

Misfolded Major Histocompatibility Complex Class I Molecules Accumulate in an Expanded ER-Golgi Intermediate Compartment

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Abstract. Misfolded membrane proteins are rapidly degraded, often shortly after their synthesis and insertion in the endoplasmic reticulum (ER), but the exact location and mechanisms of breakdown remain unclear. We have exploited the requirement of MHC class I molecules for peptide to achieve their correct conformation: peptide can be withheld by introducing a null mutation for the MHC-encoded peptide transporter, TAP. By withholding TAP-dependent peptides, the vast majority of newly synthesized class I molecules fails to leave the endoplasmic reticulum and is degraded. We used mice transgenic for HLA-B27 on a TAP1-deficient background to allow visualization by immunoelectron microscopy of misfolded HLA-B27 molecules in thymic epithelial cells. In such HLA transgenic animals, the TAP mutation can be considered a

genetic switch that allows control over the extent of folding of the protein of interest, HLA-B27, while the rate of synthesis of the constituent subunits remains unaltered. In TAP1-deficient, HLA-B27 transgenic animals, HLA-B27 molecules fail to assemble correctly, and do not undergo carbohydrate modifications associated with the Golgi apparatus, such as conversion to Endoglycosidase H resistance, and acquisition of sialic acids. We show that such molecules accumulate in an expanded network of tubular and fenestrated membranes. This compartment has its counterpart in normal thymic epithelial cells, and is identified as an ER-Golgi intermediate. We detect the presence of ubiquitin and ubiquitin-conjugating enzymes in association with this compartment, suggesting a nonlysosomal mode of degradation of its contents.

INDIVIDUAL subunits of glycoproteins are synthesized in the rough endoplasmic reticulum (RER)¹ where they fold and may undergo modifications such as disulfide bond formation and glycosylation (Hurtley and Helenius, 1989). Because the subunits may be synthesized at different rates and not necessarily in stoichiometric amount, there must be mechanisms to ensure that only properly assembled and stable complexes reach the cell surface (Rose and Doms, 1988; Hurtley and Helenius, 1989; Pelham, 1989). Studies on the assembly of multimeric proteins such as the T cell receptor (Lippincott-Schwartz et al., 1988; Chen et al., 1988), asialoglycoprotein receptor (Amara et al., 1989; Wikstrom and Lodish, 1993), acetylcholine receptors (Blount and Merlie, 1990), or immunoglobulins

(Sitia et al., 1987) show that degradation of subunits that have failed to assemble into the appropriate oligomers occurs at distinct rates and takes place in the ER or in a related compartment, possibly the ER-Golgi intermediate compartment (Klausner and Sitia, 1990; Bonifacino and Lippincott-Schwartz, 1991; Hauri and Schweizer, 1992). Misfolded proteins are not completely retained in the ER and can cycle between the *cis*-Golgi, the intermediate compartment, and the ER (Hammond and Helenius, 1994). Nevertheless, the exact location of the degradative system and the mechanisms involved are only poorly defined. Here we have taken advantage of the peculiarities of biosynthesis and assembly of major histocompatibility complex (MHC) class I molecules to address some of these issues in thymic epithelial cells. The question of where quality control of misfolded proteins occurs has, to our knowledge, not been addressed previously in situ.

MHC class I molecules bind peptides derived from degraded self and foreign antigens and display them on the surface of cells for recognition by CD8⁺ T lymphocytes. Class I molecules should be considered trimeric structures: they consist of a heavy chain (HC), the light chain, β_2 -microglobulin (β_2m), and an ~ 9 -amino acid long peptide (Yewdell and Bennink, 1992). Peptide binding is essential for the stable expression of the complex at the plasma membrane (Elliott, 1991; Bijlmakers and Ploegh, 1993). These

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1. *Abbreviations used in this paper:* 1D-IEF gel, one-dimensional iso-electric focussing gel; β_2m , β_2 -microglobulin; BFA, Brefeldin A; endo H, Endoglycosidase H; HC, class I heavy chain; h β_2m , human β_2 -microglobulin; MHC, major histocompatibility complex; PAG, protein A-gold; PDI, protein disulfide isomerase; RER, rough endoplasmic reticulum; TAP, transporter associated with antigen presentation.

peptides are generated in the cytosol by an as yet incompletely defined mechanism (Arnold et al., 1992; Momburg et al., 1992; Howard and Seelig, 1993). Ubiquitinylation and proteolysis by proteasomes have been proposed as major candidates (Michalek et al., 1993; Dick et al., 1994; Rock et al., 1994). The resultant peptides are then translocated into the lumen of the RER by the transporter associated with antigen presentation (TAP) (Powis et al., 1991; Spies and DeMars, 1991; Attaya et al., 1992). TAP is a heterodimer composed of two subunits (TAP1 and TAP2) located in the ER and *cis*-Golgi (Kleijmeer et al., 1992), and is responsible for the translocation of peptides of particular size and sequence (Androlewicz et al., 1993; Neeffjes et al., 1993; Shepherd et al., 1993; Heemels and Ploegh, 1995).

TAP-deficient cell lines such as the T lymphoma cell line RMA-S (Ljunggren and Kärre, 1985; Kärre et al., 1986) and the B lymphoblastoid cell lines 721.174 and its derivative T2 (Kavathas et al., 1980; DeMars et al., 1984) are defective in class I surface expression, although the HC and β_2m are synthesized normally and are clearly capable of assembly in the absence of TAP-dependent peptides (Alexander et al., 1989; Ljunggren et al., 1990; Baas et al., 1992). In the T2 cell line these class I molecules appear to be retained in the ER and in the Golgi region (Baas et al., 1992). In another mutant cell line, likely to be (perhaps not completely) TAP-deficient, unassembled class I molecules were shown to recycle from the Golgi to the ER through an intermediate compartment, underscoring the role of the *cis*-Golgi region in the quality control of unassembled or misfolded molecules (Hsu et al., 1991; Bonifacino and Lippincott-Schwartz, 1991).

With respect to class I biosynthesis, cells from TAP1-deficient mice were shown to have characteristics similar to those of TAP-deficient cells already analyzed (Van Kaer et al., 1992). TAP1-deficient mice are impaired in positive selection of CD8⁺ cells, as a consequence of reduced class I expression in thymic epithelial cells. Assembly of HLA-B27 in murine cells is critically dependent on peptides delivered by the TAP-dependent pathway (Anderson et al., 1993; van Santen et al., 1995).

The availability of HLA-B27 transgenic mice, together with the TAP1-deficient animals, therefore allows the generation of a model in which either properly assembled, or unassembled class I molecules may be tracked inside the cell. To obtain efficient expression at the cell surface of human class I molecules in transgenic mice, the simultaneous presence of the class I transgene and a human β_2 -microglobulin ($h\beta_2m$) transgene is required (Krimpenfort et al., 1987). We have described animals in which the HLA-B27 and $h\beta_2m$ gene were introduced on a single DNA fragment and where, consequently, these transgenes cosegregate (Baas, 1993). These transgenic animals were then used to generate HLA-B27 animals lacking a functional TAP1 gene (van Santen et al., 1995). They may be used to study the behavior of class I molecules in relation to TAP in different tissues and in particular cell types, especially because several monoclonal and polyclonal antibodies reactive with human class I molecules in ultrathin cryo-sections have been described (Stam et al., 1990; Peters et al., 1991; Baas et al., 1992).

We analyzed the influence of a properly folded state, as

determined by the presence or absence of TAP-dependent peptides, on the distribution of HLA-B27 HCs and human β_2m in thymic epithelial cells *in situ*. Thymic epithelial cells are known to express high levels of class I molecules and play a fundamental role in thymic selection. HLA-B27 is expressed in thymic epithelial cells of normal transgenic animals. In the absence of expression of TAP1, HLA-B27 HCs are largely absent from the plasma membrane, exist predominantly as free HCs as assessed biochemically, and are degraded most likely by an ER-dependent mechanism. In the TAP1-deficient cells, HLA-B27 HCs and $h\beta_2m$ are localized in an extended post-ER/pre-Golgi network or ER-Golgi intermediate compartment, consisting of tubulated and fenestrated membranes. Compartments derived from the extended network and superficially similar to lysosomes are likely involved in a nonlysosomal pathway of degradation of class I molecules, involving the ubiquitin pathway.

Materials and Methods

Mice

TAP1-deficient mice (Van Kaer et al., 1992), mice double-transgenic for HLA-B27 and human β_2m (Baas, 1993), and the cross between these two strains (van Santen et al., 1995) have been described. The mice resulting from this cross are referred to as TAP1⁻ β B27 mice. Their TAP-wild-type counterparts are referred to as TAP1⁺ β B27 mice. Nontransgenic mice obtained during the breeding of the parental strains were used as controls.

Antibodies

The following antibodies were used: mAb W6/32 (Barnstable et al., 1978), recognizing properly folded human class I complexes; mAb HC10 (Stam et al., 1990), specific for human class I free heavy chains (HCs); anti-human HC serum (Stam et al., 1986), specific for free human HCs; anti-human HC serum (Machold et al., 1995), recognizing free HCs only; anti-p8 serum (raised in our laboratory, according to Smith et al. [1986], against a synthetic peptide representing the most COOH-terminal exon of the H-2K^b molecule); anti-human β_2m serum (raised against highly purified β_2m , separated from HLA-A2 and HLA-A28 by gel filtration in acetic acid); rabbit anti-ER serum provided by Dr. D. Louvard (Institut Pasteur, Paris, France) (Louvard et al., 1982); mouse monoclonal anti-PDI (ID3) provided by Dr. S. Fuller (EMBL, Heidelberg, Germany) (Vaux et al., 1990); rabbit anti-bovine cathepsin D provided by Dr. G. Jaureguiberry (INSERM U.13, Hôpital Claude Bernard, Paris, France) (Bailly et al., 1991); mouse monoclonal anti-ERGIC 53 provided by Dr. H. P. Hauri (University of Basel, Basel, Switzerland) (Schweizer et al., 1988); rabbit polyclonal affinity purified anti-human ubiquitin and mouse monoclonal anti-E1 provided by Dr. A. Schwartz (Washington University, St. Louis, MO) (Schwartz et al., 1988, 1992). To visualize the primary antibodies nondirectly reactive with protein A, rabbit anti-mouse IgG and rabbit anti-mouse IgM (Nordic Immunochemicals, Tilburg, The Netherlands) were used.

Metabolic Labeling and Immunoprecipitations

Spleen cells were cultured for 24 h in DME medium, containing 10% FCS and 2.5 μ g/ml ConA. Cells were incubated for 45 min in methionine-free RPMI medium, labeled with 1 mCi [³⁵S]methionine/cysteine (protein labeling mix, New England Nuclear DuPont, Boston, MA) for 20 min and chased in DME medium, containing 10% FCS and 1 mM cold L-methionine and L-cysteine. Cells were lysed in NP-40 lysis mix (0.5% NP-40; 50 mM Tris, pH 7.4; 5 mM MgCl₂; 0.1 mM PMSF) and supplemented with 10 mg/ml BSA.

Thymuses were cut in four segments of approximately equal size and incubated for 45 min in methionine-free medium, labeled for 45 min with 800 μ Ci [³⁵S]methionine/cysteine, and chased for the indicated times in DME medium in presence of 1 mM cold L-methionine and L-cysteine. Thymocytes were removed by gently squeezing the thymic lobes with for-

ceps and rinsing with PBS. The remaining capsules were macerated in NP-40 lysis mix, containing 10 mg/ml BSA, and homogenized in a dounce homogenizer.

Lysates were precleared twice with normal rabbit serum and formalin fixed *Staphylococcus aureus*, followed by the indicated MHC class I specific antibodies and antisera. Precipitates, adjusted for total amount of incorporation, were run on one-dimensional isoelectric focusing gels (1D-IEF) (Neeffjes and Ploegh, 1988). Gels were fluorographed using DMSO/PPO and exposed to KODAK X-OMAT AR film (Eastman-Kodak, Rochester, NY).

