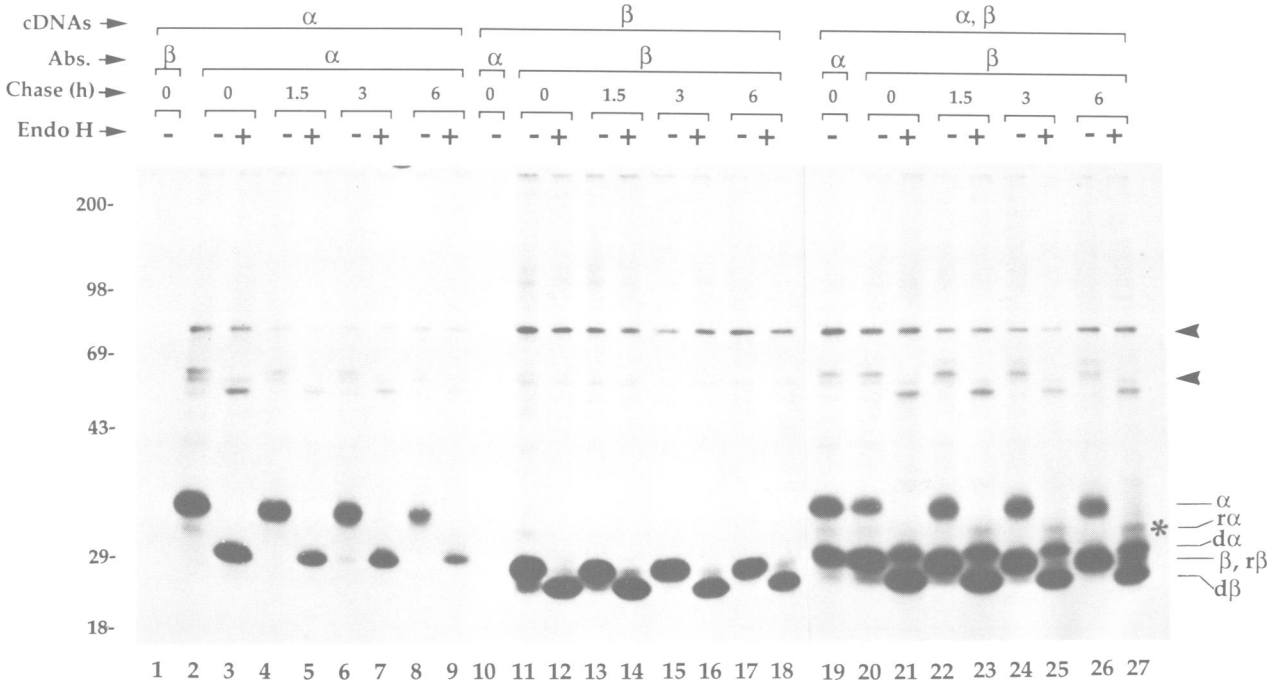

Fig. 1. Pulse–chase analysis of class II MHC chains expressed in COS-1 cells. COS-1 cells transfected with plasmids encoding the $A\alpha^k$ chain (α, lanes 1–9), the $A\beta^k$ chain (β, lanes 10–18), or both chains (α, β, lanes 19–27), were metabolically labeled with [^{35}S]methionine for 30 min and chased for different periods, as indicated in the figure. Proteins were isolated by immunoprecipitation with anti-$A\alpha^k$ (α, lanes 2–10, 19) or anti-$A\beta^k$ (β, lanes 1, 11–18, 20–27) antibodies, as described in Materials and methods. Lanes 1 and 10 represent non-specific immunoprecipitation controls. Immunoprecipitates were either treated with endo H (+) or not treated (−) before analysis by SDS–PAGE under reducing conditions on 10% acrylamide gels. The positions of molecular weight markers (expressed as $10^{-3} \times M_r$) are shown on the left. Abbreviations on the right are: α, $A\alpha^k$; rα, partially endo H-resistant $A\alpha^k$; dα, *N*-deglycosylated $A\alpha^k$; β, $A\beta^k$; rβ, endo H-resistant $A\beta^k$; dβ, *N*-deglycosylated $A\beta^k$. The asterisk on the right marks the position of the partially endo H-resistant form of $A\alpha^k$. Arrowheads indicate labelled proteins that were specifically coprecipitated with $A\alpha^k$ and $A\beta^k$.

shown). These observations suggested that unassembled class II MHC chains were largely retained within the ER, in agreement with previous studies that showed a requirement for the synthesis of both α and β for expression at the cell surface (Murphy *et al.*, 1980; Miller and Germain, 1986).

When COS-1 cells were cotransfected with plasmids encoding the $A\alpha^k$ and $A\beta^k$ chains, >90% of the transfected cells expressed both chains, as assessed by immunofluorescence microscopy (Figure 2c and d and data not shown). Pulse–chase analysis of the double transfectants showed that a fraction of $A\alpha^k$ chains that coprecipitated with $A\beta^k$ became resistant to endo H (~30% by 6 h of chase, Figure 1, lane 27, asterisk). The migration of the partially resistant $A\alpha^k$ species ($r\alpha$, Figure 1) between the fully glycosylated (α) and deglycosylated forms of the protein ($d\alpha$) is due to the fact that only one of its two N-linked carbohydrate chains is normally processed to complex carbohydrates in the Golgi system (Swiedler *et al.*, 1985; Layet and Germain, 1991). Even in this case, however, most of the coprecipitated $A\alpha^k$ chains remained sensitive to endo H digestion at 6 h after synthesis (Figure 1, lanes 19–27), suggesting that processing of $A\alpha^k$ + $A\beta^k$ carbohydrates was inefficient. To ascertain whether the inefficient processing of the newly synthesized chains was due to their overexpression in COS-1 cells, a similar analysis was performed with another African green monkey fibroblast line, CV-1. Unlike COS-1 cells, CV-1 cells do not express the SV40 T antigen, and thus are unable to replicate the transfected plasmids. Levels of gene expression are therefore less than those in COS-1 cells. The results obtained with CV-1 cells were similar to those obtained with COS-1 cells, in that only 20–50% of $A\alpha^k$ that was coprecipitated with $A\beta^k$ in different experiments was processed in the Golgi system after 6 h (data not shown). The persistence of endo H-sensitive species, even when $A\alpha^k$ and $A\beta^k$ were expressed together, suggested that $A\alpha^k$ + $A\beta^k$ complexes were not efficiently transported out of the ER.

Immunofluorescence microscopy of COS-1 cells co-expressing $A\alpha^k$ and $A\beta^k$ revealed the presence of some of the class II MHC chains at the plasma membrane, as evidenced by the staining of the cell edges, although there was also staining of an intracellular network characteristic of the ER (Figure 2c and d). Taken together, these biochemical and morphological data are consistent with previous studies suggesting that, while the Ii chain is not strictly essential for class II MHC expression at the cell surface (Miller and Germain, 1986; Sekaly *et al.*, 1986), the efficiency of $\alpha\beta$ transport from the ER is relatively low

