Major Histocompatibility Complex Class II Molecules Induce the Formation of Endocytic MIIC-like Structures

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Abstract. During biosynthesis, major histochompatibility complex class II molecules are transported to the cell surface through a late endocytic multilaminar structure with lysosomal characteristics. This structure did not resemble any of the previously described endosomal compartments and was termed MIIC. We show here that continuous protein synthesis is required for the maintenance of MIIC in B cells. Transfection of class II molecules in human embryonal kidney cells induces the formation of multilaminar endocytic structures that are morphologically analogous to MIIC in B cells. Two lysosomal proteins (CD63 and lamp-1), which are expressed in MIIC of B cells, are also present in the structures induced by expression of major histocompatibility complex class II molecules. Moreover, endocytosed HRP enters the induced structures defining them as endocytic compartments. Exchanging the transmembrane and cytoplasmic tail of the class II α and β chains for that of HLA-B27 does not result in the induction of multilaminar structures, and the chimeric class II molecules are now located in multivesicular structures. This suggests that expression of class II molecules is sufficient to induce the formation of characteristic MIIC-like multilaminar structures.

MAJOR histocompatibility complex (MHC)[1] class II molecules present peptides derived from endocytosed proteins to $CD4^+$ T cells (for reviews see Brodsky and Guagliardi, 1991; Neefjes and Ploegh, 1992*a*; Neefjes and Momburg, 1993). Therefore, class II molecules have to enter the endosomal compartments with proteolytic activity where class II-associated invariant chain (li) is removed by proteolysis (Blum and Cresswell, 1988; Nguyen et al., 1989), which renders class II molecules accessible for association with proteolytic fragments (Roche and Cresswell, 1991), including those derived from li (Chicz et al., 1993). Degradation of li is, furthermore, essential for efficient release of class II molecules from endocytic compartments (Neefjes and Ploegh, 1992*b*). It is still a matter of debate where exactly in the endocytic route class II molecules associate with peptide. Initially, early endosomes were assigned as the site where class II molecules and antigen meet (Guagliardi et al., 1990), but numerous biochemical and immunological data now indicate the involvement of late endocytic structures in the generation of peptides (Harding et al., 1991), the degradation of li, and the subsequent loading of class II molecules with peptides (Neefjes and Ploegh, 1992*b*; Harding and Geuze, 1993).

Using immunoelectronmicroscopy, Peters et al. (1991) identified a late endocytic compartment in human B cells where class II molecules accumulated en route to the cell surface. Because this compartment did not label for the late endosomal marker, mannose-6-phosphate receptor, and it lacked a number of lysosomal markers, this compartment was neither a late endosome nor a classical lysosome, and it was termed MIIC for MHC class II compartment. MIIC has a multilaminar structure and contains the lysosomal proteins lamp-1 and CD63 (Peters et al., 1991). MIIC-like structures were later identified in various other cell lines, including activated monocytes (unpublished observation), dendritic cells (Arkema et al., 1991*a*), epidermal Langerhans cells (Arkema et al., 1991*b*), and activated murine macrophages (Harding and Geuze, 1993).

Here, we have studied the possible mechanisms leading to the formation of MIIC. The observation that MIIC is neither a lysosome nor an early/late endosome (Peters et al., 1991), and the fact that MIIC is usually detected in cells expressing class II molecules (Peters et al., 1991; Arkema et al., 1991*a* and 1991*b*; Harding and Geuze, 1993), led us to consider the possibility that expression of class II molecules is a prerequi-

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1. *Abbreviations used in this paper*: li, class II-associated invariant chain; MHC, major histocompatibility complex; MIIC, MHC class II compartment; vWf, von Willebrand factor.

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site for the formation and maintenance of MIIC. We show that inhibition of translation in human B cells resulted in the gradual loss of MIIC, indicating that MIIC requires continuous protein import for its maintenance. Surprisingly, expression of class II molecules (but not of a control protein) in human embryonal kidney cells appears to be sufficient to induce endocytic multilaminar structures that resemble MIIC found in B cells, and the transmembrane and/or cytoplasmic region of class II molecules is critically involved. These data indicate that MHC class II molecules themselves trigger the formation of characteristic multilaminar endocytic structures.

Materials and Methods

Antibodies

The antibodies used were rabbit polyclonal anti-human class II α chain serum and β chain serum (Neefjes et al., 1990), rabbit anti-H2-K^b exon 8 serum (Neefjes et al., 1992*c*), mouse anti-human class II mAb Tü36 (Shaw et al., 1985), mouse anti-human CD63 mAb 435 (Knol et al., 1991), mouse anti-human lamp-1 mAb BB6 (Carlsson and Fukuda, 1989) (a kind gift from Dr. M. Fukuda, Cancer Research Center, La Jolla, CA), polyclonal rabbit anti-HRP serum (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam), and rabbit anti-human cathepsin D serum (a gift of Dr. J. M. Tager, Laboratory of Biochemistry, University of Amsterdam).

Cell Lines and Transfectants

The B-LCL JP expressing HLA-DR4, w53,-DQw3 (vanBinnendijk et al., 1992) was cultured in RPMI 1640 supplemented with 5% FCS. A transfectant of the human embryonal kidney cell line 293 (CRL 1573; American Type Culture Collection, Rockville, MD) (Graham et al., 1977) expressing wild-type HLA-DR1 and the invariant chain truncation mutant Δ15li (Bakke and Dobberstein, 1990) were generated as described (Nijenhuis et al., 1994) and maintained in DME supplemented with 7.5% FCS, 400 μg/ml G418, and 1 μM ouabain. The 293 cell line expressing chimeric class II molecules containing Δ15li and the extracellular portion of the HLA-DR α and β chains fused to the transmembrane and cytoplasmic tail portion of HLA-B27 has been made as described (Nijenhuis and Neefjes, 1994). The transfectants were maintained in DME supplemented with 7.5% FCS, 400 μg/ml G418, and 1 μM ouabain. Expression of the chimeric class II molecules was similar to the wild-type class II molecules in 293 cells as analyzed by FACS® (Becton Dickinson Immunocytometry Systems, Mountain View, CA) (not shown). The 293 cell transfectant expressing the H2-K^b heavy chain was kindly provided by Dr. H. G. Burgert, Max Planck Institute for Immunobiology (Freiburg, Germany) and was maintained in DME supplemented with 7.5% FCS and 400 μg/ml G418.

Biochemistry

293 cells transfected with either class II molecules and Δ15li, the chimeric class II molecules and Δ15li, or with the H chain of H2-K^b were labeled biosynthetically for 15 min with 25 μCi of a mixture of [^{35}S]methionine and cysteine (New England Nuclear, Boston, MA) per dish. Further incorporation of label was inhibited by addition of cycloheximide to a final concentration of 200 μM, and the cells were chased for the times indicated. Cells were lysed in NP40-containing lysismix and either class II molecules (with mAb Tü36), and the free class II α chain and β chain were isolated sequentially or the H2-K^b H chain was recovered. The chimeric class II molecules were isolated with mAb Tü36. Immunoprecipitations were performed from equal amounts of TCA-precipitable counts. Half of the immunoprecipitated wild-type class II molecules were incubated for 16 h with 2 mU Endoglycosidase H (Boehringer Mannheim Biochemicals, Indianapolis, IN) per sample, according to the manufacturer's instructions. Immunoprecipitates were analyzed by 12% SDS-PAGE.