Endo H Treatment and Immunoblotting

Whole thymus was depleted of thymocytes as described above. The remaining capsule was macerated with a razor blade and resuspended in 1% SDS/0.5% β -mercaptoethanol. This suspension was briefly heated to 95°C and vortexed twice, and sheared through a 30-G1/2 needle. The suspension was adjusted to 50 mM NaCitrate (pH 5.5), divided into two parts and treated or mock treated with 2,000 units Endoglycosidase H (endoHf) (New England Biolabs, Beverly, MA) for 2 h at 37°C. Aliquots were separated by SDS-PAGE and blotted to nitrocellulose. The blots were incubated with the indicated first antibodies, followed by horseradish peroxidase (PO)-coupled goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) or PO-coupled goat anti-rabbit Ig (Promega, Madison, WI) antiserum. Bound antibody was detected by a chemoluminescence detection kit (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and exposure to X-ray films.

Tissue Fixation, Processing, and Cryosectioning

Mice were anesthetized intraperitoneally with pentobarbital (90 mg/Kg body weight) and the thymuses were rapidly removed, immersed in fixative and immediately cut in small cubes (1 mm³). As fixatives, we used 4% paraformaldehyde (wt/vol) in 0.1 M sodium phosphate buffer pH 7.4 (PB) or a mixture of 2% paraformaldehyde (wt/vol), 1% (vol/vol) acrolein in PB. Fixation proceeded overnight and the tissue was stored in paraformaldehyde 2% (wt/vol) in PB. Processing for ultracytometry was performed as described (Raposo et al., 1995). The small fragments were embedded in gelatin (10%; wt/vol) for 30 min at 37°C, and then solidified on ice. Blocks were then immersed in 2.3 M sucrose in PB for 2 h at 4°C. Tissue blocks were mounted on specimen holders and frozen in liquid nitrogen. Semithin (300–500 nm) or ultrathin sections (60–80 nm) were cut at –80°C and –120°C, respectively, using an Ultra-CutS cryomicrotome (Leica, Biel, Switzerland) and a diamond knife (Diatome, Biel, Switzerland). Semithin sections were collected with 2.3 M sucrose and ultrathin sections with a mixture of methyl cellulose and 2.3 M sucrose (Liou and Slot, 1994).

Electron Microscopy

Ultrathin cryo-sections were collected on formvar- and carbon-coated copper grids and single or double immunogold-labeled as described (Slot et al., 1991; Raposo et al., 1995). Semiquantitative evaluation of the areas of the class I-enriched structures and determination of the density of gold labeling were performed as follows: class I-positive thymic epithelial cells were randomly selected by screening ultrathin cryo-sections immunolabeled with the antibodies directed against the human or the mouse HCs at low magnification ($\times 2,000$). For each thymus (TAP1⁺ β B27 and TAP1[–] β B27), 30 thymic epithelial cells were photographed at $\times 4,000$ in order to determine the area of the cell occupied by the class I-enriched compartments and the same cells were photographed at $\times 12,000$ for subsequent counting of gold particles representing class I HCs. The areas of the class I-enriched structures were determined by superimposing an appropriate lattice grid over the electron micrographs and by counting the number of points within each cell and within each compartment. The density of gold labeling was evaluated by counting the number of gold particles labeling a particular antigen and by relating this number to the area in μm^2 determined as described above. To evaluate the labeling for HLA-B27 HCs over the plasma membrane of thymic epithelial cells, a lattice grid was superimposed over 30 micrographs or directly on a video screen. The number of gold particles counted over the plasma membrane is related to the number of intersections with the membrane and expressed as gold/ μm plasma membrane length.

Immunofluorescence

Semithin cryosections were placed on poly-L-lysine-coated glass slides, quenched with 0.1 M glycine in PBS for 10 min, incubated with the primary antibody in 1% (wt/vol) BSA in PBS for 45 min. After several washings in 0.1% BSA, the sections were incubated with TRITC-conjugated rabbit anti-mouse IgG or goat anti-rabbit IgG (Nordic Immunochemicals) for 30 min. All incubations proceeded at room temperature. After rinsing with water, sections were mounted in Mowiol and examined by epifluorescence with a Reichert-Polyvar photomicroscope.

Results

Assembly and Intracellular Transport of HLA-B27 Is Dependent on Proper TAP Function

We analyzed the assembly, intracellular transport, and steady-state distribution of HLA-B27 in spleen cells and the thymus from TAP1-wild-type mice transgenic for HLA-B27 and $\text{h}\beta_2\text{m}$ (TAP1⁺ β B27), and their TAP1-deficient counterparts (TAP1[–] β B27). Initially, splenocytes were chosen for biochemical analysis as they can be obtained as single cell suspensions of high viability that show excellent incorporation of label. Biosynthetic labeling of thymus fragments was performed to allow a more direct comparison with the immunocytochemistry to be described below. Con A-stimulated spleen cells and thymic lobes were labeled for 20 min and 45 min, respectively, and chased for up to 4 h. Two monoclonal antibodies were used to immunoprecipitate the HLA-B27 molecules: W6/32, specific for properly assembled human class I complexes (Barnstable et al., 1978), and HC10, recognizing only free human class I heavy chains (Stam et al., 1986). The immunoprecipitates were analyzed on 1D-IEF gels to monitor addition of sialic acids upon arrival of class I molecules in the TGN, causing a shift towards a more acidic iso-electric point.

Most of the HLA-B27 heavy chains in the TAP1⁺ β B27 splenic blasts have formed a complex with $\text{h}\beta_2\text{m}$ at the onset of the chase, and can be immunoprecipitated with the W6/32 antibody (Fig. 1 A, first panel). During the chase these complexes acquire sialic acids, indicating that they have moved through the Golgi and have reached the TGN. Only a few of the HLA-B27 molecules in TAP1⁺ cells are present as free heavy chains (Fig. 1 A, third panel). In splenocytes, some of the free heavy chains acquire sialic acid and then decrease over the chase period, showing that breakdown occurs. The observation that in TAP1⁺ cells some HLA-B27 free heavy chains occur in a sialylated form, is most likely caused by dissociation of complexes formed early on due to a reduced affinity of HLA-B27 for murine self peptides, likely to be of different composition than their human counterparts. Sialylated free HCs are not observed in human cells (Neeffjes and Ploegh, 1988; Baas et al., 1992). The murine TAP complex is unable to translocate peptides that terminate in a positively charged residue, yet such residues are abundant among HLA-B27 ligands in human cells (Heemels and Ploegh, 1995).

In the TAP1[–] β B27 spleen cells, the ratio of assembled complexes vs free heavy chains is reversed: trace amounts of HLA-B27 heavy chains are present as a complex that can be recognized by W6/32, and these complexes remain unsialylated (Fig. 1 A, second panel). The majority of the HLA-B27 molecules, however, is present as free heavy

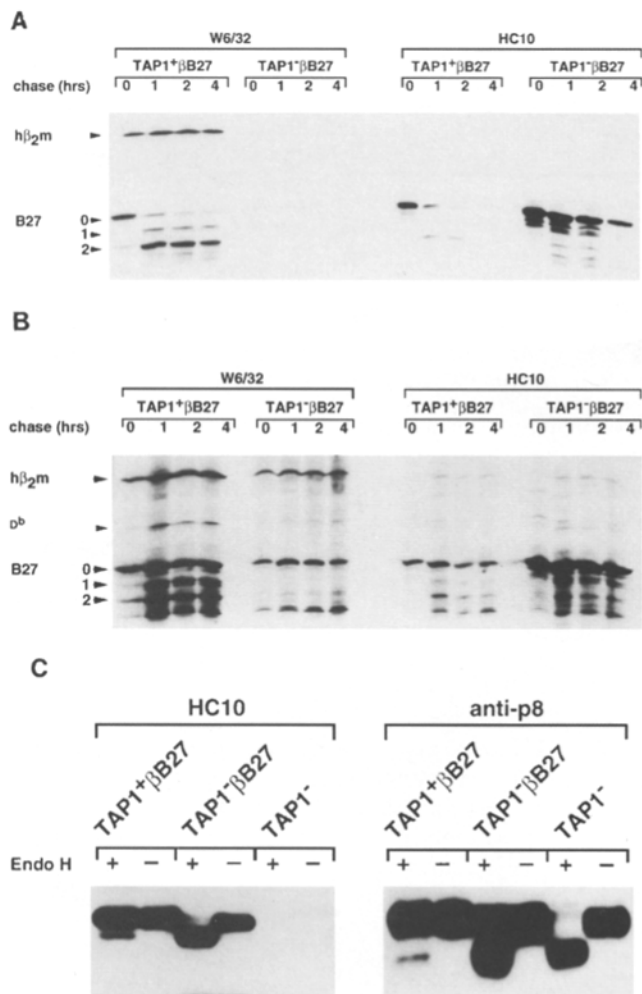


Figure 1. Assembly, intracellular transport, and endo H sensitivity of class I molecules in spleen and thymus from TAP1⁺βB27 and TAP1⁻βB27 mice. Splenocytes (*A*) and thymic lobes (*B*) were labeled biosynthetically for 20 min and 45 min, respectively, and chased for up to 4 h. Cell lysates were made and HLA-B27 heavy chains were immunoprecipitated with mAbs W6/32 or HC10. Immunoprecipitates were analyzed on 1D-IEF gels (*cathode at top, anode at bottom*). Note the slight cross-reactivity of W6/32 with D^b/hβ₂m complexes in the thymic capsules. Numbered arrowheads indicate the different sialic acid modifications of the HLA-B27 heavy chains. (*C*) Denatured and reduced lysates from TAP1⁺βB27, TAP1⁻βB27 and TAP1⁻ thymic capsules were treated or mock treated with endo H, separated by SDS-PAGE, immunoblotted, and probed with mAb HC10 or anti-p8 serum to detect HLA-B27 and K^b, respectively.

chains (Fig. 1 *A*, *fourth panel*). These heavy chains do not acquire sialic acid and are degraded during the chase ($t_{1/2}$ estimated to be 2–4 h). Class I complexes in TAP1-deficient cells may dissociate upon cell lysis (Townsend et al., 1990); the number of HLA-B27 complexes recovered by immunoprecipitation in the TAP1-deficient cells and thymus therefore represents the lower limit of the amount of complexes present. Some isoelectrically heterogeneous polypeptides coimmunoprecipitating with the HC10 antibody can be seen. They do not comigrate with the sialylated forms of HLA-B27 (see first panel), and are insensitive to neuraminidase treatment (data not shown). They

could represent breakdown products of the HLA-B27 heavy chains. This possibility has not been explored further.

In the thymus of the TAP1⁺βB27 mice, complex formation of HLA-B27 heavy chains with hβ₂m and their transport to the TGN, is observed (Fig. 1 *B*, *first panel*). Not all class I complexes are modified by sialic acid during the chase. We attribute this to the less than optimal conditions under which the labeling of thymic tissue fragments is performed, as compared to labeling of activated splenic lymphocytes in suspension. Free heavy chains are present at the onset of the chase (Fig. 1 *B*, *third panel*). These HLA-B27 free heavy chains remain unmodified. At least in Con A blasts, such free heavy chains are degraded (Fig. 1 *A*, *third and fourth panel*), and we suspect that degradation will also occur in the thymus, but difficulties of a technical nature (cell death in culturing thymic lobes) preclude us from establishing this point unambiguously using biochemical methods.