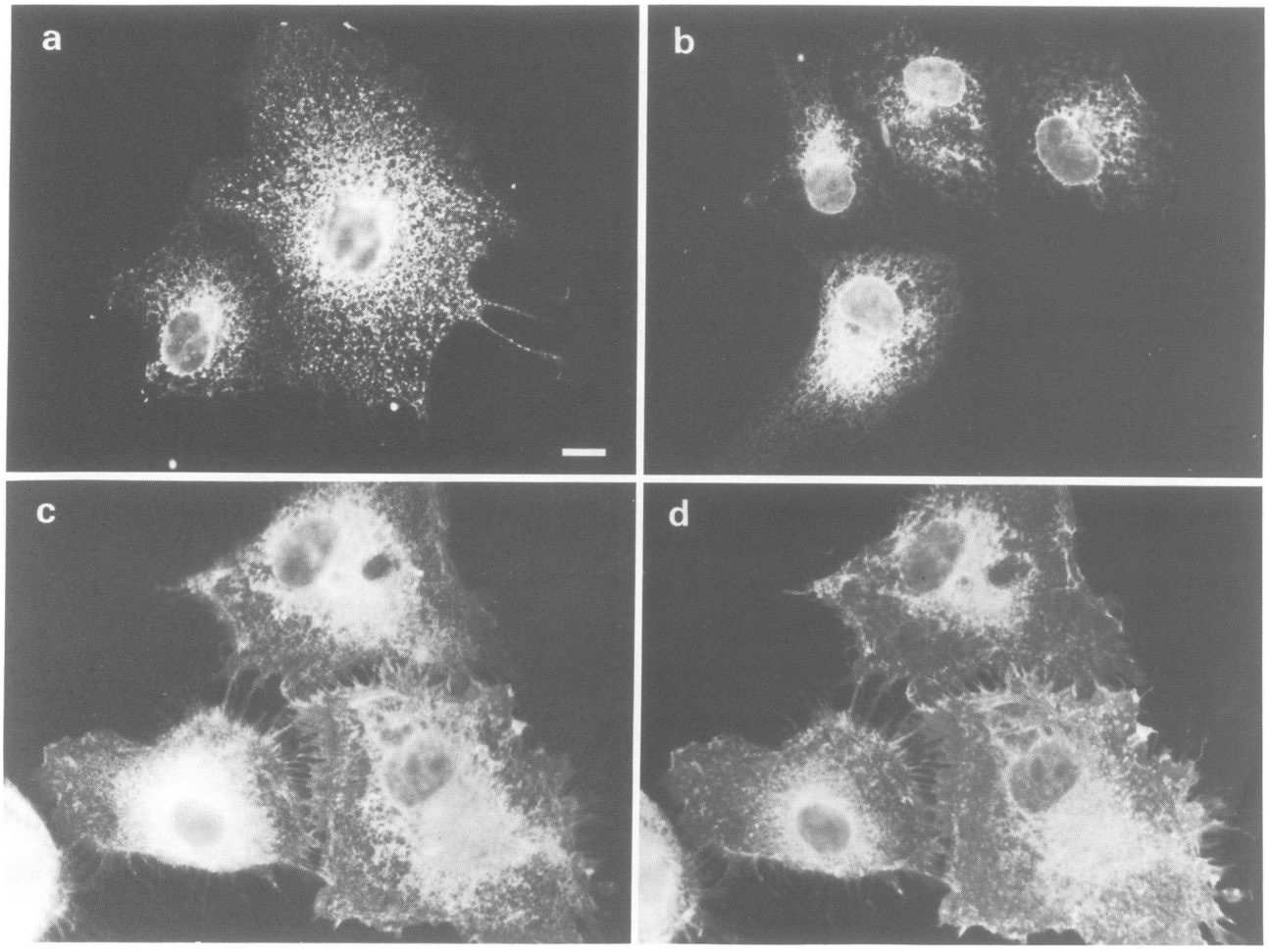

Fig. 2. Immunofluorescence microscopy of COS-1 cells expressing class II MHC chains. COS-1 cells expressing $A\alpha^k$ (**a**), $A\beta^k$ (**b**) or both chains (**c** and **d**) were stained with anti-$A\alpha^k$ (a), anti-$A\beta^k$ (b) or a mixture of both antibodies (c and d). Binding of the primary antibodies was revealed with fluorescein-conjugated donkey anti-rabbit IgG (a), rhodamine-conjugated donkey anti-mouse IgG (b) or both antibodies (c and d). (c) shows the distribution of $A\alpha^k$ and (d) the distribution of $A\beta^k$ in the same cells. Bar = 5 μm.

in the absence of Ii chain synthesis (Layet and Germain, 1991; Anderson and Miller, 1992; Bikoff *et al.*, 1993; Romagnoli *et al.*, 1993; Viville *et al.*, 1993).

Association of the ER retained class II MHC chains with BiP

To investigate the cause of the ER retention of unassembled chains or partially assembled complexes of class II MHC molecules, we first examined whether the newly synthesized chains might be binding to ER resident proteins. Interestingly, at least two protein species were found to coprecipitate specifically with class II MHC chains labelled during a 30 min pulse with [^{35}S]methionine. Aα^k immunoprecipitates contained a prominent M_r 78 000 endo H-resistant species and a group of two or three endo H-sensitive species in the M_r 62 000–64 000 range (Figure 1, lanes 2–9, arrowheads), whereas Aβ^k immunoprecipitates contained only the M_r 78 000 species (Figure 1, lanes 11–18). Similar bands were observed in immunoprecipitates from cells expressing both Aα^k and Aβ^k (lanes 19–27). The co-isolation of these species with class II MHC chains was specific, as they were not observed in non-specific immunoprecipitation controls (lanes 1 and 10) or in immunoprecipitates of Ii chain (e.g. Figure 6). While the M_r 78 000 band was clearly observed in all immunoprecipitates, the intensity of the M_r 62 000–64 000 glycoproteins was more variable from experiment to experiment. Other, fainter bands were also seen in immunoprecipitates from the Triton X-100 solubilized cells; however, their appearance was also not reproducible in all experiments. No other labelled proteins were coprecipitated with class II MHC chains when gentler solubilization agents, such as 1% digitonin, were used instead of Triton X-100, or by the addition of apyrase to deplete extracts of ATP (data not shown).

In order to examine the possible association of class II MHC chains with proteins which may not be efficiently labelled during a short pulse with [^{35}S]methionine, cells expressing Aα^k and Aβ^k were also labelled for 24 h with [^{3}H]leucine. Immunoprecipitation from these cells revealed that the M_r 78 000 protein was the only species that associated with the class II MHC chains in significant amounts under the conditions in our experiments (Figure 3, lane 3). The M_r 62 000–64 000 glycoproteins and a few other species were present in much smaller amounts in these immunoprecipitates and could only be appreciated upon prolonged exposures of the X-ray films.

Two additional experiments were performed to confirm that the species coprecipitated from pulse-labelled cells corresponded to proteins bound to class II MHC chains in the ER. In the first experiment, we prepared microsomes from metabolically labelled cells expressing Aα^k and Aβ^k. The microsomes were incubated with or without proteinase K to digest any cytosolic proteins that might have been adsorbed to the microsomal vesicles. Following solubilization with Triton X-100, Aα^k + Aβ^k complexes were isolated by immunoprecipitation. Both the M_r 78 000 protein and the M_r 62 000–64 000 glycoproteins were coprecipitated with class II MHC chains from the solubilized microsomal fractions (Figure 4a, lanes 1–4). The migration of the associated proteins did not change upon treatment of the labelled microsomes with proteinase K, indicating that the proteins were not cytoplasmically exposed, but were instead contained within the lumen of the microsomal vesicles

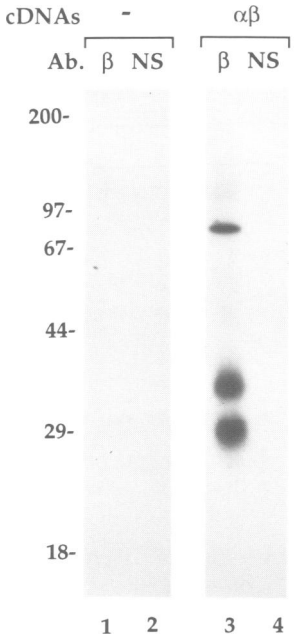

Fig. 3. Immunoprecipitation of class II MHC chains from cells labelled for 24 h with [^{3}H]leucine. COS-1 cells transfected with either an irrelevant plasmid (pCDM8, lanes 1 and 2) or with plasmids encoding the Aα^k and Aβ^k chains ($\alpha\beta$, lanes 3 and 4) were metabolically labelled for 24 h with [^{3}H]leucine, as described in Materials and methods. Proteins were isolated by immunoprecipitation with either an irrelevant antibody (NS, lanes 2 and 4) or with the anti-β^k antibody, 10-2.16 (β, lanes 1 and 3) and resolved by SDS–PAGE under reducing conditions on a 10.5% acrylamide gel. The positions of molecular weight markers (expressed as $10^{-3} \times M_r$) are shown on the left.