Immunoelectron Microscopy

Transfected 293 cells and the human B-LCL JP cells were fixed in a mixture of 4% (wt/vol) paraformaldehyde and 0.5% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and embedded in 10% (wt/vol) gelatin in PBS. The cell line JP was treated with 200 μM cycloheximide for different periods before fixation. Ultrathin frozen sections were incubated first with a mixture of rabbit anti-class II serum (1/500) and mouse monoclonal anti-CD63 (1/100) or anti-human lamp-1 monoclonal BB6 (1/200) antibodies, followed by incubation with a mixture of goat anti-rabbit IgG linked to 10-nm gold (1/40) and goat anti-mouse IgG linked to 5-nm or 15-nm gold (1/40) (Amersham, 's-Hertogenbosch, the Netherlands). Incubations lasted for 1 h at room temperature. After immunolabeling, the cryosections were embedded in a mixture of methylcellulose and uranyl acetate. Alternatively, the morphology of MIIC was studied on thin sections of 293 cells expressing wild-type class II molecules and Δ15li, fixed with 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in a mixture of LX112/Araldite. All sections were examined with an electron microscope (CM 10; Philips Electronic Instruments, Inc., Mahwah, NJ).

Serial sections were made from 293 cells transfected with class II molecules and Δ15li that were first fixed in 2.5% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), postfixed in 1% osmium tetroxide in the same buffer, stained en bloc with uranyl acetate, and embedded in LX112/Araldite. The electron microscopical images were pictured with the public domain program NIH-Image, which was developed by Wayne Rasband, National Institutes of Health (Bethesda, MD). The alignment of the slices was performed with this program. For the three-dimensional reconstruction, the data set was prepared for visualization with the program MacStereology by Dr. Victor A. Moss (Institute of Physiology, University of Glasgow, Glasgow, Scotland). The final pictures have been processed with the program AVS on a SUN workstation by Dr. M. van Herk (The Netherlands Cancer Institute, Amsterdam).

Results

MIIC Compartments Exist Transiently in B Cells

MIIC compartments were originally detected in human EBV-transformed B cells (Peters et al., 1991). As before, we analyzed the intracellular distribution of MHC class II molecules in an EBV-transformed B cell line by immunoelectron-microscopy. Sections of the B cell line JP were double labeled with anti-class II α chain serum and anti-CD63 mAb. Confirming our earlier report (Peters et al., 1991), extensive labeling of class II molecules was observed in the *trans*-Golgi reticulum area (Fig. 1 *a*, *left panel*). Note the presence of a vesicular structure (*open arrow*) that labels for CD63. As described by Peters et al. (1991), MHC class II molecules colocalized with the CD63 antigen in vesicular structures (Fig. 1 *a*, *right panel*). Occasionally, these structures had a multivesicular morphology, but they usually contained many internal membranes, or they were multilaminar. Labeling of sections of the cell line JP with anti-class II α chain and anti-lamp-1 antibodies confirmed the presence of lamp-1 in MIIC (Fig. 1 *b*) (Peters et al., 1991). At the cell surface, only labeling for class II molecules was observed.

We then examined whether MIIC is a preexisting compartment, or whether MIIC requires continuous protein synthesis for maintenance. Protein synthesis in JP cells was inhibited with the drug cycloheximide, and cells were fixed after varying periods of culture in the presence of cycloheximide. Sections were labeled with anti-class II α chain antibodies, and the amount of MIIC observed in randomly chosen sections of 20 cells, as well as the number of gold particles in MIIC structures, was determined. MIIC compartments are easily detectable by their multilaminar/vesicular ultrastructure and their electron-dense appearance. The amount of MIIC per section gradually decreased upon prolonged incubation with cycloheximide (Fig. 1 *c*, *open squares*). Also, the number of MHC class II molecules present in MIIC decreased (Fig. 1 *c*, *closed circles*). This suggests that continuous protein synthesis is required for the maintenance of MIIC in B cells.