Some HLA-B27 heavy chains in the TAP1⁻βB27 thymus form a complex with hβ₂m (Fig. 1 *B*, *second panel*), but remain unsialylated. This complex formation is more stable than in activated spleen cells of these mice. However, the majority of the HLA-B27 heavy chains is still present as free heavy chains (Fig. 1 *B*, *fourth panel*). These data show that stable complex formation and transport of the HLA-B27 heavy chains in spleen and thymus is dependent on an intact TAP complex.

We performed endo H digestions on class I molecules in the thymus, to determine more accurately where in the secretory pathway the block in transport of HLA-B27 in TAP1-deficient cells manifests itself. Thymic capsules were directly lysed under denaturing conditions, treated with endo H and immunoblotted to determine the steady-state distribution of the class I molecules between the ER/*cis*-Golgi and more distal compartments. We determined the distribution of both the HLA-B27 molecules and the endogenous K^b molecules by using the HC10 antibody and a serum specific for the cytoplasmic tail of K^b (anti-p8), respectively. The HC10 antibody does not react with the thymic extract of TAP1⁻ mice, indicating that the antibody recognizes only the HLA-B27 molecules (Fig. 1 *C*, *HC10 panel*). Most HLA-B27 molecules detected in the TAP1⁺βB27 thymic capsule are endo H resistant, indicating that at steady state most of these molecules are located beyond the ER/*cis*-Golgi area. This observation is also consistent with the assertion that the occurrence of unsialylated HLA-B27 complexes observed in labeling experiments (Fig. 1 *B*) is due to suboptimal conditions of labeling tissue fragments and does not correspond to a sizable pool of unsialylated HLA-B27 HCs at steady state. In contrast, all HLA-B27 molecules detected in the TAP1⁻βB27 thymus remain endo H sensitive, showing that the majority of molecules has failed to reach the medial Golgi. Unlike the HLA-B27 molecules, a significant number of K^b molecules in the TAP1⁻βB27 thymus is resistant to endo H treatment, in agreement with previous data (Alexander et al., 1989; Anderson et al., 1993; van Santen et al., 1995), which have shown that heterodimers formed between hβ₂m and murine class I heavy chains are transported to the cell surface in a TAP-deficient background, owing to the greater stability of these heterodimers compared to their mouse–mouse counterparts.

These data underscore that HLA-B27 relies heavily on a functional TAP complex for proper assembly, folding, and transport. The availability of the TAP1⁻βB27 and TAP1⁺βB27 mice therefore allows us to compare the fate of assembled and unassembled MHC class I molecules *in vivo* by cytochemical methods.

Distribution of HLA-B27 in the Thymus

To examine the pattern of expression of HLA-B27 in the thymus of the transgenic TAP1⁺βB27 mice and compare it to that of endogenous class I molecules, we performed immunofluorescence on cryosections from thymic tissue.

To visualize the human class I HC we used the monoclonal antibody HC10. The murine class I molecules were visualized with the rabbit polyclonal antibody anti-p8. Murine class I molecules in nontransgenic thymus are localized in a reticular staining in the cortical region (Fig. 2 A), characteristic of epithelial reticular cells. The intercellular spaces are filled by thymocytes which stain poorly if at all, because thymocytes, as a rule, are low in class I expression (Van Ewijk, 1984). The medullary area displays a more confluent staining of thymic stromal cells (Fig. 2 A). The labeling observed with the anti-p8 antibody is similar to that described for class I molecules in murine or human thymuses (Rouse et al., 1979; Van Ewijk et al., 1980; Janossy et al., 1980). Staining for HLA-B27 in the thymus of the TAP1⁺βB27 mice is strong in cells of the cortex and medulla with a pattern of expression indistinguishable from that of the endogenous murine class I molecules (Fig. 2 B). Sections of thymus from a nontransgenic animal did not stain with HC10, confirming that this antibody is specific for human class I HCs (Fig. 2 C).

Immunofluorescence patterns with HC10 and anti-p8 antibodies in the thymus of TAP1⁻βB27 mice was similar to those observed in TAP1⁺ animals, though the labeling intensity was lower (see below).

Subcellular Localization of HLA-B27 in Thymic Epithelial Cells

When comparing the steady-state levels of HLA-B27 by immunoblot, we observed that in TAP1⁻ animals the HLA-B27 HCs remain fully Endo H sensitive (Fig. 1 C). This observation indicates that the intracellular transport of HLA-B27 synthesized in the absence of TAP-dependent peptides is blocked before medial-Golgi. To analyze the subcellular localization of HLA-B27 in the absence or in the presence of TAP1, we used immunogold cytochemistry and electron microscopy.

At the ultrastructural level thymic epithelial cells can be easily distinguished by their characteristic nucleus with dispersed chromatin, as well as by the presence of connecting desmosomes and bundles of cytokeratins, the so-called tonofilaments (Clark, 1963; Bearman et al., 1978). To immunolocalize HLA-B27, we used a rabbit polyclonal antibody recognizing class I heavy chains (R anti-hHC) to avoid having to use a bridging antibody (rabbit anti-mouse IgG), necessary when using the HC10 antibody because of its relatively poor direct reactivity with protein A-gold conjugates. To establish the specificity of the R anti-hHC antibody, ultrathin cryosections of thymus from

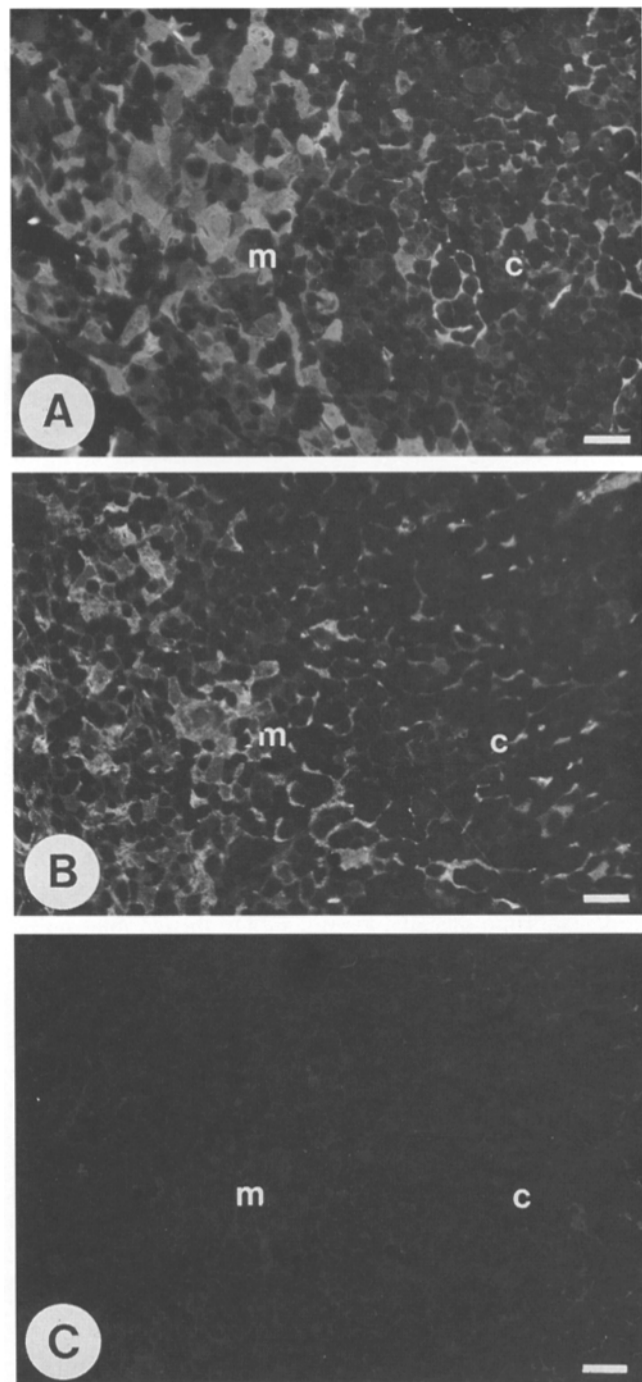


Figure 2. Immunofluorescence localization of endogenous K^b (anti-p8 antibody) and transgenic HLA-B27 molecules (HC10 antibody) in thymic tissue from nontransgenic (A and C) and TAP1⁺βB27 (B) mice. (A) K^b positive cells are detected in the thymic cortex (c) and in the thymic medulla (m). (B) HLA-B27 shows a distribution similar to that of K^b positive cells in cortical and medullary cells. (C) No staining is detected in nontransgenic thymic tissue after immunolabeling with the monoclonal HC10. Bars, 10 μm.

a nontransgenic mouse were immunolabeled (Fig. 3 A). No labeling above background was detected, confirming the lack of cross-reaction with endogenous class I molecules. When using the R anti-hHC antibody, it is not possible to attribute staining to free HC, or to HC that were

part of a complex with β_2m but that acquired reactivity with R anti-hHC antibody upon fixation of the tissue. When reference is made to the distribution of HLA-B27 HCs, this distribution therefore necessarily includes both free HCs and assembled complexes. In thymic tissue from a TAP1⁺ β B27 mouse, specific labeling is observed over the folded plasma membranes of epithelial cells (Fig. 3 B). When the thymus from a TAP1⁻ β B27 animal was labeled with R anti-hHC, a decrease in staining was observed on the plasma membrane of thymic epithelial cells (Fig. 3 C), indicating that in the absence of bound peptide, the majority of HLA-B27 HCs do not reach the plasma membrane. Quantitation of the labeling for HLA-B27 HCs over the plasma membrane of thymic epithelial cells from TAP1⁺ β B27 and TAP1⁻ β B27 animals indicate that this reduction is at least 20-fold (Table I) (see also van Santen et al., 1995).

HLA-B27 in thymic epithelial cells is also detected intracellularly. In thymic epithelial cells of a TAP1⁺ β B27 mouse, HLA-B27 HCs are present in intracellular compartments with a distinctive morphology, consisting of fenestrated areas and electron-dense, tubulated membranes (Fig. 4 B). In thymic epithelial cells from TAP1-deficient animals these structures are more abundant and appear expanded (Fig. 4 A). They, too, consist of electron-dense anastomosing tubular membranes and fenestrated regions enriched in class I HCs, and occupy a substantial area of the cytoplasm. Endoplasmic reticulum, labeled with an antibody directed against protein disulfide isomerase (PDI), is only poorly labeled for HLA-B27 HCs (Fig. 4 A). The HLA-B27 enriched compartments observed in thymic epithelial cells from TAP1⁺ and TAP1⁻ thymuses are very similar morphologically, yet the area of the cytoplasm that they occupy is drastically increased in TAP1-deficient cells. Morphometric analysis shows that in TAP1-deficient cells, the area of the network almost doubled (Table I), largely the consequence of expansion of the electron-dense tubular membranes. The density of gold particles specific for HLA-B27 HCs is also doubled. A total three-fold increase in the labeling for HLA-B27 HCs per cell was observed (not shown). Interestingly, in control animals, compartments with similar morphology, though less expanded and in continuity with electron-dense tubules, contain endogenous class I molecules visualized with an anti-murine HC antibody raised in a manner similar to that used for the R anti-hHC (Machold et al., 1995) (Fig. 4 C). These observations, together with the finding that the majority of HLA-B27 in TAP1-deficient cells remains Endo H sensitive, lead us to conclude that improperly assembled class I molecules are massively retained in an expanded compartment.