(Figure 4a, lanes 1–4). In a second experiment, we mixed a detergent extract from unlabelled cells expressing Aα^k and Aβ^k with an extract from labelled but untransfected cells. The unlabelled Aα^k + Aβ^k complexes were isolated with specific antibodies. A few faintly labelled bands were recovered in the immunoprecipitates (Figure 4b, lanes 5–8). However, as defined by molecular weight, glycosylation state and intensity of labelling, none of these bands corresponded to the M_r 78 000 and M_r 62 000–64 000 species. From these experiments we concluded that the association of class II MHC molecules with the M_r 78 000 and M_r 62 000–64 000 species was not an artefact of solubilization or immunoprecipitation, but rather reflected an association occurring in the ER of intact cells.

The size of the M_r 78 000 species, its lack of N-linked glycans, and its association with unprocessed forms of the class II MHC chains suggested to us that it might be the immunoglobulin heavy chain binding protein, BiP (Haas and Wabl, 1983; Bole *et al.*, 1986). BiP is an ER resident protein that has previously been shown to interact with many newly synthesized proteins (reviewed by Gething and Sambrook, 1992). The exact physiological role of BiP is not known with certainty, although a number of studies have suggested functions in protein folding and assembly (reviewed by Rothman, 1989; Gething and Sambrook, 1992), translocation across the ER membrane (Vogel *et al.*, 1990; Nguyen *et al.*, 1991) and retention of abnormal proteins in the ER (Haas and Wabl, 1983; Dorner *et al.*, 1988; Hurtley *et al.*, 1989; Suzuki *et al.*, 1991). One- and two-dimensional electrophoretic analysis of class II MHC chains immunoprecipitated from a labelled microsomal fraction confirmed the identity of the M_r 78 000 protein as BiP by demonstrating

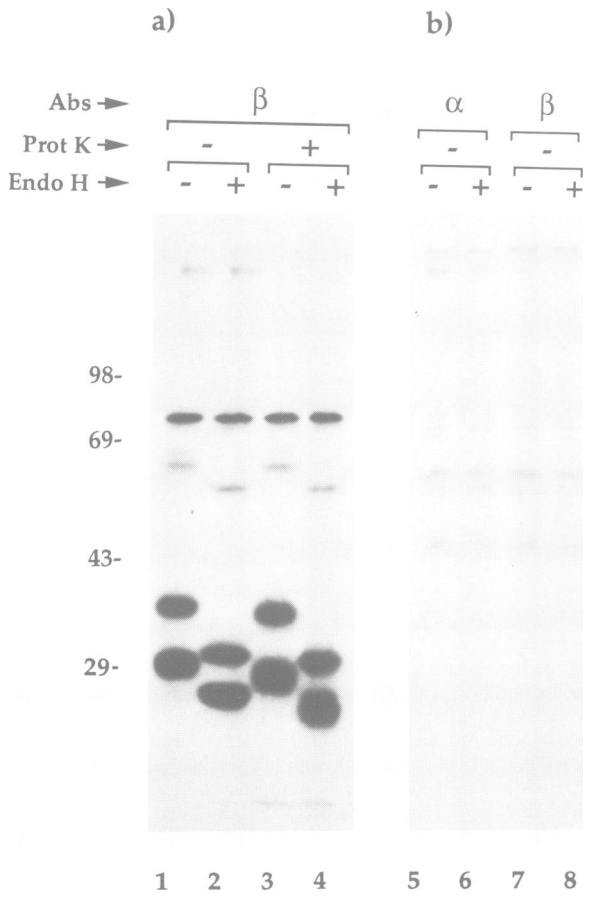

Fig. 4. Specificity of proteins coprecipitated with class II MHC chains. (**a**) COS-1 cells expressing the $A\alpha^k$ and $A\beta^k$ chains were metabolically labelled with [^{35}S]methionine for 30 min. Fractions enriched in ER vesicles were prepared by centrifugation on discontinuous sucrose gradients (Bole *et al.*, 1986) and incubated in the absence or presence of proteinase K (Prot K in the figure), as described (Bonifacino *et al.*, 1991). Class II MHC chains were isolated by immunoprecipitation with the anti-$A\beta^k$ antibody 10-2.16 and incubated without (−) or with endo H (+) before analysis by SDS−PAGE under reducing conditions on 10% acrylamide gels. Notice the slight shift in molecular weight of the $A\alpha^k$ and $A\beta^k$ chains upon incubation with proteinase K and the resistance of the associated proteins to such treatment. (**b**) Untransfected COS-1 cells labelled as in (a) were mixed with unlabelled, $A\alpha^k$ + $A\beta^k$-expressing COS-1 cells. The mixed cells were lysed and processed for immunoprecipitation with anti-$A\alpha^k$ or anti-$A\beta^k$ antibodies. Immunoprecipitates were incubated without (−) or with endo H (+), as indicated, and resolved by SDS−PAGE under reducing conditions on 10% acrylamide gels. The positions of molecular weight markers (expressed as $10^{-3} \times M_r$) are shown on the left.

comigration with BiP isolated using a specific monoclonal antibody (Figure 5). The identity of the M_r 62 000−64 000 glycoprotein, hereafter referred to as gp62, remains to be established. We considered the possibility that gp62 could be a dimeric form of $A\alpha^k$. However, two-dimensional gel electrophoresis analyses showed that the gp62 glycoprotein was slightly less acidic than $A\alpha^k$ (Figure 5), and limited digestion with *Staphylococcus aureus* V8 protease generated a different pattern of proteolytic fragments than those derived from $A\alpha^k$ (data not shown).

Analysis of the aggregation state of class II MHC chains by sedimentation velocity on sucrose gradients

In the two-dimensional analysis of the anti-$A\beta^k$ immunoprecipitate shown in Figure 5, we noticed the presence of a significant amount of labelled material that did not enter the first dimension, non-equilibrium pH gradient electrophoresis gel. This observation suggested that, in addition to interacting with ER resident proteins, the class II MHC chains retained in the ER might be forming high molecular weight aggregates. To investigate this possibility we examined the size of class II MHC chains by sedimentation on sucrose gradients. In these experiments, cells were labelled for 1 h with [^{35}S]methionine, treated for 20 min on ice with either iodoacetamide or *N*-ethylmaleimide, and extracted with Triton X-100. The detergent-solubilized proteins were then separated by sedimentation on 8−35% sucrose gradients. A crude estimation of the molecular weights of the class II MHC species was obtained by comparison with the sedimentation behaviour of the transferrin receptor (M_r ~180 000 for the native dimeric species; McClelland *et al.*, 1984), following the procedure of Martin and Ames (1961) (see Materials and methods section). Estimations of M_r were made under the assumption that the unknown and standard proteins have a spherical shape and similar partial specific volumes (i.e. implying similar amounts of bound detergent) (Martin and Ames, 1961).