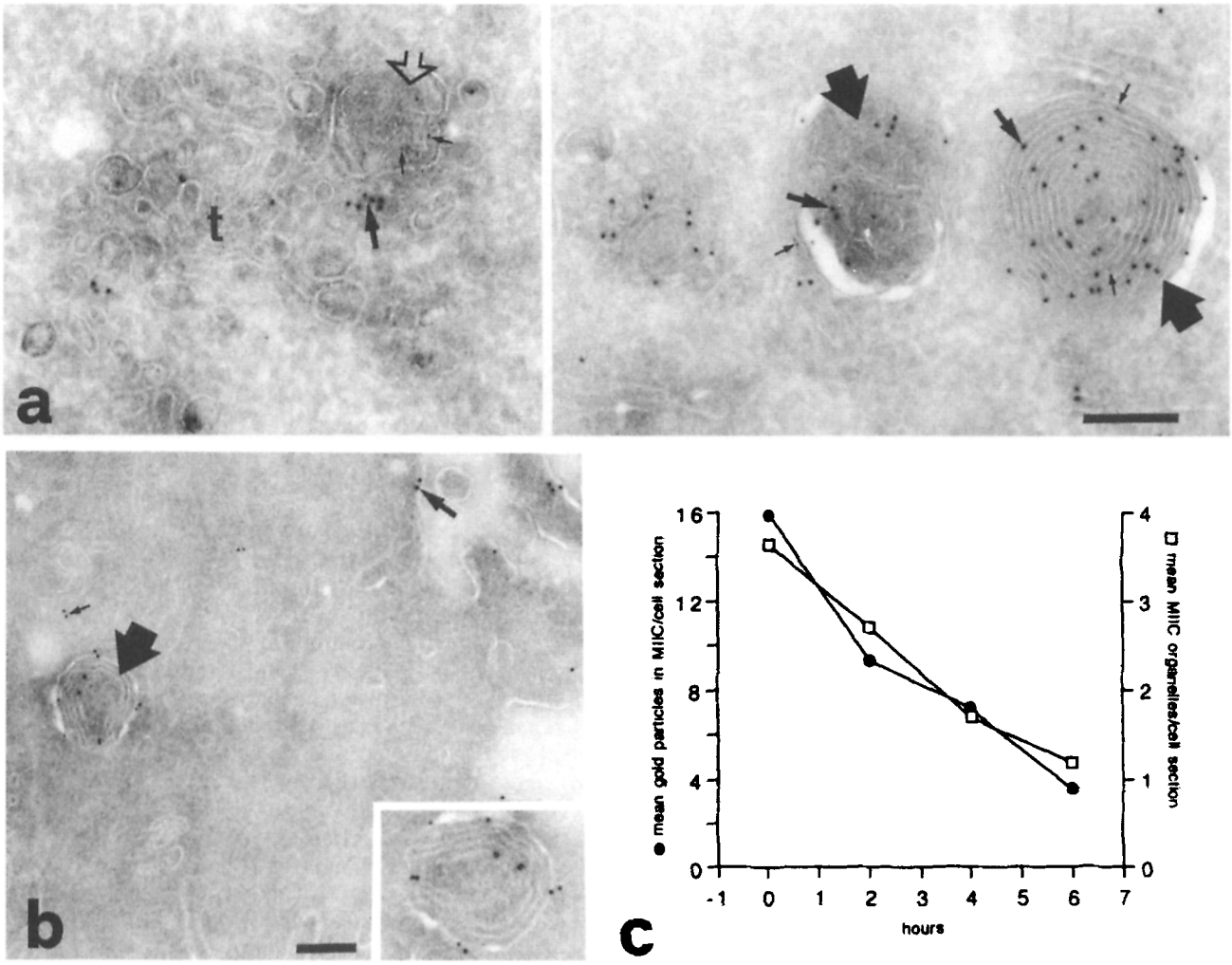


Figure 1. MIIC in B cells and the requirement of protein synthesis. (*a*) Colocalization of MHC class II molecules and CD63 in MIIC of B cells. Ultrathin cryosections of B-lymphoblastoid JP cells were labeled with anti-class II α-chain serum (10 nm gold, *large arrows*) and anti-CD63 mAb (5 nm gold, *small arrows*). The left panel shows a TGR domain (*t*) with vesicles labeling only for MHC class II molecules and a large vesicle containing CD63 (*open arrow*). The right panel shows MIIC structures (*thick arrows*) containing abundant internal membrane sheets, arranged in a concentric shape or containing multiple vesicles. These structures label both for class II molecules and for CD63. Bar, 200 nm. (*b*) Colocalization of MHC class II molecules and lamp-1 in MIIC. Ultrathin cryosections of B-lymphoblastoid JP cells were labeled with anti-class II α-chain serum (15 nm gold) and anti-lamp-10 (10 nm gold). MIIC (*thick arrow*; at higher magnification in inset) contains both class II molecules and lamp-1. (*Small arrow*) A vesicle labeling for lamp-1. Class II molecules are present at the cell surface (*large arrow*). Bar, 200 nm, inset the same magnification as in *a*. (*C*) The effect of cycloheximide (CHX) treatment on the number of MHC class II molecules in MIICs and on the number of MIICs per cell profile. JP cells were cultured in the presence of CHX for the periods indicated in the figure. At each time point, cells were fixed and sections were labeled with anti-class II α chain serum. The total number of gold particles in MIIC and the number of these organelles in 20 randomly chosen sections of cells was determined (indicated on vertical axis). The corresponding period of culture in the presence of CHX is given on the horizontal axis. The number of MIIC gradually decreased after CHX treatment. Furthermore, the amount of MHC class II molecules in these compartments decreased with continuing CHX treatment.

Biochemical Characterization of Cells Transfected with Wild-type Class II Molecules or H2-K^b H Chains

A possible candidate protein required for the maintenance of MIIC is the class II molecule, particularly because the level of expression of class II molecules seemed to be linked to the number of MIIC (Fig. 1 *c*). To test this hypothesis, human 293 kidney cells were transfected with the α and β chains of class II HLA-DR1. To induce rapid exit of class II molecules from the ER, li lacking 15 NH_2-terminal amino acids (Δ15li) was cotransfected (Nijenhuis and Neefjes, 1994). As a control, 293 cells were transfected with the H chain of class I H2-K^b molecules. To characterize the transfectants for the expression of the subunits of MHC class I or II molecules, the transfectants were biosynthetically labeled for 15 min and chased for the times indicated (Fig. 2). Either class II molecules were isolated from the lysates followed by free class II α and β chains, or H2-K^b H chains were recovered (as indicated). To show that class II molecules were readily released from the ER, half of the isolated class II molecules was incubated with Endo H. Class II molecules are rapidly released from the ER and converted to an Endo H-resistant form. Δ15li (31 kD), as well as the class II β

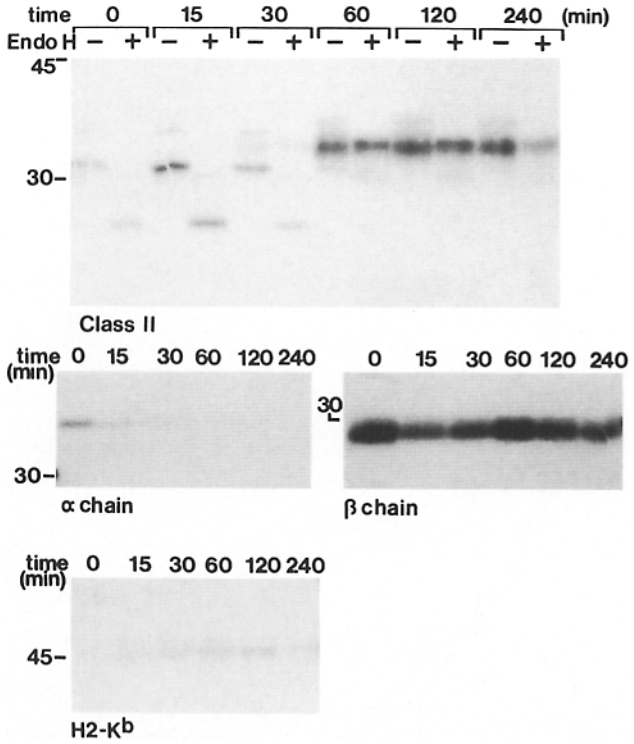


Figure 2. Biochemical analysis of the class II and control transfectants. 293 cells transfected either with class II molecules and Δ15li or with the H-chain of H2-K^b molecules were labeled biosynthetically for 15 min. Incorporation of label was inhibited with cycloheximide, and cells were cultured for the times indicated. Class II molecules and the free class II α and β chains were isolated sequentially from the class II transfectants and the H2-K^b H chain was recovered from the control transfectants. The immunoprecipitates were analyzed by 12% SDS-PAGE, as indicated. To follow exit from the ER, the isolated class II molecules were analyzed by SDS-PAGE before or after Endo H digestion, as indicated. Class II molecules are rapidly converted to an Endo-H-resistant form, indicative for exit from the ER. The class II β chain is expressed in excess over the class II α chain. The H2-K^b H chain is expressed in the control transfectant.

chain (30 kD), are already Endo H-resistant after 60 min. The mature class II α chain (35 kD) contains one high mannose and one complex carbohydrate and is, therefore, partially Endo H resistant. The cell surface expression of class II molecules was analyzed by FACS®, and it was found to be similar to that of EBV-transformed B cells (Nijenhuis et al., 1994). Free class II α and β chains can be recovered after isolation of the class II molecules, and the free class II β chain is expressed in excess. H2-K^b H chains are recovered from lysates of the control transfectant.