Morphological and Immunocytochemical Characterization of the Site of Accumulation of HLA-B27 in TAP1-deficient Thymic Epithelial Cells

In epon-embedded thymus sections of TAP1⁻ β B27 animals, epithelial cells show a network of tubular and anastomosing electron-lucent areas of a morphology and intracellular position equivalent to that seen in cryosections. In the same region, ER cisternae displaying rough and smooth

regions are present, suggesting that the network of smooth tubules is part of a post-ER/pre-Golgi region (Fig. 5). The morphology of the class I-enriched reticulum, as observed in plastic-embedded and positively contrasted material, is reminiscent of the site of assembly and accumulation of virus or unassembled viral glycoproteins observed in the CV1 and CHO cell lines (Kartenbeck et al., 1989; Hobman et al., 1992). An antibody that recognizes four ER membrane proteins and is known to label post-ER derivatives (Louvard et al., 1982; Noda and Farquar, 1992), stains the dense tubules and the vesiculated areas enriched in class I HCs (Fig. 6 A), confirming that the tubulo-vesicular profiles are likely derived from, or are part of the ER. Double labeling allowing the visualization of human HCs and ERGIC-53, a known marker of the ER-Golgi intermediate compartment (Schweizer et al., 1988, 1990), further confirms this interpretation. Although this antibody has been used to immunolocalize the human or primate molecule (Hobman et al., 1992), specific labeling is detected in the ER derived electron-dense tubules and fenestrated areas in mouse tissue as well (Fig. 6 B). These results show that the extended network enriched for HLA-B27 molecules is indeed a post-ER/pre-Golgi network.

Compartments Resembling Lysosomes Originate from the Extended post-ER/pre-Golgi Network

A major question concerns the role of the extended network in which class I molecules accumulate: is it an intermediate station in the recycling of class I molecules between the Golgi and the ER? Is it implicated in the disposal of the accumulated class I molecules? Class I HCs are rarely observed in the Golgi stacks, whereas h β_2m is observed throughout the Golgi cisternae, even in TAP1-deficient cells (Fig. 7 A). The β_2m subunit is normally synthesized in excess in relation to the HC, and is in fact a bona fida secretory protein. The observation that in TAP1-deficient cells β_2m penetrates the secretory pathway into the Golgi complex, whereas the HC do not, illustrates the selective retention of improperly assembled (peptide-free) class I molecules. In intact thymic lobes, biosynthetic labeling reveals the presence of W6/32 reactive, assembled heterodimers, like the situation encountered in T2 cells (Baas et al., 1992). Therefore, we may conclude that HLA-B27 class I heterodimers exist in TAP1⁻ thymic epithelial cells, even though intracellular transport to the Golgi is negligible compared to their TAP1⁺ counterparts. This fraction likely corresponds to the material detected in the post-ER/pre-Golgi compartment after double labeling for HLA-B27 HC and h β_2m .

The HLA-B27 HC and h β_2m are also present in electron-dense and smaller compartments consisting of condensed tubular membranes and vesiculated regions (Fig. 7 B). These compartments are observed in the same cells in which we observe the extended *cis*-Golgi reticulum. More electron-dense compartments showing low labeling for HCs and β_2m are also present (Fig. 7 B). These compartments also display proteins characteristic of intermediate compartments, illustrated by labeling performed with the antibody directed against ERGIC-53 (not shown). The densest compartments observed are morphologically similar to electron-dense lysosomes.

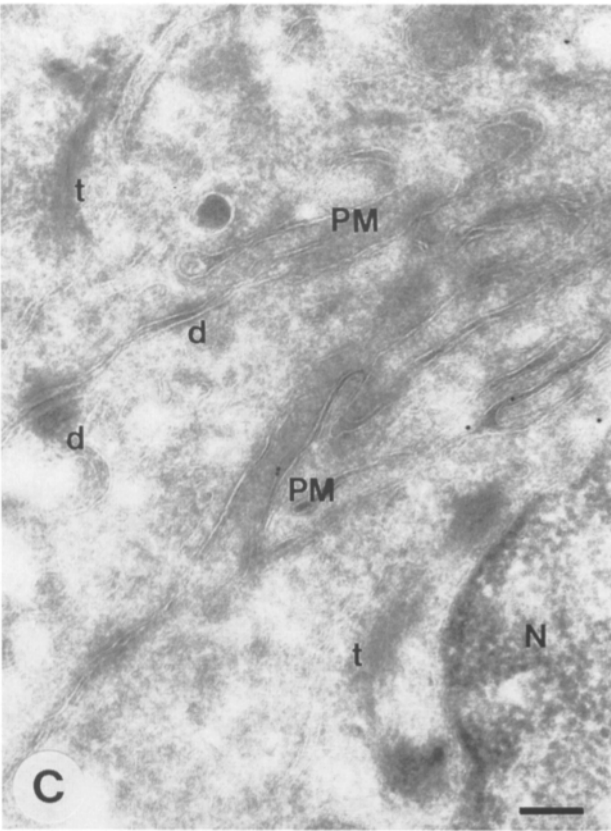
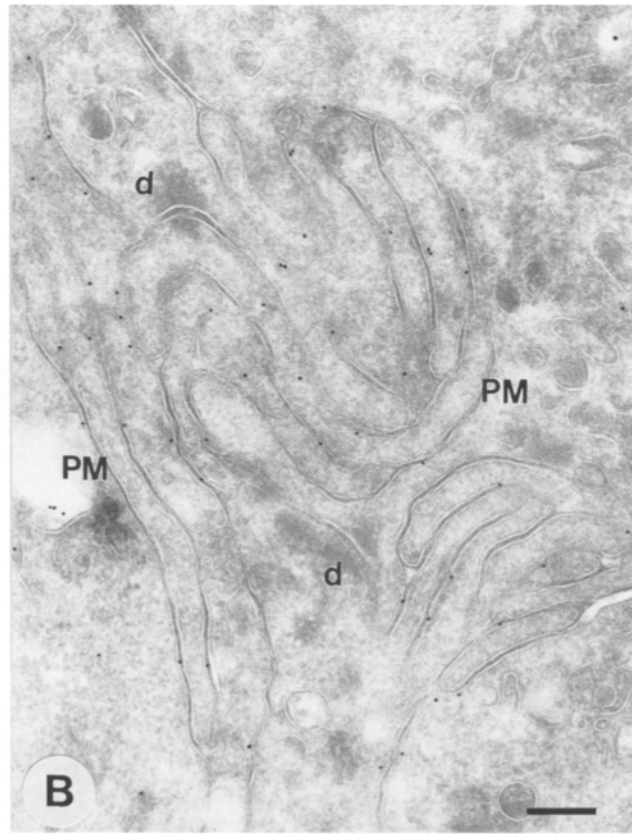
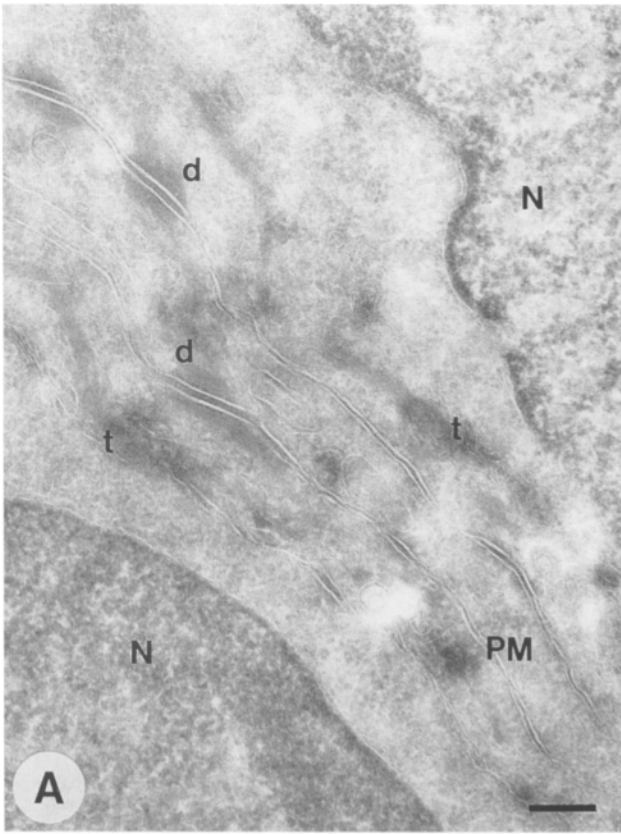


Figure 3. Plasma membrane localization of HLA-B27 in thymic epithelial cells from TAP1⁺βB27 and TAP1⁻βB27 mice. Ultrathin cryosections from thymic tissue of a nontransgenic (A), TAP1⁺βB27 (B) and TAP1⁻βB27 mice (C) were immunogold-labeled with the rabbit polyclonal serum raised against human HCs and protein A-10-nm gold (PAG 10). (A) No labeling is observed with this antibody on thymic epithelial cells of nontransgenic mice. (B) HLA-B27 HCs are detected on the plasma membrane (PM) of two adjacent cells. (C) Little labeling for HLA-B27 HCs is detected on the plasma membrane (PM) of TAP1-deficient thymic epithelial cells. (d, desmosomes; t, tonofilaments; PM, plasma membrane; N, nucleus.) Bars, 200 nm.

Table 1.

	TAP1 ⁺ βB27	TAP ⁻ βB27
Plasma membrane (HLA-B27 HC) gold/μm	14	<1
Post-ER/pre-Golgi network (HLA-B27 HC) gold/μm ²	63	110
Relative volume*	8%	15%

Quantitation of HLA-B27 labeling at the plasma membrane and in post-ER/pre-Golgi compartments in thymic epithelial cells from TAP1⁺βB27 and TAP1⁻βB27 mice. The number of gold particles per μm plasma membrane and the number of gold particles per surface area were determined as described in Materials and Methods.

*Relative volume occupied by the HLA-B27 enriched compartment.

Ubiquitin and Ubiquitin-activating Enzymes Are Associated with the post-ER/pre-Golgi Network

Studies on the disposal of intracisternal granules in thymotrophs previously showed a nonautophagic pathway for conversion of ER cisternae to lysosomal like-organelles (Noda and Farquhar, 1992). A similar mechanism could operate in thymic epithelial cells for accumulated class I molecules.

To investigate this possibility, we performed double labeling, allowing the visualization of cathepsin D at the sites of accumulation of HLA-B27 HCs. The extended compartment with tubulated/fenestrated morphology is not significantly labeled for cathepsin D (Fig. 9 A), nor are the denser organelles (Fig. 8). In the same area of the cytoplasm, cathepsin D-rich lysosomes are clearly present. Equivalent results were obtained with antibodies directed against LAMP1 or Igp120 (not shown). Nonlysosomal proteolysis may operate in parallel, and the ubiquitin-dependent pathway of protein degradation is the major candidate (Hershko and Ciechanover, 1992; Ciechanover, 1994). To test this hypothesis we explored whether ubiquitin and ubiquitin-activating enzyme E1 were associated with the post-ER/pre-Golgi network. Clearly, ubiquitin is associated with the electron-dense tubules and fenestrated areas stained for the human HC (Fig. 9 A) and with the densest compartments (Fig. 9 B). The ubiquitin-activating enzyme E1 is also associated with electron-dense tubules (Fig. 9 C) and it is detected in association with organelles displaying condensed class I-positive tubulated membranes (Fig. 9 D). As compared with its presence in the cytosol, ubiquitin is enriched in the tubular areas. The dimension of the dense tubules and the size of the immunolabel preclude a determination of whether ubiquitin and E1 are inside the lumen of the tubules or at the cytosolic face of the tubule membranes (Fig. 9, A and C). However these proteins clearly reside in the interior of the dense compartments (Fig. 9, B and D). These observations are in agreement with the hypothesis that the pre-Golgi compartments can converge with the ubiquitin pathway of protein degrada-

tion. In Fig. 10, a model is proposed for the disposal of HLA-B27 HCs.