The Ii chain (monomeric M_r 33 000), expressed in the absence of α and β chains, was detected as a sharp peak near the top of the gradient (Figure 6I). The molecular weight of this species could not be determined with accuracy in this type of gradient, but its migration was compatible with the reported trimeric structure of the Ii chain (Marks *et al.*, 1990). Expression of $A\alpha^k$ (monomeric M_r 34 000) in the absence of other components of the complex resulted in a heterogeneous mixture of protein species, with most forms having molecular weights in the range of 100 000−600 000 (Figure 6α). The $A\beta^k$ chain (monomeric M_r 28 000) was even more aggregated; the aggregated species ranged in molecular weight from 100 000 to at least 1 000 000 (Figure 6β). $A\alpha^k$ + $A\beta^k$ complexes were similarly found throughout the gradient (Figure 6$\alpha\beta$). A fraction of the $A\alpha^k$ + $A\beta^k$ complexes peaked with molecular weight of ~180 000, while faster sedimenting species had molecular weights of up to at least 1 000 000 (Figure 6$\alpha\beta$). Some variability in the size of the different species was observed from experiment to experiment, although in all cases the sedimentation rate of the proteins was disproportionately higher than expected for a monomer or a dimer. These observations indicated that both the unassembled $A\alpha^k$ and $A\beta^k$ chains, as well as $A\alpha^k$ + $A\beta^k$ complexes, existed as high molecular weight aggregates. BiP and gp62 cosedimented with the individually expressed $A\alpha^k$ and $A\beta^k$ species, indicating that they were part of the aggregates (Figure 6α and β). In cells expressing both $A\alpha^k$ and $A\beta^k$, BiP and gp62 cosedimented preferentially with the larger $A\alpha^k$ + $A\beta^k$ species, and to a lesser extent with the smaller species (Figure 6$\alpha\beta$).

We next examined whether the aggregation of $A\alpha^k$ + $A\beta^k$ complexes could also be observed in fibroblasts that express lower levels of the individual chains. Analysis of CV-1 cells expressing $A\alpha^k$ + $A\beta^k$ showed that the class II MHC chains were also aggregated in these cells, and that the aggregates contained BiP and gp62 (Figure 7). Analysis of the immunoprecipitates by SDS−PAGE under non-reducing conditions revealed that some of the aggregates were linked by intermolecular disulfide bonds, as evidenced by the increased amount of material at or near the top of the separating gel (Figure 7, bracket). Similar disulfide-

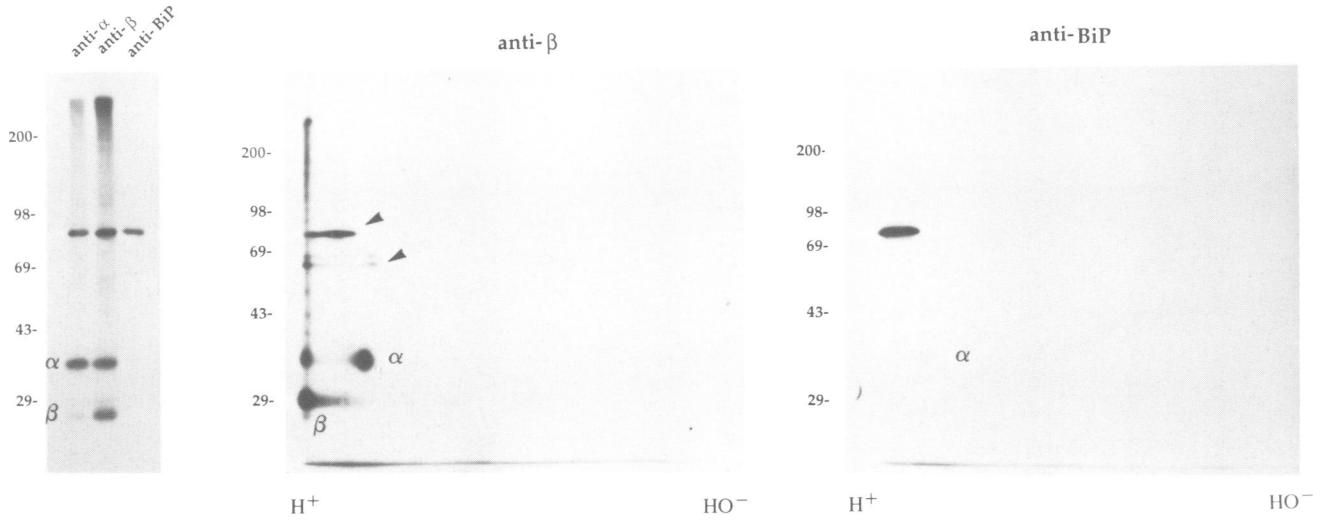

Fig. 5. Identification of the M_r 78 000 protein as BiP. COS-1 cells expressing $A\alpha^k$ and $A\beta^k$ were metabolically labelled for 1 h with [^{35}S]methionine and a microsomal fraction was prepared as described by Bole *et al.* (1986). Proteins were isolated from Triton X-100-solubilized microsomes by immunoprecipitation with anti-$A\alpha^k$, anti-$A\beta^k$ or anti-BiP, as indicated in each panel. Immunoprecipitates were resolved by one- or two-dimensional electrophoresis under reducing conditions. The positions of the M_r 78 000 and M_r 62 000–64 000 proteins are indicated by arrowheads. For unknown reasons, the M_r 62 000–64 000 species was only barely visible in the experiment shown in the left panel and could only be detected upon long exposure of the autoradiograms. Prolonged exposures of the gels showed a small amount of α chains in the anti-BiP immunoprecipitates. The positions of molecular weight markers (expressed as $10^{-3} \times M_r$) are shown on the left of each panel.

linked aggregates were also observed in some experiments using COS-1 cells (data not shown). From the experiment using CV-1 cells, we concluded that the formation of high molecular weight aggregates was not dependent merely on overexpression of the transfected gene products but was likely an intrinsic property of the incompletely assembled class II MHC chains.

Aggregation of class II MHC chains in splenic cells from Ii chain deficient mice

All of the experiments described above were performed in fibroblast cell lines that normally do not express class II MHC molecules. In order to determine whether our observations would also be applicable to class II MHC-expressing, 'professional' antigen-presenting cells, we decided to examine the aggregation state of newly synthesized class II MHC chains in splenic cells from mice carrying a disruption of the Ii chain gene (Bikoff *et al.*, 1993). The Ii chain mutant mice used in these experiments expressed MHC molecules of the A^b haplotype, which also allowed us to test whether our findings with A^k molecules could be extended to other class II MHC chains. The size of class II MHC molecules labelled during a 30 min pulse with [^{3}H]leucine was examined by sedimentation on sucrose gradients in comparison with the migration of two other endogenously expressed integral membrane proteins: the class I MHC molecule H-2 K^b (M_r ~57 000, Ploegh *et al.*, 1981) and the CD45 antigen (M_r ~200 000, Springer *et al.*, 1978). A substantial amount of $A\beta^b$ chains were found not to be assembled with labelled $A\alpha^b$ chains (Figure 8A), in agreement with previous observations (Bikoff *et al.*, 1993). This pool of $A\beta^b$ migrated as a heterogeneous species of M_r ~200 000–500 000, whereas the co-precipitated $A\alpha^b$ chain (a more direct measure of $A\alpha^b$ + $A\beta^b$ complexes) peaked at M_r ~200 000 (Figure 8, upper panel). Thus, these experiments demonstrated that newly synthesized class II MHC chains also have a tendency to aggregate in splenic cells from Ii chain deficient mice. The species migrating on the SDS–PAGE gels at M_r ~20 000 is likely to be a fragment of the $A\beta^b$ chain, as demonstrated by re-precipitation with antibodies to the β chain from SDS-denatured samples (not shown).