Expression of MHC Class II Molecules Induces MIIC-like Endosomal Compartments

The intracellular location of MHC class II molecules was analyzed in the class II transfectant. At the same time, the intracellular distribution of two markers for MIIC (Peters et al., 1991), CD63 (Fig. 3), and lamp-1 (Fig. 4) was determined in the class II transfectant, as well as in the H2-K^b H-chain transfectant. In the class II transfectant, MHC class II molecules were present at the cell surface and in characteristic multilaminar structures (Fig. 3 *a*) that were morphologically similar to MIIC in B cells (compare with Fig. 1). As is the case with MIIC in B cells, these multilaminar structures contained the CD63 antigen. In contrast, CD63 molecules were observed in vesicles with no or few internal membranes, and they were abundantly present in lysosomal-like structures in 293 cells transfected with H2-K^b H chains (Fig. 3 *b*). Multilaminar structures were usually not observed. In the class II transfectants, lamp-1 colabeled with MHC class II molecules in structures with a multilaminar morphology (Fig. 4 *a*), again similar to MIIC in B cells (Fig. 1; Peters et al., 1991). On the other hand, in the control transfectant, lamp-1 was observed in vesicles with few or one internal membranes (Fig. 4 *b*), but compartments with a morphology similar to MIIC in B cells were not detected. The multilaminar structures induced in 293 cells transfected with class II molecules and li also labeled for cathepsin D (Fig. 4 *b*, *lower panel*). Thus, the induced compartments contained at least three lysosomal markers (CD63, lamp-1, and cathepsin D; see also Table I).

To test whether the MHC class II-containing compartments observed in the class II transfectant were part of the endocytic route, as MIIC in B cells (Fig. 1; Peters et al., 1991), we cultured the control and the class II transfectants for 15 min with the fluid-phase endocytosis marker HRP before fixation. Sections were double labeled with anti-class II and anti-HRP antibodies. HRP was observed in vesicles with electron-lucent lumen in the control transfectant (Fig. 5 *b*), whereas HRP was localized in multilaminar/vesicular, electron-dense, MIIC-like compartments containing class II molecules in the class II transfectant (Fig. 5 *a*). Thus, the MIIC-like compartments induced by expression of class II molecules are endocytic structures that contain certain lysosomal marker proteins, as described for MIIC in human B cells (Peters et al., 1991). Surprisingly, HRP reaches MIIC-like structures after already 15 min in class II-transfected 293 cells, whereas another fluid phase marker (cationized ferritin) required >30 min to enter similar compartments in EBV-transformed B cells (Peters et al., 1991), suggesting that some cell-type specific differences in accessibility of endosomal/lysosomal compartments do exist.

The Three-dimensional Structure of the Induced MIIC-like Compartment

The ultrastructure of the multilaminar structure induced by expression of class II molecules was studied on thin sections of the class II transfectant embedded in a mixture of LX112/Araldite. Fig. 6 shows several of the induced multilaminar structures at high magnification. Each leaflet has a triple layered organization ("unit membrane") characteristic of an intact membrane (see also the membrane of the ER and the plasma membrane [pm]). In this section, the thickness of the unit membrane of the induced MIIC compartment and the ER was 72 Å, and that of the cell surface was 77 Å, which is in line with published observations (Benedetti and Emmelot, 1968; Sjöstrand, 1968). The structures induced by expression of class II molecules are different from myelin figures that develop from residual phospholipids (Glauert and Lucy, 1968) because the latter consists of closely spaced lamellae, giving a fine striated pattern of light and dense lines with a repeat period of 40 Å in embedded specimens

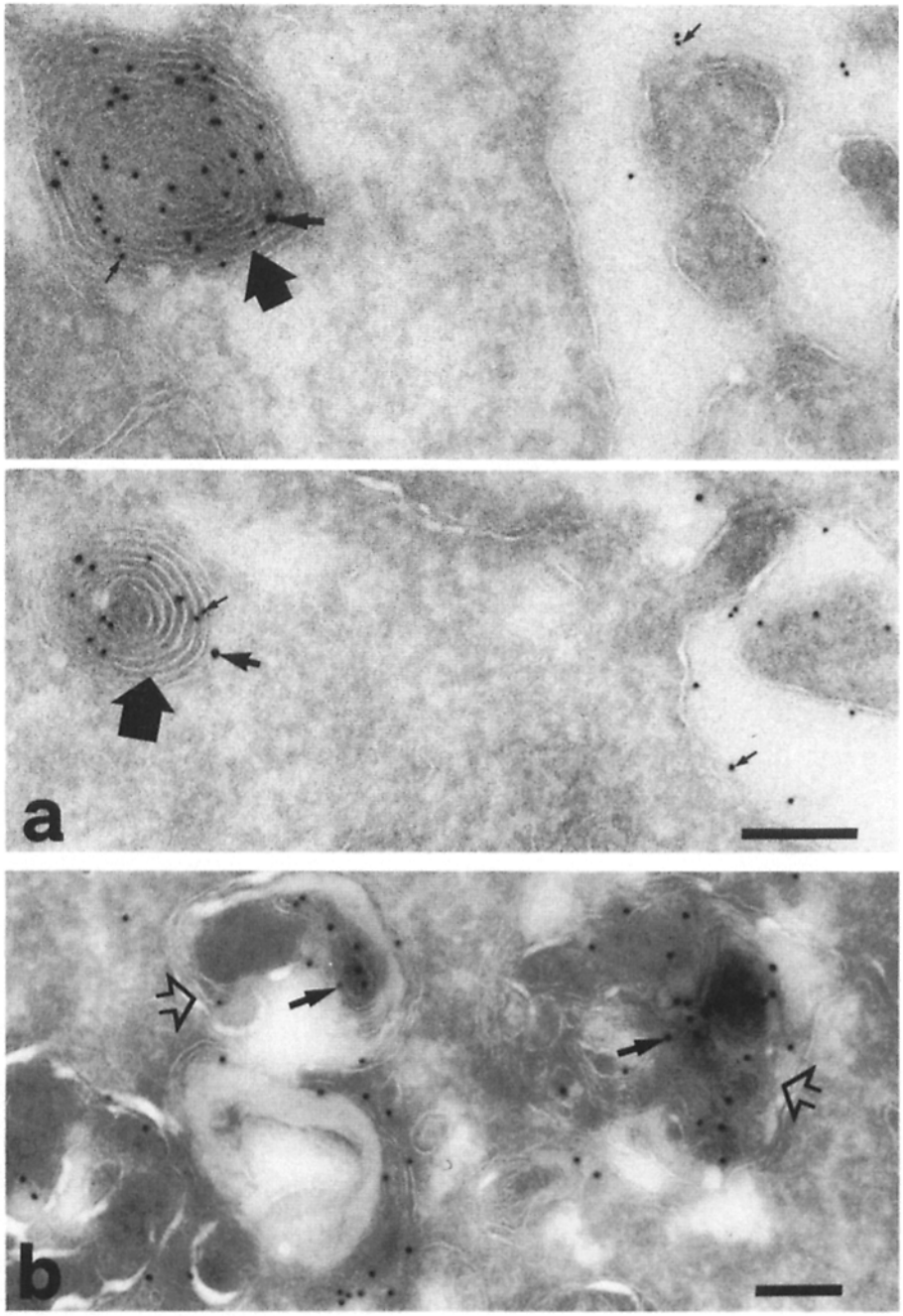


Figure 3. Morphology of CD63-containing structures in control and class II transfectants. (*a*) CD63 and MHC class II molecules are present in multilaminar MIIC-like structures in 293 cells transfected with class II molecules. Sections of 293 cells transfected with class II molecules and Δ15li were labeled with anti-class II α-chain serum (*small arrows*, 10-nm gold) and anti-CD63 mAb (*large arrows*, 15-nm gold). Multilaminar MIIC-like organelles (*thick arrows*) are observed that labeled with anti-class II and anti-CD63 antibodies. At the cell surface, only staining for class II molecules is observed. (*b*) In control transfectants, CD63 is present in lysosomal-like structures lacking a multilaminar appearance. Sections of 293 cells transfected with the H chain of H2-K^b were incubated as described under *a*. Labeling with anti-CD63 antibodies (*arrows*) is mainly observed in lysosomal-like structures that are morphologically distinct from the CD63-positive structures in the class II transfectant. No labeling is observed with anti-class II antibodies. Bars, 200 nm.