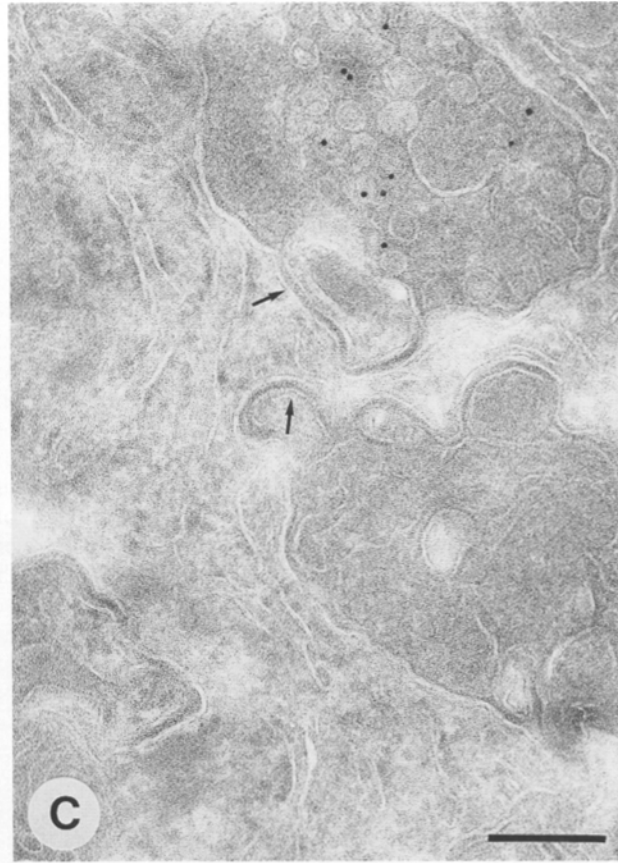
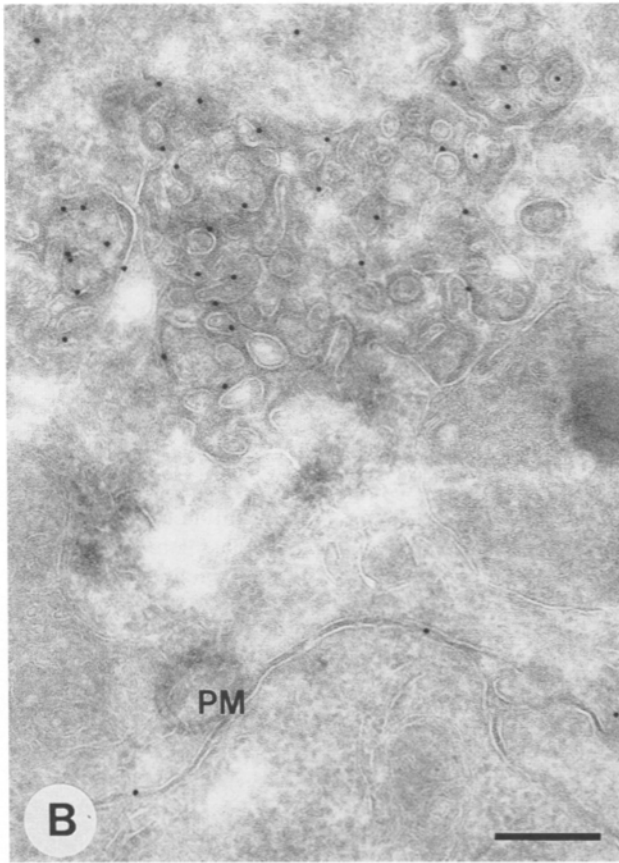
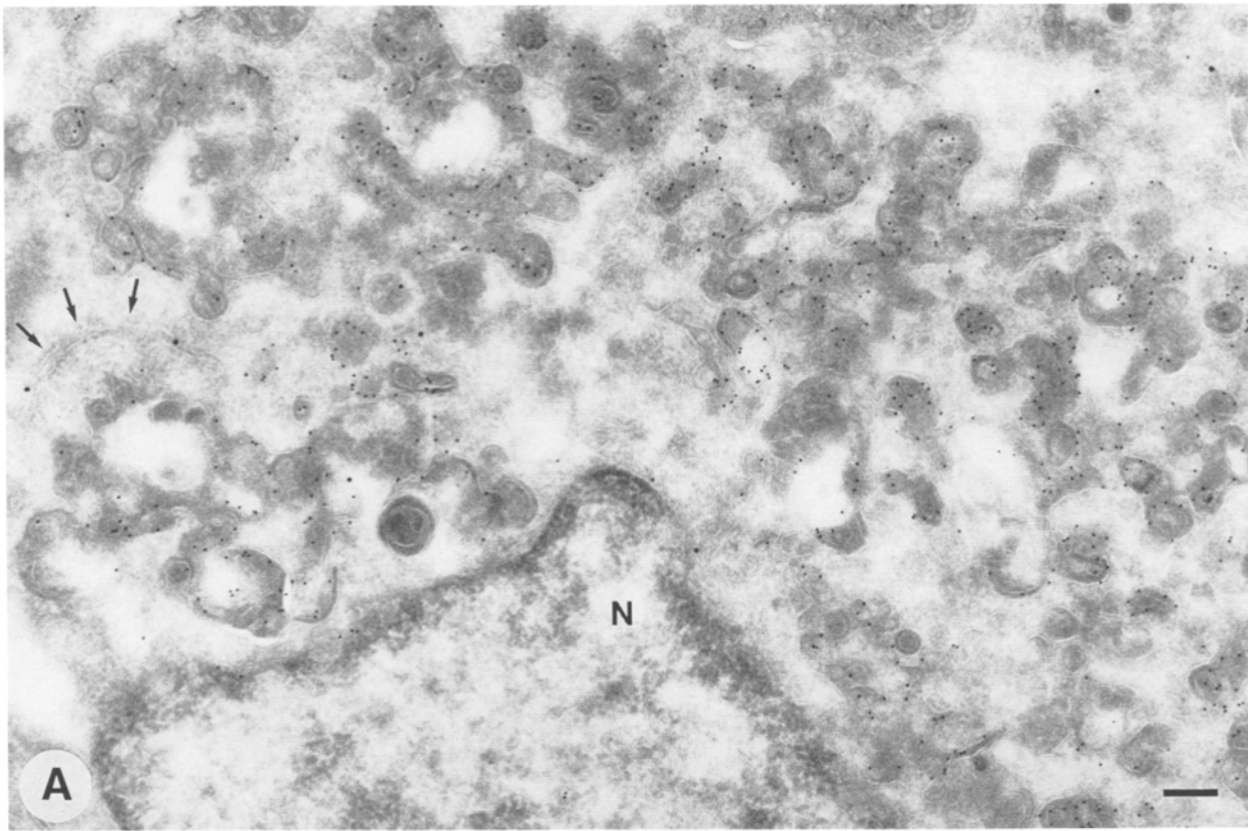
Discussion

In the present study, we have used HLA-B27 transgenic mice and their TAP1 knockout counterparts (van Santen et al., 1995) to approach in an in vivo model and in situ the fate and subcellular localization of misfolded class I molecules. Using polyclonal and monoclonal antibodies recognizing human HC and human β₂m, the localization of HLA-B27 can be followed specifically without cross-reaction with endogenous murine class I molecules.

The results show that in TAP1-deficient mice, (a) unavailability of peptide causes the vast majority of HLA-B27 HC and hβ₂m to remain unassembled. The HLA-B27 HCs do not acquire sialic acids, are endo H sensitive, and in Con A blasts are degraded before arrival in the Golgi. In the thymus, we very much suspect a similar sequence of events, but cell death in the course of culturing thymus fragments does not allow a detailed biochemical analysis of such heavy chain breakdown in the thymus. (b) HLA-B27 HC and β₂m accumulate in an expanded ER-Golgi intermediate compartment displaying anastomosing tubulated and fenestrated membranes. (c) This compartment has its counterpart in nonmutant cells and (d) is associated with ubiquitin and the ubiquitin-activating enzyme E1.

In HLA-B27 transgenic mice, HLA-B27 is expressed at high levels in the thymus and has a cellular distribution similar to that of the endogenous class I molecules in cells of both cortex and medulla, in agreement with previous studies on the localization of MHC molecules in thymic tissue (Rouse et al., 1979; Van Ewijk et al., 1980; Janossy et al., 1980; Van Ewijk, 1984). In thymic epithelial cells from TAP1⁺βB27 mice, at steady-state class I molecules are localized mainly at the plasma membrane, and to a lesser extent in intracellular compartments such as the ER and Golgi complex. In TAP1-deficient cells, HLA-B27 HCs at the plasma membrane are reduced at least 20-fold, confirming that their stable expression at the cell surface is dependent on peptides translocated by TAP (Anderson et al., 1993; van Santen, 1995). TAP1-deficient cells accumulate HLA-B27 HCs and β₂m intracellularly in structures similar to those observed in the TAP1⁺ animals. However, in TAP1-deficient animals, the dense tubulated membranes and fenestrated regions are extended (almost doubled in volume), suggesting a direct link between the inhibition of intracellular transport of class I molecules and the appearance and size of the compartment. In the T2 cell line, class I molecules are retained in the rough endoplasmic reticulum (RER) and in the Golgi region (Baas et al., 1992). In another murine mutant cell line (CMT), class I molecules seem to be retained in smooth parts of the ER and recycle through the cis-Golgi reticulum (Hsu et al.,

Figure 4. Intracellular localization of HLA-B27 and murine class I HCs in thymic epithelial cells from TAP1⁻βB27 (A), TAP1⁺βB27 (B), and nontransgenic (C) mice. Ultrathin cryosections from thymic tissue were immunogold-labeled with the rabbit anti-serum raised against human HCs (PAG 10) (A and B) and with the rabbit anti-serum raised against murine HCs (PAG 10) (C). In A, the ultrathin cryosection was also labeled with an antibody directed against protein disulfide isomerase (PDI) (PAG 15). (A) In TAP1-deficient cells the perinuclear region is filled with tubulated and fenestrated membranes heavily labeled for HLA-B27 HCs (PAG 10). Protein disul-



fide isomerase (PDI) (PAG15) is detected in the RER (*arrows*). (*B*) In thymic epithelial cells from a TAP1⁺βB27 mouse, HLA-B27 HCs (PAG 10) are detected in intracellular compartments of similar morphology but smaller than in *A*. (*C*) In thymic epithelial cells from a nontransgenic mouse, murine HCs (PAG 10) can be detected in compartments with a fenestrated appearance continuous with electron dense tubules (*arrows*). *N*, nucleus; *PM*, plasma membrane. Bars, 200 nm.