Recent observations have demonstrated that expression of the Ii chain increases the efficiency with which class II MHC molecules are transported out of the ER (Layet and Germain, 1991; Anderson and Miller, 1992; Bikoff *et al.*, 1993; Viville *et al.*, 1993; Elliott *et al.*, 1994). In order to examine whether this effect of the Ii chain correlated with a change in the aggregation state of the class II MHC chains, we analyzed the size distribution of class II MHC chains in spleen cells from normal mice labelled for 30 min with [^{3}H]leucine (Figure 8B). In these cells, the $A\alpha^b$ and $A\beta^b$ chains were found to form discrete complexes with the Ii chain, migrating as a homogeneous species of M_r ~200 000–300 000. This is consistent with the size of class II–Ii chain complexes observed in human lymphoblastoid cell lines (Roche *et al.*, 1991). Thus, expression of the Ii chain led to the assembly of stoichiometric complexes with class II MHC chains and thereby prevented the accumulation of aggregated class II MHC chains in the ER. This effect may be an important factor for the ability of the Ii chain to promote trafficking of class II MHC molecules from the ER.

Discussion

Abnormal physico-chemical properties of incompletely assembled class II MHC molecules

Protein misfolding, as a consequence of alterations in the primary structure of proteins or of a failure to acquire certain post-translational modifications, has been proposed to be the underlying cause for the retention of many abnormal proteins in the ER (reviewed by Pfeffer and Rothman, 1987; Rose and Doms, 1988; Hurtley and Helenius, 1989). Proteins that misfold in the ER tend to associate with ER resident proteins and to form macromolecular aggregates (Machamer, 1988; Hurtley *et al.*, 1989; Singh *et al.*, 1990; Marquardt and

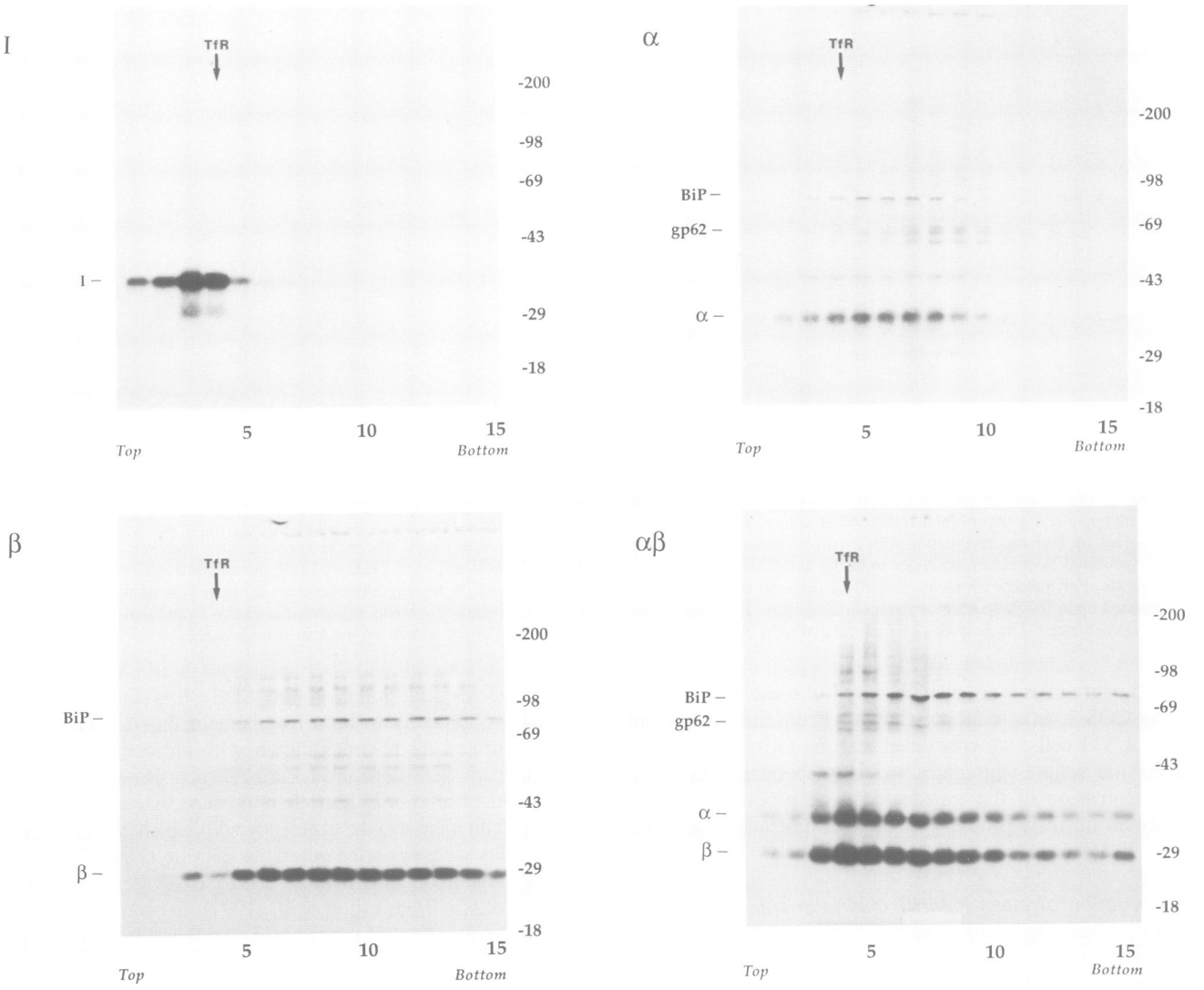

Fig. 6. Sedimentation velocity analysis of newly synthesized class II MHC chains expressed in COS-1 cells. COS-1 cells expressing the Ii chain (**I**), $A\alpha^k$ (α), $A\beta^k$ (β), or $A\alpha^k$ and $A\beta^k$ ($\alpha\beta$) were labelled for 1 h with [^{35}S]methionine and preincubated and detergent-solubilized in the presence of 1.8 mg/ml iodoacetamide. Lysates were resolved by sedimentation on 8–35% linear sucrose gradients, as described in Materials and methods. Each gradient was divided into 14 fractions, with fraction 15 corresponding to the pellet, and class II chains were isolated by immunoprecipitation with anti-Ii (I), anti-$A\alpha^k$ (α) or anti-$A\beta^k$ (β and $\alpha\beta$). The human transferrin receptor (TfR) was isolated by immunoprecipitation from parallel gradients of metabolically labelled extracts of the human erythroleukemia cell line, K562. The position of the human transferrin receptor in these gradients is indicated by the vertical arrows. The positions of the Ii and class II MHC chains are indicated on the left of each panel, along with the positions of BiP and gp62. The positions of molecular weight markers (expressed as $10^{-3} \times M_r$) on SDS–PAGE are indicated to the right of each panel. Fraction numbers are indicated at the bottom of each panel.

Helenius, 1992). These phenomena are likely to be the basis for the 'quality control' function of the ER, which is manifested at a cellular level as a block in the export of abnormal proteins from the ER (Hurtley and Helenius, 1989).

The results of the present study have shown that unassembled, or partially assembled, chains of class II MHC molecules expressed in both transfected fibroblasts and splenic cells can likewise display abnormal physico-chemical properties, analogous to those of mutated or aberrantly processed proteins. Incomplete class II MHC species expressed in fibroblasts were found to interact with BiP. In addition, the unassembled α and β chains, as well as a fraction of $\alpha + \beta$ complexes synthesized in the absence of the Ii chain, were observed to form macromolecular aggregates in both transfected fibroblasts and splenic cells from Ii chain deficient mice. These associations are likely to be a reflection of the status of class II MHC molecules in the ER of intact cells, since mixing experiments failed to show artefactual association of class II MHC molecules with BiP (Figure 4) or association of individually expressed $A\alpha^k$ and $A\beta^k$ occurring upon solubilization of the cells (data not shown). If the mechanism of retention of abnormal proteins in the ER indeed involves interaction with ER resident proteins and/or aggregation, as previously proposed, then there may not be anything intrinsically different about the way misfolded polypeptides and unassembled subunits of multiprotein complexes become retained in the ER. The only differences among subunits from different oligomeric complexes may be in their tendency to aggregate, the size and stability of the aggregates, their affinity for binding to ER resident proteins, and the relative contribution of each of these factors to the retention process.