(Glauert and Lucy, 1968). The MIIC-like structures shown here contain an electron-lucent space between the leaflets ranging from 72 to 92 Å in width.

A three-dimensional structure of MIIC-like structure was constructed on the basis of serial sections of class II transfectants embedded in LX112/Araldite. We never observed any connection with other structures in the cells. An MIIC-like structure was comprised in ~12 serial sections, each section having a thickness of ~70 nm (Fig. 7 *A*). The morphology of a single compartment varied from multilaminar with internal vesicles (sections 2–5) to almost entirely multilaminar (section 8). The bottom and top section did not show any detailed structure.

The three-dimensional surface structure of the induced MIIC-like structure was determined by introducing the coordinates of the circumference of the respective sections. It shows that the MIIC-like structure has an irregular globular structure with a diameter of ~800 nm (Fig. 7 *B*). Removal of the first four sections gives an overview of the surface and the content of the MIIC-like compartment (Fig. 7 *C*). The multilaminar structures are found under the outer membrane surface and surround an area of small vesicles.

The Induced Multilaminar MIIC-like Structure is Induced by the Transmembrane and Cytoplasmic Region of Class II Molecules

To analyze which part of the MHC class II αβli heterotrimer was reponsible for the induction of the MIIC-like structure, we first analyzed 293 cells transfected with the class II α

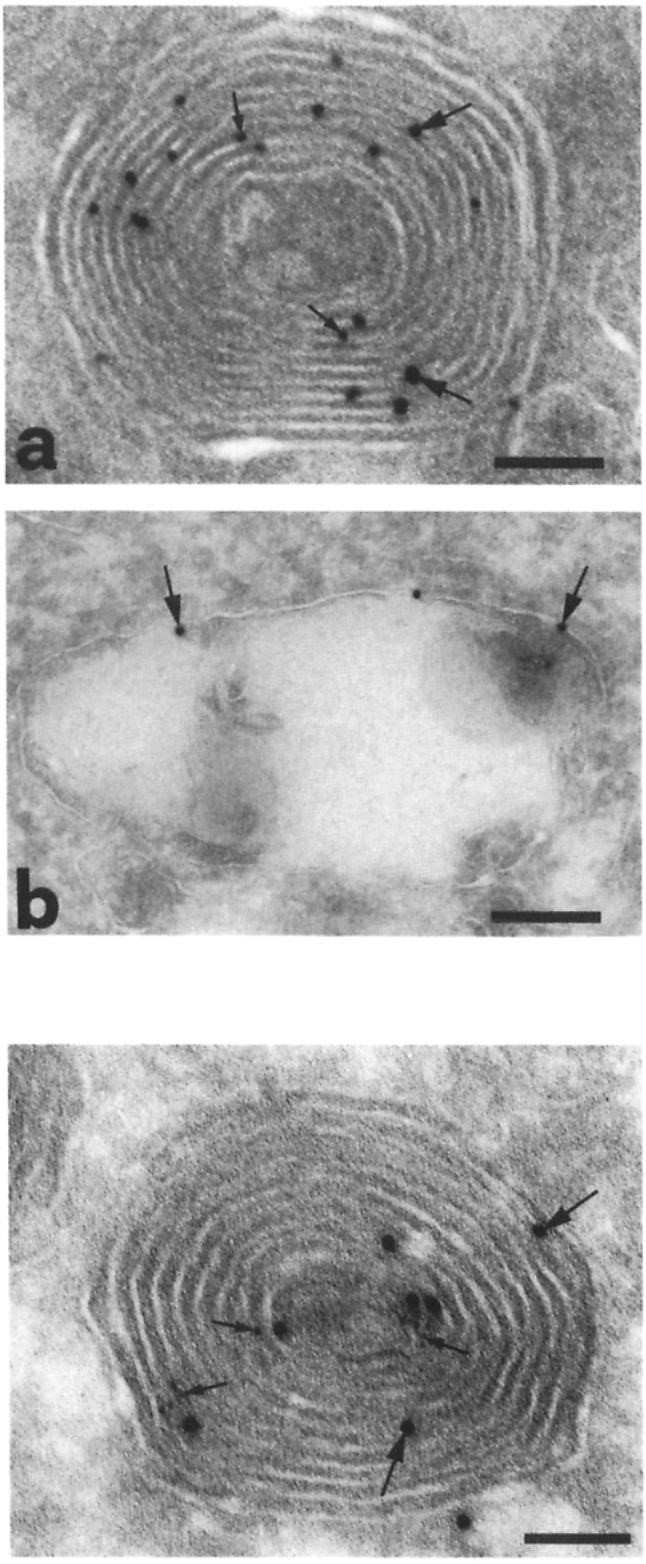


Figure 4. Morphology of lamp-1-containing structures in class II and control transfectants. (*a*) Lamp-1 and MHC class II molecules are present in membrane-rich multilaminar structures in the class II transfectants. Sections of 293 cells transfected with class II molecules and Δ15li were incubated with anti-class II (*small arrows*, 10-nm gold) and anti-lamp-1 (*large arrows*, 15-nm gold) antibodies. A MIIC-like multilaminar structure is labeled for both class II molecules and lamp-1 molecules. Bar, 100 nm. (*b*) Lamp-1 molecules are present in vesicular structures without multiple internal membranes in control transfectants. Sections of 293 cells transfected with the H chain of H2-K^b were incubated as in *a*. The anti-lamp 1 antibody (10-nm gold) stains the membrane of a vesicle that is morphologically different from the lamp 1 (or CD63)-positive structures in class II transfectants. No labeling was found with anti-class II antibodies. Bar, 200 nm. The panel below *b* shows colocalization of MHC class II molecules and cathepsin D. Sections of 293 cells transfected with class II molecules and Δ15li were incubated with anti-class II antibodies (*large arrows*, 10-nm gold) and anti-cathepsin D antibodies (*small arrows*, 5-nm gold). The multilaminar MIIC structure labels for both antibodies. Bar, 100 nm.