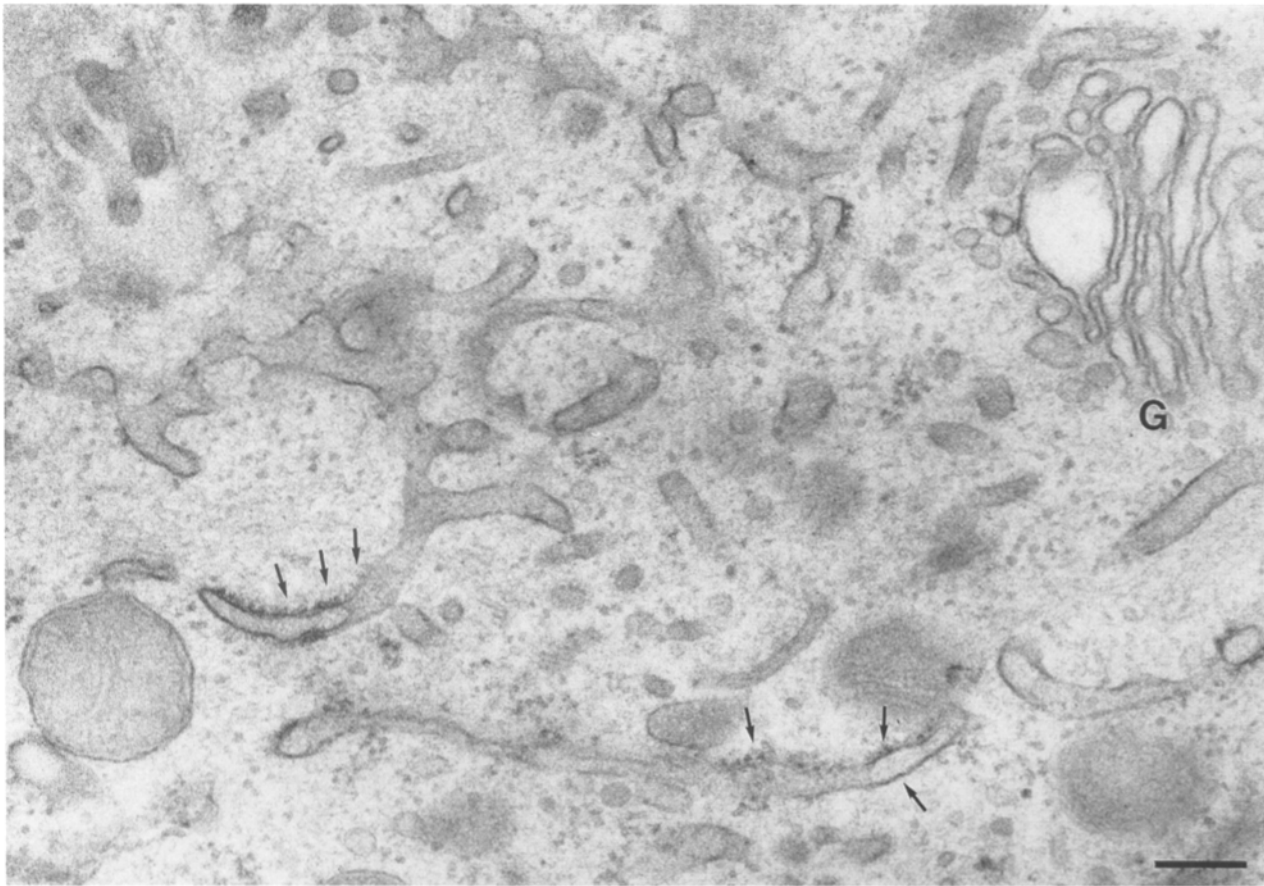


Figure 5. Morphology of the tubulo-vesicular network in plastic-embedded thymic tissue of a TAP1⁻βB27 mouse. Note the tubulated membranes close to the Golgi complex (G). In the same area rough ER cisternae show smooth surfaced subdomains (arrows). Bar, 200 nm.

1990). Morphologically the smooth tubules with electron dense content are reminiscent of “smooth ER” close to, or in continuity with RER (Hobman et al., 1992). The vesiculated areas that we observe in continuity with the tubules are similar to transitional elements described in pancreatic cells (Jamieson and Palade, 1967; Oprins et al., 1993). To characterize the extended network enriched in class I molecules, we used known markers allowing the identification of ER-Golgi intermediate compartments. Our results show that the dense tubulated areas are ER-derived, display ER membrane proteins, and are part of an extended intermediate compartment identified by the presence of ERGIC-53 (Schweizer et al., 1990). Tubulo-vesiculated areas localized between the Golgi and the ER have been identified as the site of assembly of vaccinia and hepatitis virus (Sodeik et al., 1992; Krijnse-Locker et al., 1993), but they are not developed as extensively as is the site of accumulation of the E1 glycoprotein of the rubella virus in CHO cells (Hobman et al., 1992). A post-ER, pre-Golgi compartment was identified as a differentiated proximal portion of the intermediate compartment (Hobman et al., 1992) and the complex tubular network accumulating class I HCs and β₂m’s described in the present study is morphologically similar. A region of anastomosing tubular membranes in continuity with the RER, similar to the compartments referred to above, was also described as the site of accumulation of endocytosed Simian virus 40 (Kartenbeck et al., 1989). Our

observations extend the findings on virus or viral glycoproteins to other multimeric proteins such as class I molecules, and do so in primary cells in situ. A similar ER-derived structure, displaying dense-tubulated regions surrounding electron-lucent areas, was observed upon treatment of pancreatic cells with Brefeldin A (BFA) and was shown to accumulate coatomers (Orci et al., 1994). Our results show that such subcompartmentalization of the ER can also be observed in cells not exposed to BFA. On the other hand, morphologically different ER-derived structures have been shown to accumulate high amounts of HMG-CoA reductase in CHO cells (Chin et al., 1982) and to concentrate chondroitin sulfate proteoglycan precursors in chondrocytes (Vertel et al., 1989).

In thymic epithelial cells from TAP1⁺βB27 mice, class I molecules are found in similar ER-Golgi intermediate compartments but of a smaller size. Also, in nontransgenic normal animals class I molecules can be detected in similar structures. The ability of HLA-B27 to accumulate in smooth tubulated membranes is not a feature unique to thymic epithelial cells because in L-fibroblasts transfected with HLA-B27 in the absence of human β₂m, HCs are also localized in electron-dense tubules in the *cis*-Golgi region (Peters, P. J., N. J. Stam, and H. L. Ploegh, unpublished observations). The presence of class I molecules in the intermediate compartment of thymic epithelial cells from TAP1⁺ animals may be due to high expression of HLA-B27,

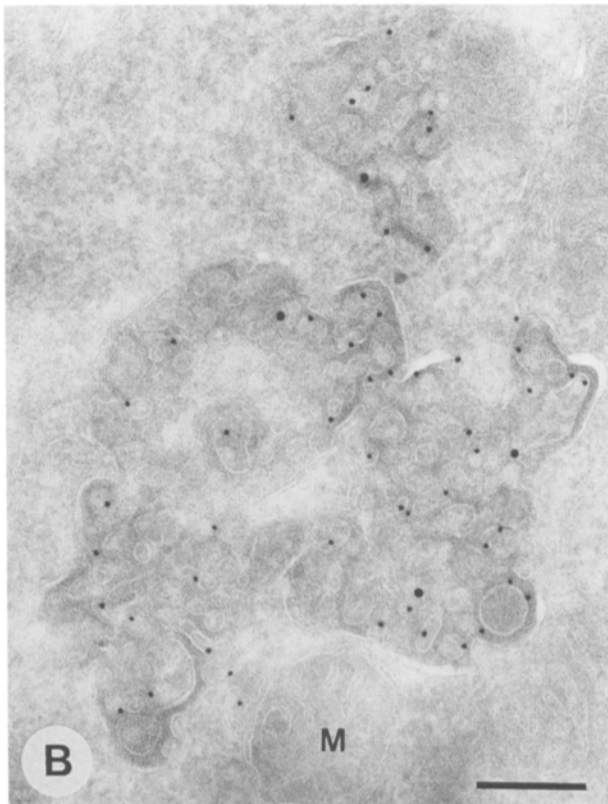
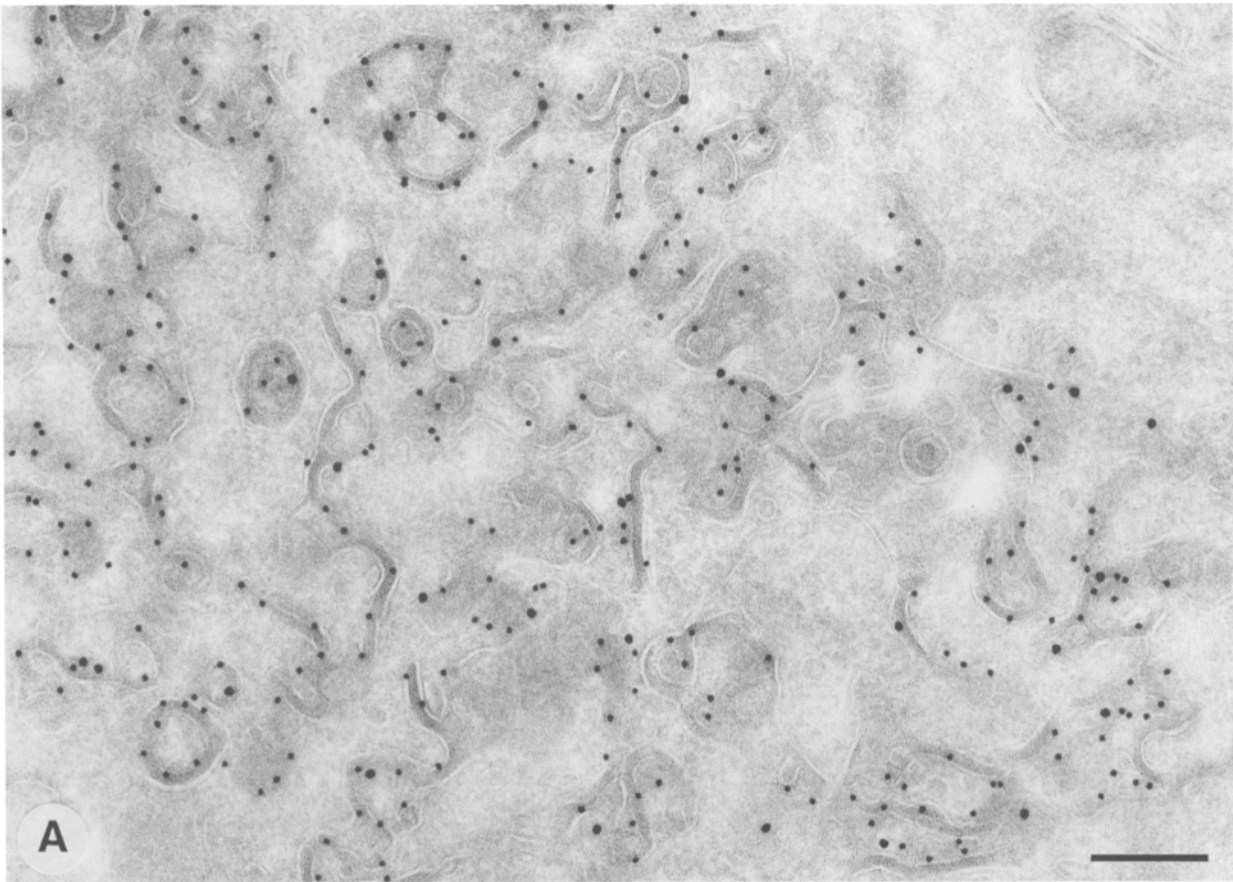


Figure 6. The class I-enriched tubulo-vesicular network contains ER membrane proteins and the intermediate compartment marker protein (ERGIC-53). Ultrathin cryosections from thymic tissue of a TAP^{-βB27} animal were double immunogold-labeled with the rabbit anti-serum raised against human HCs and with a rabbit anti-serum directed against ER membrane proteins (A) or a monoclonal directed against ERGIC-53 (B). (A) The electron dense tubulated membranes intensely labeled with the anti-hHC antibody (PAG 10) are also reactive with the anti-ER antibody (PAG 15 nm). (B) HLA-B27 HCs (PAG 10) colocalize with ERGIC-53 (PAG 15) in tubulated and fenestrated membranes. Bars, 200 nm.