While binding of BiP, and potentially of other ER resident proteins (Anderson and Miller, 1992; Schaiff *et al.*, 1992), to the class II MHC chains is likely to contribute to the large

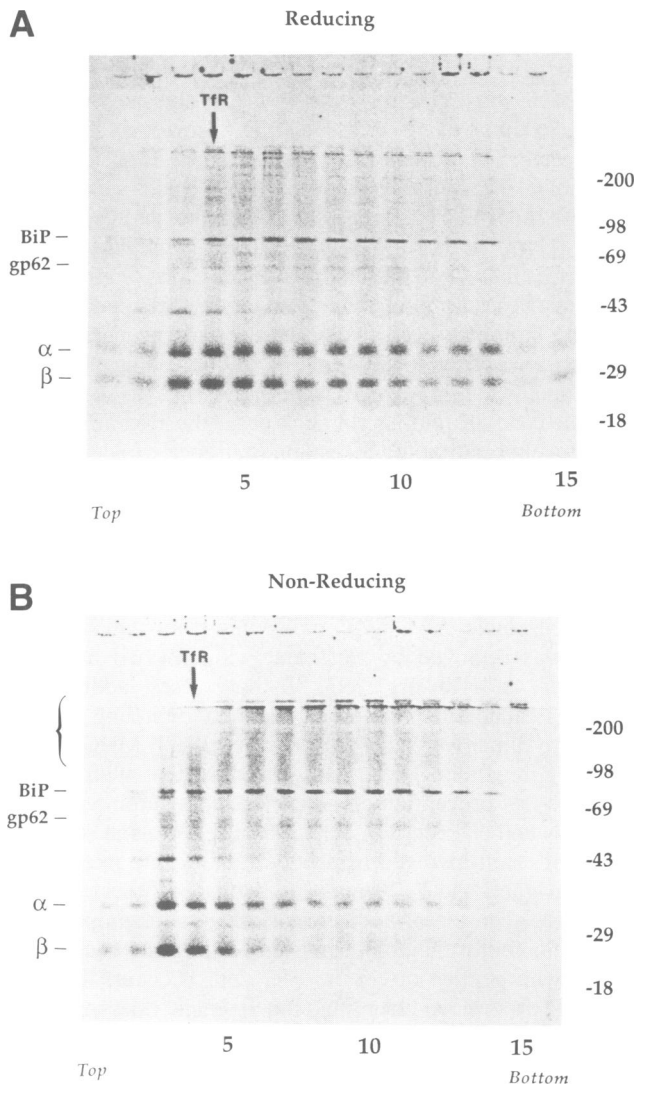

Fig. 7. Sedimentation velocity analysis of newly synthesized class II MHC chains expressed in CV-1 cells. CV-1 cells expressing the $A\alpha^k$ and $A\beta^k$ chains were labelled for 1 h with [^{35}S]methionine, and extracted with lysis buffer in the presence of 20 mM NEM. Lysates were resolved by sedimentation on 8–35% linear sucrose gradients, as described in Materials and methods. Class II MHC chains were isolated by immunoprecipitation with an anti-$A\beta^k$ antibody. Immunoprecipitates were resolved by SDS–PAGE under either reducing (**A**) or non-reducing conditions (**B**) on 11% acrylamide gels. The image shown in the figure was produced in a phosphorimager. The positions of the class II MHC chains and of BiP and gp62 are indicated to the left of each panel. The position of the TfR is indicated by the vertical arrows. The bracket in (B) indicates disulfide-linked aggregates. Molecular weight markers (expressed as $10^{-3} \times M_r$) on SDS–PAGE are indicated to the right of each panel. Fraction numbers are indicated at the bottom of each panel.

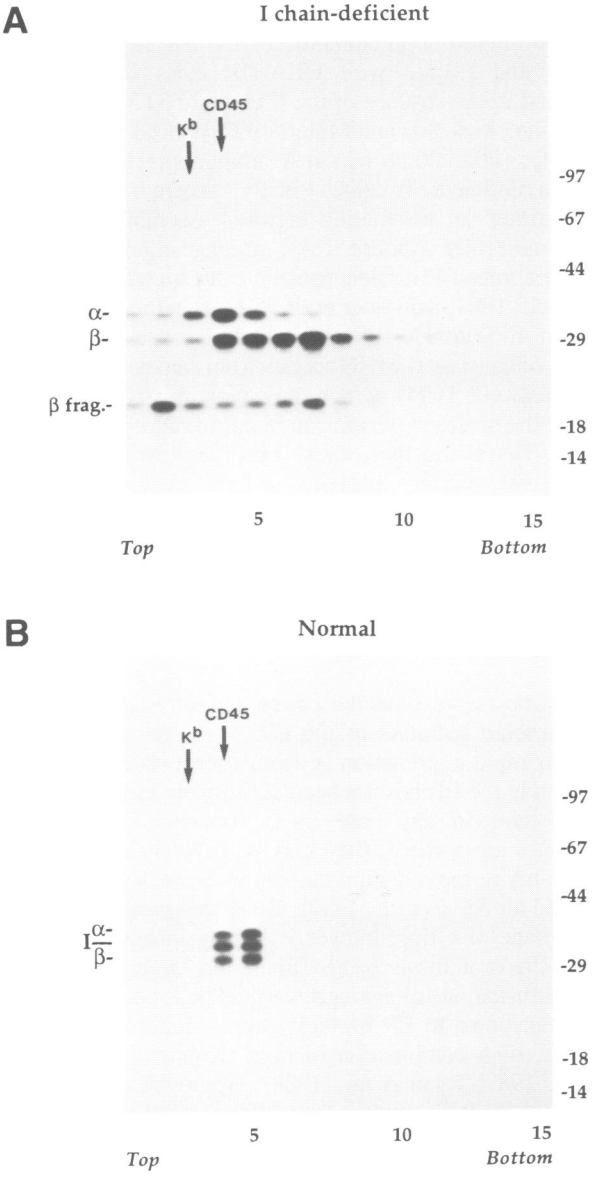

Fig. 8. Sedimentation velocity analysis of endogenous class II MHC chains expressed in splenic cells from Ii chain deficient and normal mice. Mice used in this experiment expressed class II MHC molecules of the A^b haplotype. Splenic cells from mice carrying a disruption of the Ii chain gene (**A**) or normal mice (**B**) were labelled for 30 min with [^{3}H]leucine and extracted with lysis buffer in the presence of 20 mM NEM. Extracts were resolved by sedimentation on 8–35% linear sucrose gradients and class II MHC chains isolated by immunoprecipitation with an antibody to the cytoplasmic domain of the β chain. Immunoprecipitates were resolved by SDS–PAGE under reducing conditions on 11% acrylamide gels. The positions of the class II MHC and Ii chains are indicated to the left of each panel. The positions of H-2 K^b (M_r ~57 000) and CD45 (M_r ~200 000) immunoprecipitated from the same gradients are indicated by the vertical arrows. Longer exposures of the X-ray films showed coprecipitation of a M_r 78 000 protein with class II chains from the Ii chain deficient mice. This protein may correspond to BiP. Molecular weight markers (expressed as $10^{-3} \times M_r$) on SDS–PAGE are indicated to the right of each panel. Fraction numbers are indicated at the bottom of each panel.