Table I. Semiquantitative Analysis of the Intracellular Distribution of Analyzed Proteins

293 Cells transfected with wild-type class II molecules and Δ15li

	RER	MIIC	MVB	Ves.	Pl.mem
MHC class II	+	++	+	+	++
Cathepsin D	−	+	−	−	−
CD63	−	++	+	+	−
Lamp-1	−	++	+	+	−

293 Cells transfected with chimeric class II molecules and Δ15li

	RER	MVB	Ves	Pl.mem
MHC class II	++	++	+	++
CD63	−	+	+	−
Lamp-1	−	+	+	−

The distribution of the marker proteins was established by determining the relative labeling of a particular antigen. Ves., vesicles other than MVB or MIIC; Pl.mem, plasma membrane.

and β chain. No li was expressed in these cells (Nijenhuis and Neefjes, 1994). Sections were labeled with anti-class II and anti-CD63 antibodies. We observed multilaminar structures that labeled for both antigens (Fig. 8 *A*), indicating that li was not essential for the induction of these structures.

We then analyzed whether the information for the induction of multilaminar structures was located in the extracellular portion of class II molecules. Therefore, the transmembrane and cytoplasmic region of class II molecules was exchanged for that of class I HLA-B27, and 293 cells were transfected with these chimeric molecules and Δ15li (Nijenhuis and Neefjes, 1994). The surface expression of the chimeric class II molecules was similar to the wild-type class II molecules, as determined by FACS® analysis (not shown). Intracellular transport of the chimeric class II molecules was analyzed by labeling the transfectants with [^{35}S]methionine/cysteine for 15 min followed by culture for the times indicated (Fig. 8 *C*). Class II molecules were isolated with the mAb Tü36, recognizing properly folded class II molecules, and they were analyzed by SDS-PAGE. The chimeric class II molecules and associated Δ15li are efficiently transported with kinetics similar to the wild-type class II molecules (Fig. 2).

We then determined the intracellular localization of the chimeric class II molecules by labeling sections of the transfectant with anti-class II and anti-lamp-1 antibodies (Fig. 8 *B*). Surprisingly, multilaminar MIIC-like structures were not observed in the 293 cells transfected with the chimeric class II molecules (see also Table I). Instead, only multivesicular structures were observed that labeled both for

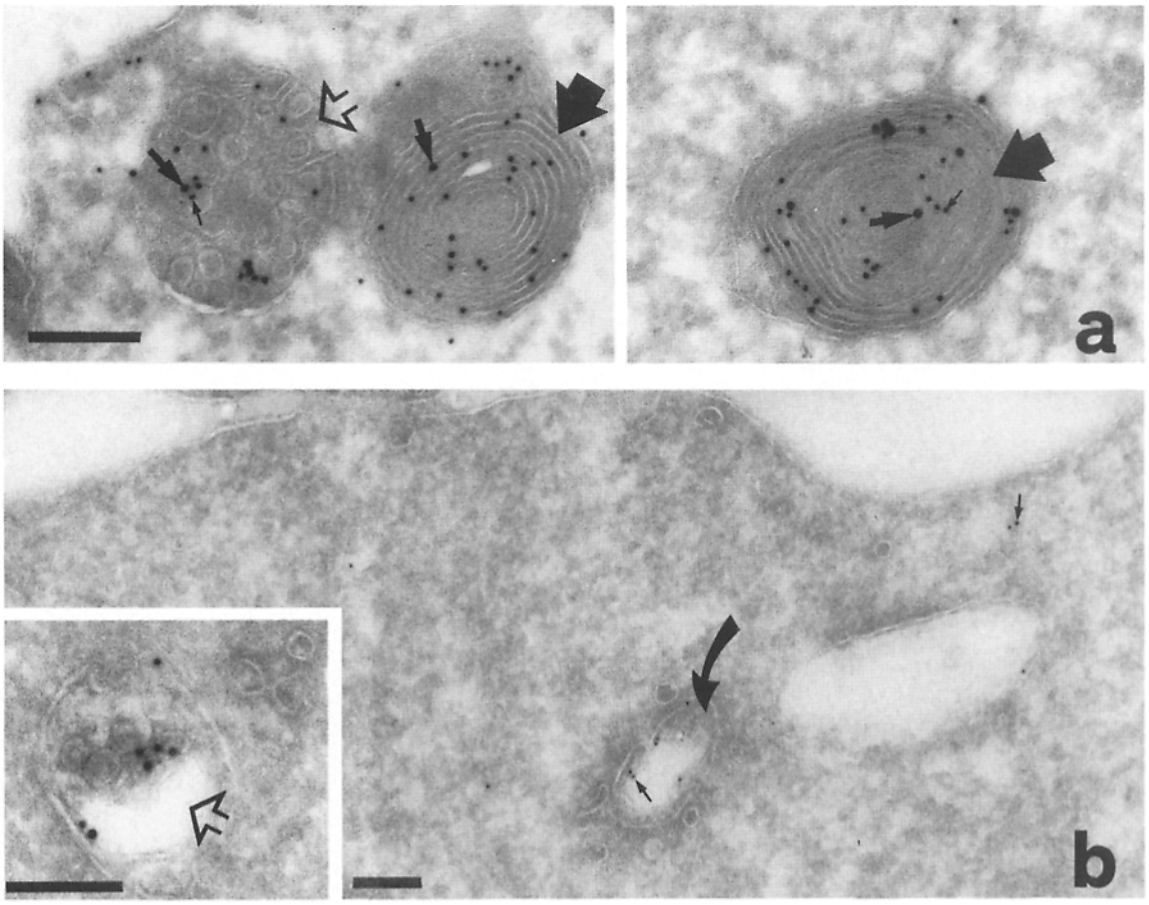


Figure 5. Exogenously added HRP enters structures with a morphology different in control and class II transfectants. (*a*) In class II transfectants, internalized HRP enters multilaminar and multivesicular structures that contain class II molecules. 293 cells expressing class II molecules and Δ15li were cultured in the presence of HRP for 15 min before fixation, and sections were stained with anti-class II (*small arrows*, 10-nm gold) and anti-HRP (*large arrows*, 15-nm gold) antibodies. Electron-dense multilaminar MIIC-like structures (*closed thick arrows*) and a dense multivesicular body (*open thick arrow*) are labeled for both antigens. (*b*) Internalized HRP enters membrane-delimited structures in control transfectants. 293 cells transfected with the H2-K^b H chain were cultured for 15 min in the presence of HRP and stained as in *a*. Electron-lucent structures (*bent arrow*) are labeled with anti-HRP antibodies. (*Inset*) higher magnification of a HRP-containing endosomal structure (*open thick* arrow) labelled with anti-HRP antibodies. No labeling with anti-class II antibodies is observed. Bars, 200 nm.

lamp-1 (and CD63) and class II molecules, suggesting that the transmembrane and cytoplasmic region of class II molecules is essential for the induction of the characteristic multilaminar morphology of the MIIC-like structure.

Discussion

To associate with fragments of endocytosed antigen, MHC class II molecules enter endocytic compartments during biosynthesis. In B cells and other antigen presenting cells, intracellular class II molecules are preferentially found in endocytic compartments with a multilaminar morphology. Peters et al. (1991) observed that this particular compartment did not have the characteristics of an early/late endosome nor of a lysosome and named this endocytic compartment: MIIC. Most probably, class II molecules associate with antigenic peptides in this compartment.