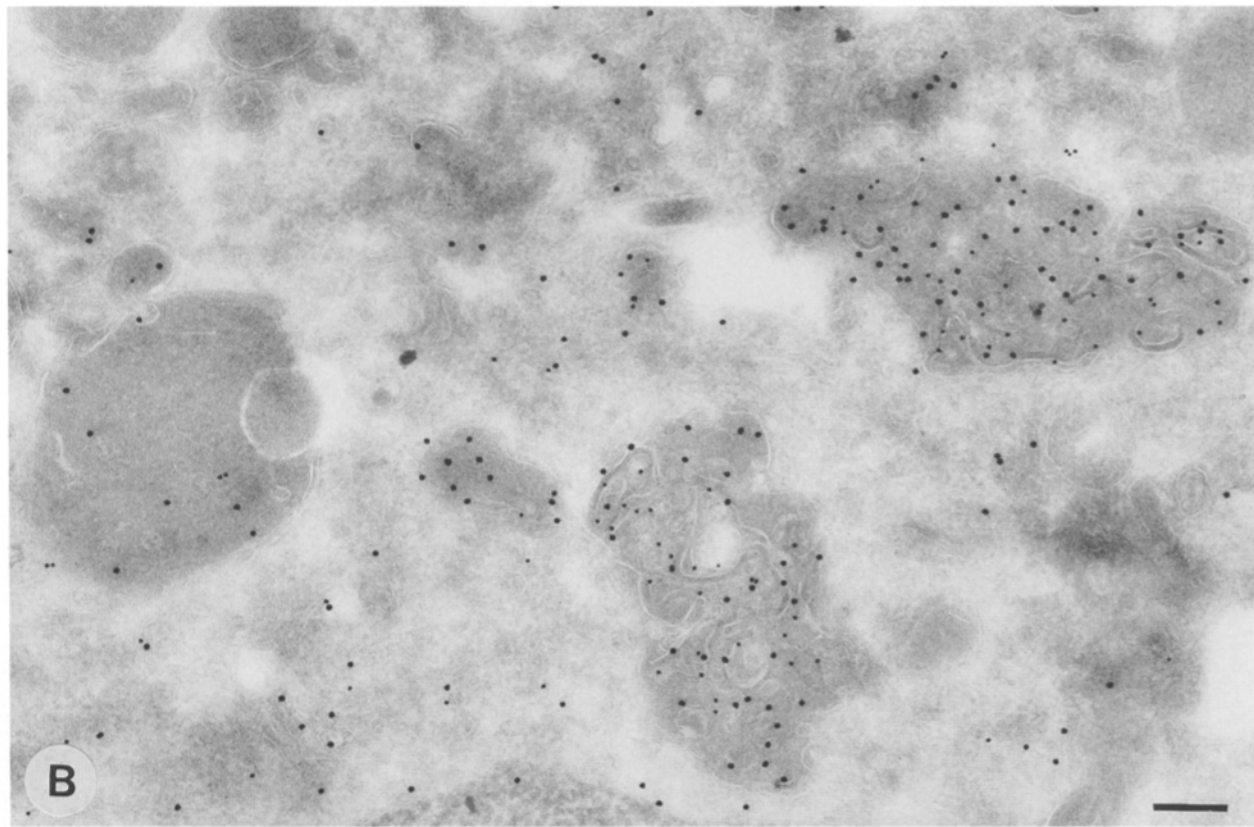
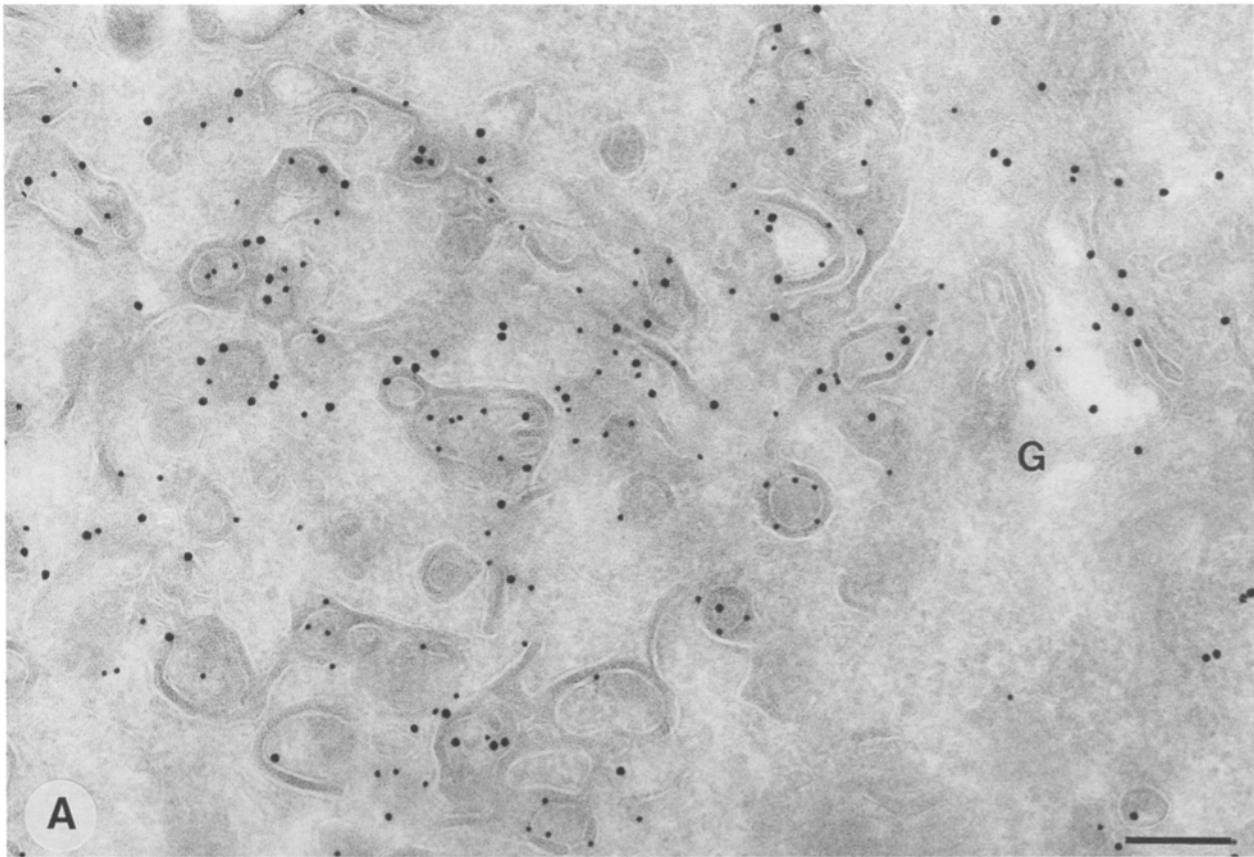


Figure 7. In thymic epithelial cells from TAP⁻βB27 mice HLA-B27 HCs and hβ₂m are localized in electron-dense compartments displaying tubulated membranes. (A) HLA-B27 hHCs (PAG 10) and hβ₂m (PAG 15) colocalize in tubulated and fenestrated membranes close to the Golgi complex (G). Only the β₂m subunit is detected in the Golgi complex. (B) HLA-B27 HCs (PAG 10) and hβ₂m (PAG 15) are detected in compartments displaying tubulated and vesiculated regions reminiscent of the extended tubulo-vesiculated network as well as in electron-dense organelles. Bars, 200 nm.

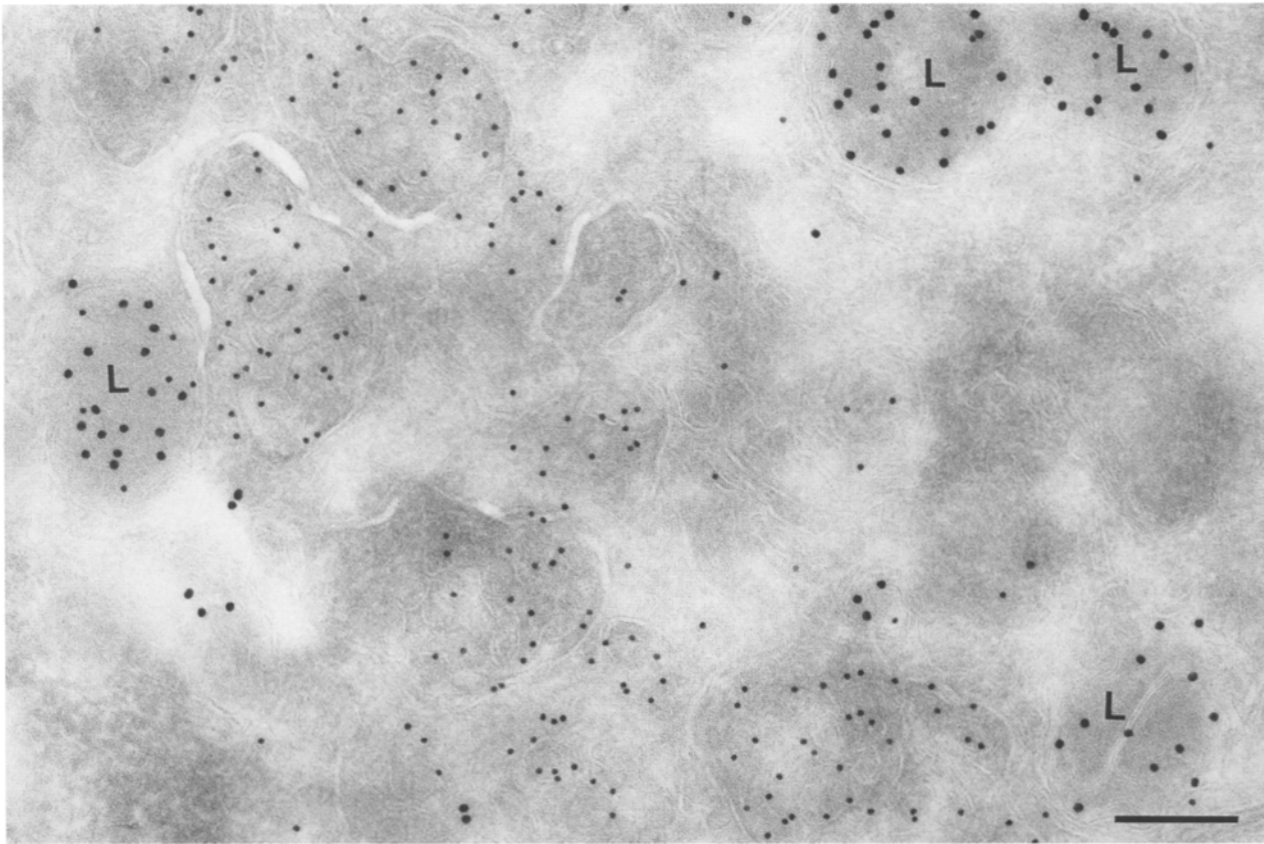


Figure 8. The HLA-B27-enriched compartments are devoid of lysosomal markers. Ultrathin cryosections from thymic tissue of a TAP β B27 mouse were double immunogold-labeled with the rabbit anti-serum raised against human HCs and a rabbit anti-serum directed against cathepsin D. The labeling for HLA-B27 HC (PAG 10) and cathepsin D (PAG 15) is clearly segregated, the lysosomal enzyme being detected only in lysosomes (L). Bar, 200 nm.

in addition to the endogenous murine class I molecules already present in this region. For example, we do not know whether the amounts of class I molecules present in a transgenic animal might saturate a component necessary for their ordered progression from the ER to the Golgi. The accumulation of HLA-B27 HCs could be compounded by the inability of HLA-B27 to bind murine self peptides with affinities comparable to those observed for human self peptides (Peters, P. J., N. J. Stam, and H. L. Ploegh, unpublished observations). Consequently, “empty” molecules may accumulate in the course of biosynthesis in a sub-compartment of the ER.

The exact role of the intermediate compartment between the Golgi and the ER remains to be unraveled. Studies on virus assembly show the importance of the *cis*-Golgi region in the sorting of mature virions (Sodeik et al., 1993; Krijnse-Locker et al., 1994). Intermediate compartments may also play an important role in fatty-acylation of proteins (palmitoylation), in the recycling of proteins from the Golgi to the ER, in the quality control of misfolded or unassembled proteins, and may be directly implicated in ER-degradation mechanisms (Hammond and Helenius, 1994; Hauri and Schweizer, 1992). In TAP1-deficient cells a clear accumulation of class I HCs and β_2m occurs in such a compartment; they are predominantly present as free subunits, as analyzed biochemically in thymic cells and splenocytes.