size of the macromolecular complexes, some of our observations suggest that homotypic associations between the class II MHC chains also play a role in the formation of the aggregates. First, a fraction of $A\alpha^k + A\beta^k$ complexes in CV-1 cells were found to exist as disulfide-linked aggregates, even after complete dissociation of BiP and gp62 (Figure 7). Second, cell solubilization in the presence of ATP results in the dissociation of most of the BiP, but the class II MHC chains still remain aggregated, as determined by sedimentation velocity experiments (data not shown). Since no other proteins are coprecipitated with class II MHC chains in stoichiometric amounts, even after long-term labelling with [^{3}H]leucine (Figure 3), these observations are most consistent with an aggregate of class II MHC chains to which BiP, and perhaps other proteins, bind in a non-covalent fashion. The fact that we did not observe other associated proteins under our experimental conditions does not rule out the possibility that additional ER resident proteins might bind

to class II MHC chains in the ER. Indeed, Schaiff *et al.* (1992) observed coprecipitation of the ER stress proteins GRP94 and ERp72 with HLA-DR class II molecules expressed in the absence of the Ii chain in 3T3 fibroblasts. In addition, Anderson and Miller (1992) noticed the presence of an M_r ~200 000 protein in A^d immunoprecipitates from Ii chain deficient T cells. Finally, recent studies have demonstrated an association between assembling class II MHC molecules (mouse I-A^k, among others) and the transmembrane ER resident protein, calnexin (Anderson and Cresswell, 1994; Schreiber *et al.*, 1994). In the latter study, addition of Triton X-100 was found to cause dissociation of most of the class II MHC species from calnexin (Anderson and Cresswell, 1994), which may explain why we did not observe the presence of calnexin in our immunoprecipitation assays. Taken together, these observations suggest that several ER resident proteins, which may function as molecular chaperones, are capable of interacting with incompletely assembled class II MHC molecules. These interactions may participate in the mechanism of folding or assembly of normal class II MHC chains, or otherwise contribute to the retention of the unassembled chains in the ER.

Aggregation as a possible cause for retention of unassembled subunits in the ER

Whether rapid aggregation is a cause or a consequence of retention in the ER has not been definitively established for any protein. In any case, it is conceivable that once aggregates are formed, they may be severely restricted in their ability to move within the ER cisternae, as previously proposed (de Silva *et al.*, 1990). Given that protein diffusion in the plane of a lipid bilayer is largely insensitive to the size of the protein *per se* (Saffman and Delbrück, 1975), a low diffusion rate of aggregates would be expected to result from attachment to ER proteins, steric hindrance by other proteins, or a combination of both (Koppel *et al.*, 1981; Saxton, 1987; Ryan *et al.*, 1988). Aggregates of class II MHC chains (this study), as well as of other abnormal proteins (Doms *et al.*, 1988; Machamer, 1988; Hurtley *et al.*, 1989; Singh *et al.*, 1990; Marquardt and Helenius, 1992), have indeed been observed in association with BiP and other ER resident proteins. Retention of the aggregates would thus be an indirect effect of mechanisms that function to maintain resident proteins in the ER (Pelham, 1989). Even ER membrane proteins that do not directly interact with the aggregates could further obstruct their movement. In combination, these factors would prevent migration of the aggregates towards sites of exit from the ER, thus resulting in accumulation of the proteins within the ER cisternae.

Role of Ii chain in class II MHC assembly and transport

It is now well established that the Ii chain facilitates egress of class II MHC molecules from the ER (Layet and Germain, 1991; Anderson and Miller, 1992; Bikoff *et al.*, 1993; Viville *et al.*, 1993; Elliott *et al.*, 1994). Our data suggest that this function may be a direct consequence of the ability of the Ii chain to generate transport-competent assemblies of the class II MHC molecules. In assembling with newly synthesized class II MHC chains, the Ii chain is likely to promote proper folding of the individual chains, which in turn would lead to the production of correctly assembled, conformationally mature complexes capable of exiting the ER. In the absence of the Ii chain, the class II MHC molecules would remain partially folded and would tend to aggregate into species that are unable to exit the ER. It is unclear at present whether the Ii chain exerts its effects by preventing aggregation of the newly synthesized class II MHC chains or by dissociating previously formed aggregates. The former possibility would be consistent with the known excess of Ii chain over α and β chains synthesized by class II MHC-expressing cells (Machamer and Cresswell, 1982; Marks *et al.*, 1990), since fewer aggregates would form if Ii chain were present in large amounts. On the other hand, the vesicular stomatitis virus G protein has been shown to form large aggregates prior to achieving proper folding and oligomerization (de Silva *et al.*, 1993), lending precedent to a pathway in which aggregates act as transient intermediates in folding. In such case, the Ii chain would presumably catalyze the formation of properly folded forms of the class II MHC chains by rescuing them from the aggregates.

A tendency of assembled class II $\alpha\beta$ dimers to aggregate *in vitro* has been noted for human class II MHC proteins secreted from insect cells (Stern and Wiley, 1992) and for mature, splenic A^k and A^d molecules from which the Ii chain was removed by treatment with low pH at 37°C (Germain and Rinker, 1993). In both cases, addition of peptides capable of binding to the peptide-binding groove of the $\alpha\beta$ dimer were able to rescue the class II MHC chains from aggregation. These data are consistent with recent findings that prevention of $\alpha\beta$ dimer aggregation with free β chains and efficient ER egress require the same segment of the Ii chain involved in peptide binding site interaction (P.Romagnoli and P.Germain, unpublished observations). Taken together, these various results suggest that proper class II protein conformation depends on binding site occupancy either with peptide or, in the ER, with Ii chain.

While it is now clear that the Ii chain does promote transport of newly synthesized proteins out of the ER, there is also abundant evidence that a fraction of class II $\alpha\beta$ complexes can reach distal compartments of the secretory pathway even in the absence of the Ii chain (Miller and Germain, 1986; Sekaly *et al.*, 1986; Anderson and Miller, 1992; Cosson and Bonifacino, 1992; Bikoff *et al.*, 1993; Viville *et al.*, 1993; Elliott *et al.*, 1994; this study). The explanation of this phenomenon is not known. It remains to be established to what extent the coprecipitation of α with β in Ii chain deficient cells observed in our studies is a true reflection of the normal assembly process or whether it simply represents the co-aggregation of two incompletely folded species in intact cells. However, the fact that a fraction of the class II chains synthesized in the absence of the Ii chain are recognized by antibodies such as 11-5.2 (Oi *et al.*, 1978) and 40B (Pierres *et al.*, 1980), which only recognize assembled $\alpha\beta$ complexes, argues that at least some stages of folding and conformational maturation can occur in this situation (Cosson and Bonifacino, 1992). It is then conceivable that class II MHC chains have some ability to fold and assemble correctly, albeit inefficiently, even without the Ii chain. Whether the class II MHC chains that escape ER retention belong to a discrete group of molecules with physico-chemical properties different from the rest will have to be addressed in future studies.

Materials and methods

Cells

The African green monkey fibroblast lines, COS-1 and CV-1, were obtained from the American Type Culture Collection (Rockville, MD). These cells

were cultured in Dulbecco's modified Eagle's medium (DMEM, Biofluids, Rockville, MD) with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (complete medium). The human erythroleukemia cell line K562 (a gift of Joe Harford, National Institutes of Health, Bethesda, MD) was cultured in RPMI 1640 medium, also supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin.

Plasmids

Complementary DNAs encoding the mouse class II MHC chains, $A\alpha^k$ (Benoist *et al.*, 1983) and $A\beta^k$ (Estess *et al.*, 1986) were kindly provided by Eric Long (National Institutes of Health). A cDNA encoding the p31 form of the Ii chain was described by Miller and Germain (1986). The cDNAs were cloned into the mammalian expression vector pCDM8 (Seed, 1987).

Transfections

COS-1 or CV-1 cells, grown to 40–60% confluence in 100 mm tissue culture plates, were transiently transfected by the calcium phosphate precipitation method (Graham and van der Eb, 1973), as previously described (Bonifacino *et al.*, 1989). Transfections with plasmids encoding one or two chains were routinely done with 20–30 μg total plasmid DNA. Cells were generally used 40–48 h after transfection.