In the present study, we show that MIIC in B cells are not stable compartments, but they require continuous protein synthesis for existence. The proteins involved in the maintenance of MIIC were thereby not identified but transfection of class II molecules and li in 293 cells turned out to be sufficient to induce compartments with a morphology analogous to that of MIIC in B cells. Whether the multilaminar membranes are arranged like concentric rings or whether they have a spiral-like configuration cannot be established with certainty at the present level of resolution.

li is essential for rapid release of class II molecules from the ER, but it is not involved in the induction of the MIIC-like structures because MIIC-like structures are also observed in 293 cells transfected with only class II molecules (Fig. 8 *A*; Nijenhuis et al., 1994). Indeed, expression of li is not essential per se for appearance of class II molecules in endocytic compartments (Simonsen et al., 1993; Humbert et al., 1993), in fact these class II molecules may enter the endocytic route after cell surface internalization (Nijenhuis et al., 1994). However, cell type-specific differences in expression of class II molecules in the endosomal route in the absence of li have been observed (Simonsen et al., 1993),

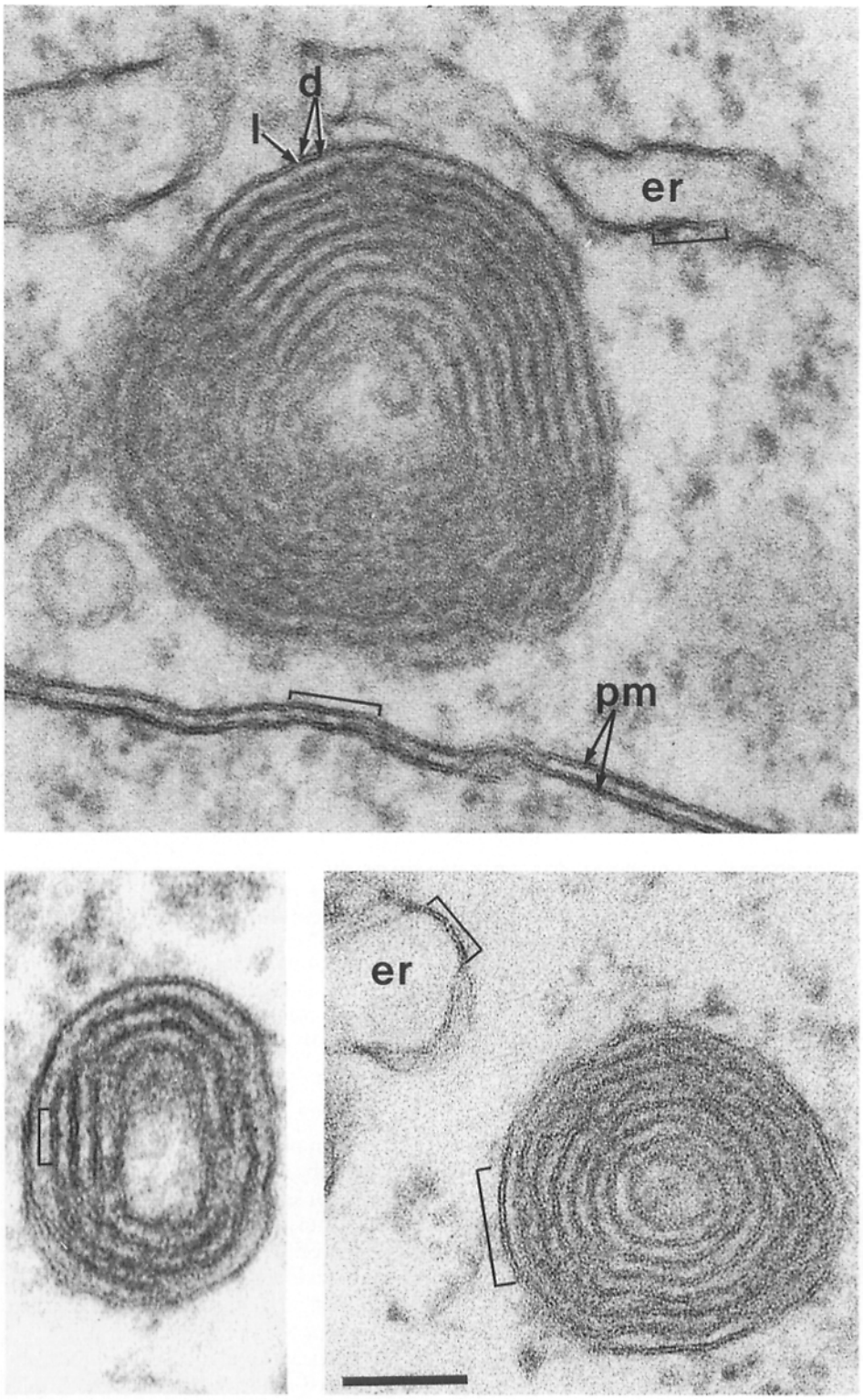


Figure 6. High-resolution analysis of the structures induced by expression of class II molecules. 293 cells transfected with class II molecules and Δ15li were embedded in a mixture of LX112/ Araldite and sections were analyzed. Different induced MIIC-like structures are shown at high magnification. The structure of the leaflet (*marked areas*) is visible for the induced MIIC-like structure, the ER and the plasma membrane (*pm*). Each leaflet has the characteristics of an intact membrane: two electron-dense strata (*d*) separated by a light zone (*l*). Bar, 100 nm.

which may be caused by different stability of these molecules in endosomal structures from different cells. The class II–containing compartments label for CD63 and lamp-1, which are markers for MIIC in B cells (Peters et al., 1991). Thus, expression of class II molecules induces compartments similar to MIIC in B cells, explaining why MIIC-like structures are generally observed in cells expressing class II molecules. The MIIC-like structures are globular and have, in general, a multilaminar morphology with some internal vesicles. Also, but less prominently, class II molecules are observed in multivesicular structures, which are also observed in other cells (Table I). It is unclear how the multilaminar morphology of the structures induced by expression of class II molecules is generated but it may be the result