Our studies do not address the possible association of misfolded HLA-B27 molecules with chaperones such as BIP or calnexin, as has been reported to occur for MHC class I, MHC class II or for viral glycoproteins (Degen and Williams, 1991; Bonnerot et al., 1994; Hammond and Helenius, 1994). As degradation occurs at a pre-Golgi stage, the intermediate compartment itself likely plays a role in the disposal of class I molecules.

In agreement with the assumption that the extended post-ER/pre-Golgi network may be involved in diversion to or convergence with other intracellular routes, we have observed class I HCs and β_2m 's in more electron-dense organelles. These lysosome-like structures display internal tubular membranes reminiscent of the electron-dense, smooth-ER tubules, suggesting that they originate from the condensation of the class I-enriched reticulum. A non-autophagic pathway for diversion of ER proteins to lysosomes has been described for disposal of intracisternal granules in rat thyrotrophs (Noda and Farquhar, 1992). In this ER-derived degradative pathway, RER elements lose their ribosomes and acquire a lysosome-like membrane as well as lysosomal enzymes. Even if the extended network we observe differs from the first steps of accumulation of intracisternal granules and from autophagy (Noda and Farquhar, 1992; Dunn, 1990; Rabouille et al., 1993), we wonder whether the more electron-dense compartments rep-

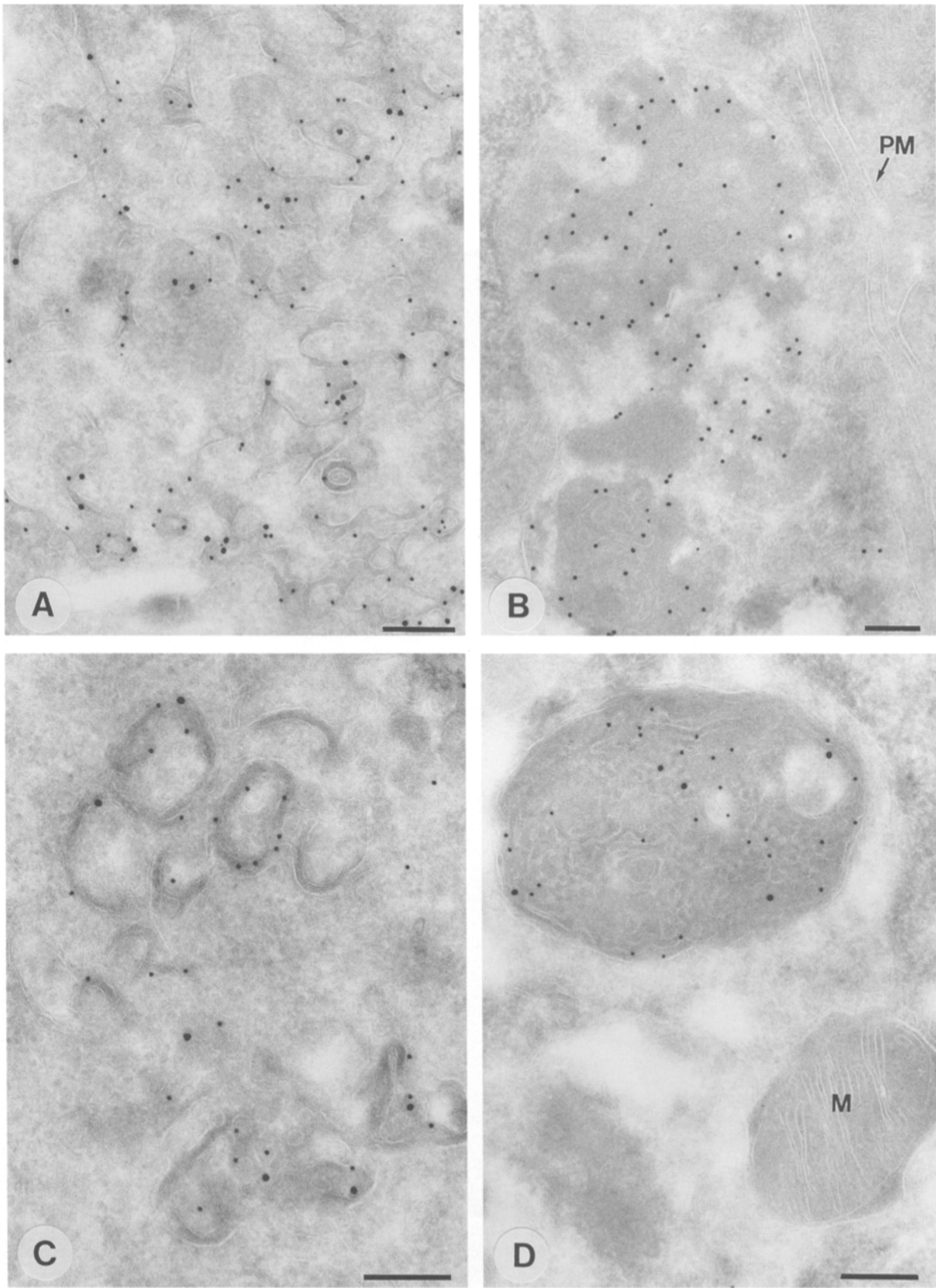


Figure 9. The HLA-B27-enriched compartments contain ubiquitin and the ubiquitin-activating enzyme E1. Ultrathin cryosections from thymic tissue of TAP⁻βB27 mice were double- and triple-immunogold-labeled with the rabbit anti-serum raised against human HCs, with a monoclonal antibody directed against ubiquitin and with a rabbit anti-serum directed against cathepsin D (*A* and *B*) or with a

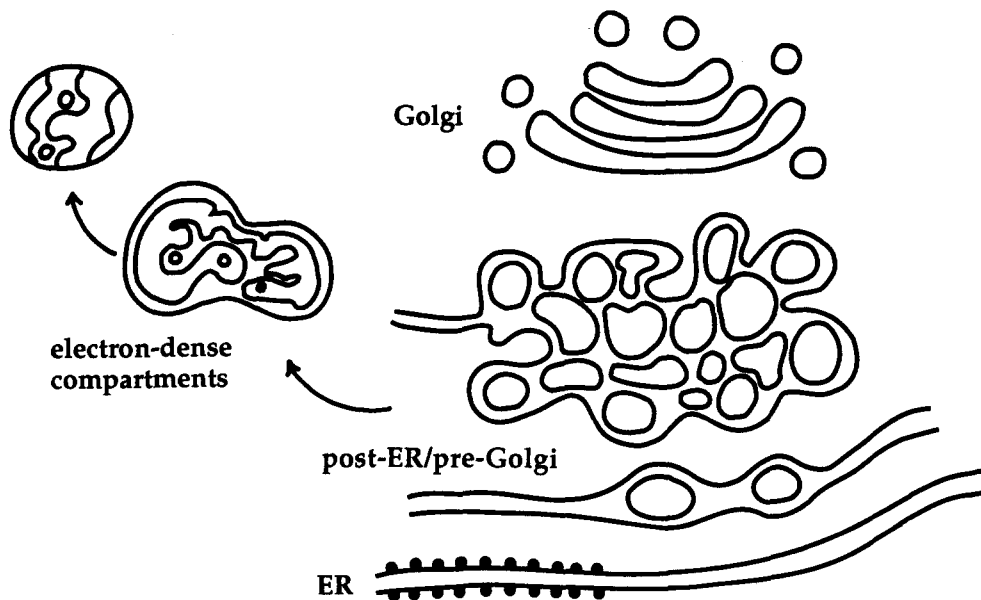


Figure 10. Diagram depicting the proposed ubiquitin-dependent pre-Golgi degradation pathway for class I molecules in TAP1-deficient thymic epithelial cells. In the absence of bound peptides HC and β_2m accumulate in an extended network of tubulated membranes in continuity with the ER and close to the Golgi complex. Electron-dense compartments originate from the compression of the extended network. Ubiquitin and ubiquitin-activating enzymes are progressively enriched in the different steps of compression of the post-ER-pre-Golgi network.

represent a precursor stage in the diversion to lysosome-like organelles. The electron-dense compartments display proteins characteristic of intermediate compartments, such as ERGIC-53. This agrees with the suggestion that they are derived from the extended reticulum and with their localization in the pre-Golgi region. Interestingly, these lysosome-like structures are devoid of lysosomal enzymes or other lysosomal proteins. We investigated whether such compartments could represent a diversion to a nonlysosomal pathway of degradation, where ubiquitin-dependent proteolysis would be a major candidate. There is at present no direct experimental evidence for involvement of ubiquitin in degradation of class I molecules. Ubiquitin is covalently ligated to protein substrates in an ATP-dependent reaction and ubiquitin ligation commits proteins to degradation (Ciechanover, 1994; Hochstrasser, 1995). Using antibodies recognizing ubiquitin and the ubiquitin-activating enzyme (E1), respectively, ubiquitin and E1 have been localized in the cell cytosol, in the nucleus, and in lysosomes and autophagic vacuoles (Schwartz et al., 1990, 1992). These observations suggest that the ubiquitin pathway is operative at different intracellular locations. We observed ubiquitin and the ubiquitin-activating enzyme E1 in association with the class I-enriched post-ER/pre-Golgi network and in electron dense organelles, suggesting the possibility of a link between this compartment and the ubiquitin pathway of degradation. In thymic epithelial cells, ubiquitin is present in the nucleus and to a lesser extent in the cytoplasm, but appears to be concentrated in the class I-enriched profiles. In BHK cells overexpressing the cation-independent mannose-6-phosphate receptor, electron-dense ER-derived tubules occur which accumulate MPR and

are E1 positive (Klumperman, J., and H. J. Geuze, unpublished observations). As proposed by Schwartz (1990), our observations are in agreement with the notion of compartmentalization of the ubiquitin pathway of degradation, as opposed to being a homogeneously cytosolic phenomenon.

ER-degradation has been the subject of numerous studies. Several polypeptides including the asialoglycoprotein receptor, ribophorin, or T cell receptor subunits are known to be degraded by a nonlysosomal endoproteolytic cleavage that takes place in the ER itself or in ER-derived subcompartments (Amara et al., 1989; Wikstrom and Lodish, 1993; Tsao et al., 1992; Klausner and Sitia, 1990; Bonifacino and Lippincott-Schwartz, 1991). Our observations on thymic epithelial cells show that the ER-Golgi intermediate station is complex and can be induced to expand, in response to the quantity and quality of passenger proteins. In the ER, misfolded and unassembled proteins are often relatively long-lived. Frequently, a lag period with little degradation is followed by rapid degradation, further suggesting that the proteins need to be transferred into specific compartments or subregions of the ER (Hurtley and Helenius, 1989; Klausner and Sitia, 1990). In agreement with these observations, our data reveal the existence of ER-derived organelles most likely involved in the disposal of accumulated molecules (Fig. 10). The unique morphological properties of this compartment suggest the possibility that it may be purified away from ER and Golgi membranes by subcellular fractionation. The ability to exert strict control over the folding of HLA-B27 in the model described here should be a useful adjunct to such an endeavor and may assist in the further characterization of this compartment.

monoclonal antibody directed against the ubiquitin-activating enzyme E1 (C and D). (A) Ubiquitin (PAG 15) is detected in electron-dense tubules and fenestrated membranes positive for HLA-B27 HC (PAG 10). No labeling is observed for cathepsin D (PAG 5). (B) electron-dense compartment containing ubiquitin (PAG15) and some HLA-B27 HC (PAG 10). The ubiquitin-activating enzyme E1 (PAG 15) is detected in HLA-B27 HC (PAG 10)-positive tubules (C) and in electron-dense compartments displaying internal tubulated membranes (D). M, mitochondrion; PM, plasma membrane. Bars, 200 nm.

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