Mice

Normal C57BL/6 mice (H-2^b) and Ii chain mutant mice bred to the C57BL/6J background were maintained as described (Bikoff *et al.*, 1993). Female mice between 2 and 4 months of age were used. For metabolic labelling experiments, spleens were isolated from freshly sacrificed animals and cell suspensions prepared by disruption in complete DMEM or RPMI 1640 media.

Antibodies

The anti-class II MHC monoclonal antibody 10-2.16 (Oi *et al.*, 1978), that binds both unassembled and assembled forms of $A\beta^k$, and the anti-class I MHC (H-2 K^b) monoclonal antibody Y3 (Jones and Janeway, 1981) were prepared from hybridoma lines obtained from the American Type Culture Collection. Rabbit antisera to the cytoplasmic tails of class II MHC α ($A\alpha^k$ and $A\alpha^b$) and β chains ($A\beta^k$ and $A\beta^b$) have been described previously (Sant *et al.*, 1991). A monoclonal antibody to the mouse Ii chain (P4H5, Mehringer *et al.*, 1991) was the gift of Sue Cullen (Washington University, St Louis, MO). A monoclonal anti-BiP antibody (Bole *et al.*, 1986) was obtained from David Bole (University of Michigan, Ann Arbor, MI). The monoclonal anti-human transferrin receptor antibody B3/25 (Boehringer Mannheim, Indianapolis, IN) was the gift of Joe Harford (National Institutes of Health). A monoclonal antibody to mouse CD45 (M1/89, Springer *et al.*, 1978) was the gift of Barbara Niklinska and Jonathan Ashwell (National Institutes of Health). For immunoprecipitation, most antibodies were bound to protein A–Sepharose (Pharmacia, Piscataway, NJ), except for M1/89 which was bound to protein G–Sepharose (Pharmacia). Fluorescently labelled donkey anti-mouse IgG and anti-rabbit IgG antibodies, suitable for multiple labelling experiments, were purchased from Jackson Immuno-Research (West Grove, PA).

Metabolic labelling and immunoprecipitation

Transfected fibroblasts were washed twice in PBS and incubated for 10–30 min at 37°C in 15 ml of methionine-free DMEM containing 15 mM EDTA to release cells from the dish. The suspended cells were washed twice in methionine-free DMEM, and incubated for 30–60 min at 37°C in 2 ml of 0.25–0.5 mCi/ml [^{35}S]methionine (Tran ^{35}S-Label, ICN Radiochemicals, Irvine, CA) in methionine-free DMEM containing 2–5% dialyzed FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Whenever indicated, pulse-labelled cells were chased for different periods at 37°C in complete medium. Mouse spleen cells were incubated for at least 1 h with leucine-free DMEM containing 5% dialyzed FBS, penicillin and streptomycin, and then pulse-labelled by incubation for 30 min at 37°C with 1.7 mCi/ml [^{3}H]leucine (New England Nuclear, Boston, MA) in leucine-free DMEM, containing 5% dialyzed FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Long-term labelling of transfected fibroblasts was done for 24 h at 37°C with 1 mCi/ml [^{3}H]leucine in DMEM containing 1/10 the normal amount of unlabelled leucine, plus 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. In all cases, at the end of the labelling or chase periods, cells were washed twice with ice-cold PBS and incubated for an additional 20 min period at 4°C in PBS containing 20 mM *N*-ethylmaleimide (NEM) or 1.8 mg/ml iodoacetamide to prevent artificial formation of disulfide bonds (Braakman *et al.*, 1991) and a mixture of protease inhibitors (10 μg/ml leupeptin, 5 μg/ml E-64, 30 μg/ml aprotinin, 0.1 mM TLCK, 0.1 mM TPCK, 10 μg/ml pepstatin A, 0.5 mM AEBSF). In most experiments, cells were solubilized in lysis buffer [1% (w/v) Triton X-100, 0.3 M NaCl, 50 mM Tris–HCl, pH 7.4] containing NEM or iodoacetamide and a mixture of protease inhibitors, as described above. Lysates were cleared by centrifugation for 15 min at 12 000 *g* in a microfuge (Beckman Instruments, Palo Alto, CA), and supernatants were added to antibodies previously bound to either protein A–Sepharose or protein G–Sepharose beads. Lysates from spleen cells were pre-cleared at least twice with 10 μg/sample of affinity purified rabbit anti-mouse IgG (Zymed, San Francisco, CA) bound to protein A–Sepharose and once with protein A–Sepharose alone. After incubation for 1–2 h at 4°C, immunoprecipitates were washed four or five times with wash buffer [0.1% (w/v) Triton X-100, 0.3 M NaCl, 50 mM Tris–HCl, pH 7.4] and once with PBS. Some immunoprecipitates were treated with endo H, as previously described (Chen *et al.*, 1988). In most experiments, samples were heated for 10 min at 95°C in electrophoresis sample buffer with or without 3% β-mercaptoethanol (reducing or non-reducing conditions, respectively), and resolved by SDS–polyacrylamide gel electrophoresis (PAGE) on 10–13% acrylamide gels. Two-dimensional non-equilibrium pH gradient electrophoresis/SDS–PAGE was performed by the method of O'Farrell *et al.* (1977). Labelled proteins were revealed by either conventional fluorography, or by analysis in a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Sedimentation velocity analyses

Detergent lysates of metabolically labelled cells (0.6 ml) were loaded on top of 8–35% linear sucrose gradients (12 ml total volume) containing 0.2% (w/v) Triton X-100, 0.3 M NaCl and 50 mM Tris–HCl, pH 7.4. Gradients were spun for 16 h at 10°C in an SW41 rotor (Beckman Instruments) at 39 000 r.p.m. Gradients containing marker proteins (different combinations of cytochrome *c*, ovalbumin, bovine serum albumin, immunoglobulin, aldolase and thyroglobulin) were run alongside for reference. The human transferrin receptor (M_r ~180 000) from lysates of metabolically labelled K562 cells and H-2 K^b (M_r ~57 000) and CD45 (M_r ~200 000) from splenic cells were used as standards for estimation of molecular weights. Gradients were divided into 15 fractions. Proteins were isolated by immunoprecipitation and separated by SDS–PAGE on 10–12% acrylamide gels. Estimates of molecular weights were obtained as described by Martin and Ames (1961), according to the formula:

$$d_1/d_2 = (M_{r1}/M_{r2})^{2/3}$$

where d_1 and d_2 are the distances travelled by the unknown and standard macromolecules, respectively, and M_{r1} and M_{r2} are the relative molecular weights of the unknown and standard macromolecules, respectively. Fraction 1 of the gradients corresponds to the applied sample and is therefore considered to represent distance 0. Calculations were made under the assumptions that the proteins are roughly spherical in shape and that they have similar partial specific volumes (i.e. meaning that they have similar proportions of bound detergent). The relative molecular weights calculated using this method correspond to the glycoprotein part of the molecules and exclude the contribution of bound detergent for both the unknown and standard species. Because of the assumptions involved in these calculations, the relative molecular weights given in the text should be taken as crude approximations, and are only provided in order to simplify the description of the data.

Immunofluorescence microscopy

Transfected COS-1 cells grown on glass cover slips were fixed for 15 min at room temperature in 2% formaldehyde in PBS. After washing twice with PBS, the cells were incubated for 1 h at room temperature with rabbit anti-$A\alpha^k$ or mouse anti-$A\beta^k$ (10-2.16) antibodies in PBS containing 0.1% bovine serum albumin and 0.1% saponin (AS buffer). Excess antibody was removed by a 15 min incubation in PBS, after which the cells were incubated for 30 min at room temperature with fluorescently labelled secondary antibodies (1:200 dilution in AS buffer). Cells were washed again in PBS, and then mounted onto glass slides with Fluoromount G (Southern Biotechnology Associates, Birmingham, AL). Samples were examined under a Zeiss inverted microscope.

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