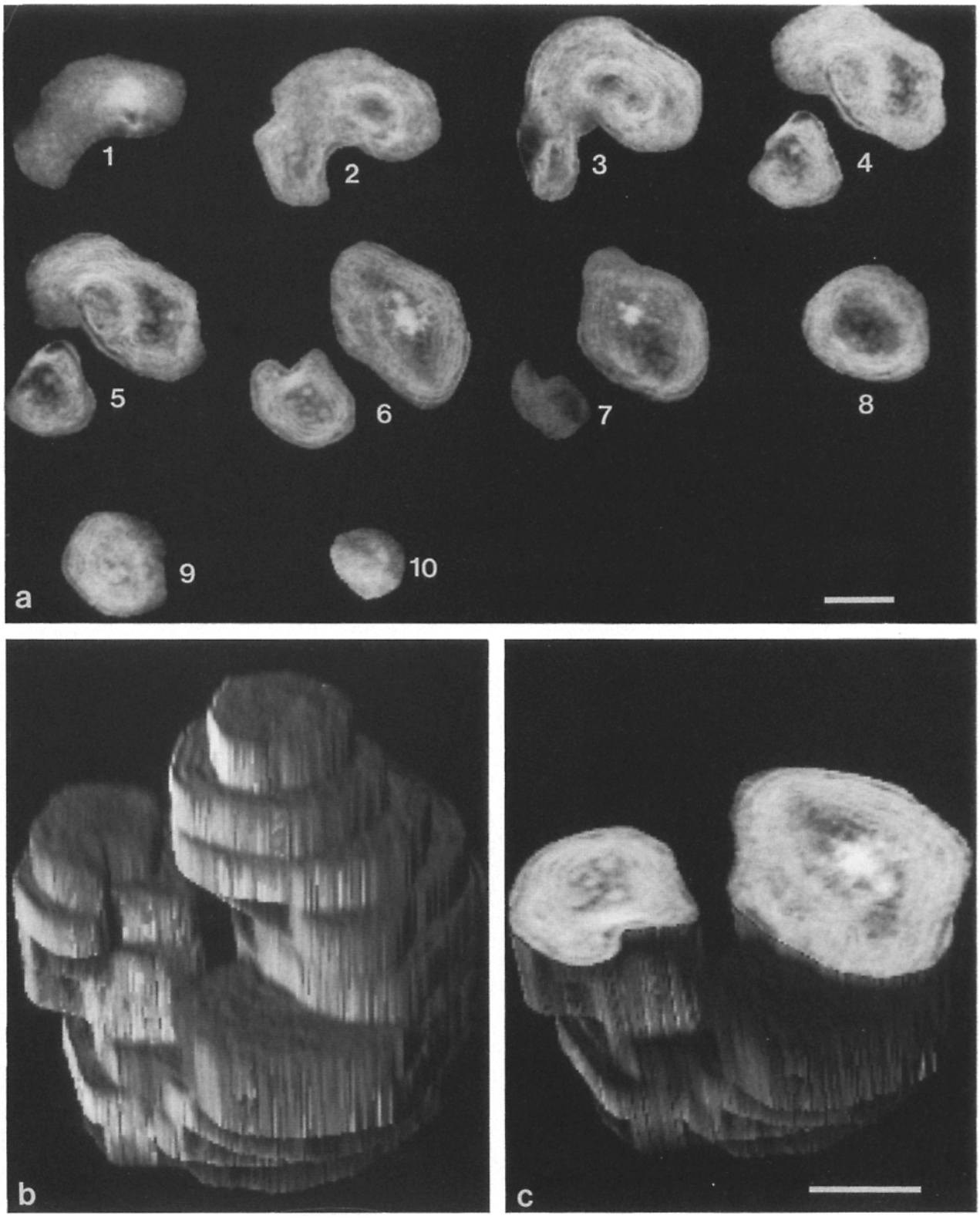
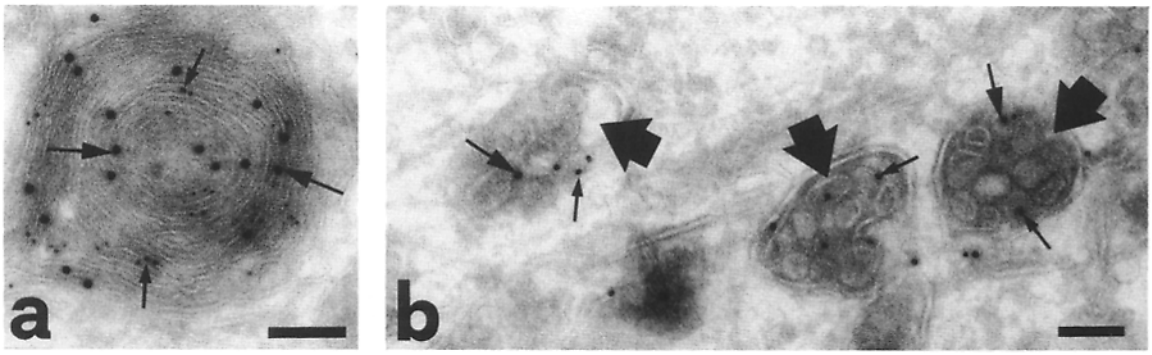


Figure 7. The three-dimensional composition of the MIIC-like structure in class II transfectants. (*a*) Serial sections of a MIIC-like structure. 293 cells transfected with class II molecules and Δ15li were embedded in a mixture of LX112/Araldite, and the same MIIC-like structure was identified in serial sections and ordered from top to bottom. These structures were not present in 293 cells transfected with the H2-K^b H chain. The different sections indicate that one MIIC-like structure has a multilaminar exterior with internal vesicles. (*b*) Surface structure of an induced MIIC. The surface structure of the respective sections was determined. Since the magnification and the thickness of the sections (~70 nm) is known, the three-dimensional structure could be calculated. MIIC has a globular structure with, in this case, two extensions. The diameter of the MIIC structure is ~800 nm. (*c*) View on the bottom half of MIIC. Like *b*, but the upper four sections have been removed to visualize the fifth section. The exterior of MIIC contains multilaminar membranes with multiple small vesicles in the interior. Bars, 200 nm.



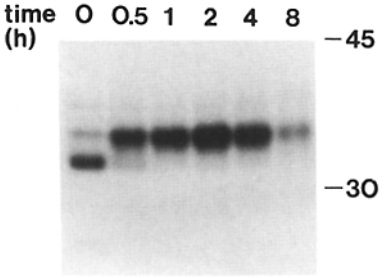


Figure 8. The involvement of the cytoplasmic/transmembrane region of class II molecules in the formation of multilaminar MIIC-like structures. (*a*) li is not involved in the formation of MIIC-like structures. Sections of 293 cells transfected only with MHC class II molecules were incubated with anti-class II α chain serum (*large arrows*, 10 nm gold) and anti-CD63 mAb (*small arrows*, 5-nm gold). Class II molecules are detected in multilaminar CD63-containing structures in the absence of li. (*b*) The transmembrane and cytoplasmic region of class II molecules and formation of MIIC. 293 cells were transfected with class II molecules that contained the transmembrane and cytoplasmic region of MHC class I HLA-B27 molecules and Δ15li. Sections were incubated with anti-class II α chain serum (*small arrows*, 10-nm gold) and anti-lamp-1 mAb (*large arrow*, 15-nm gold). The chimeric class II molecules were located in multivesicular bodies (*thick arrows*) that weakly labeled for lamp-1. The characteristic multilaminar MIIC-like structures were not observed in these transfectants (see also Table I). Bars, 100 nm. (*c*) Biochemical analysis of 293 cells transfected with chimeric class II molecules and Δ15li. The transfectant analyzed under *b* was biosynthetically labeled for 15 min, cultured for the times indicated above the figure, and class II molecules were isolated and analyzed by 12% SDS-PAGE. The positions of the 30- and 45-kD protein markers are shown. The chimeric class II molecules and Δ15li were rapidly transported after already 30 min, as indicated by the shift in molecular weight caused by carbohydrate modifications.

of fusion of the vesicles that are usually found in the centre of MIIC. There may even exist an equilibrium between the multivesicular and the multilaminar state, which may be affected by factors like membrane density and protein content, including the local density of class II molecules. Interestingly, the transmembrane and/or cytoplasmic region of class II molecules appears to be involved in the generation of the multilaminar morphology. If this region is exchanged for that of class I HLA-B27, class II molecules accumulate in multivesicular structures that label for CD63. It is still unclear how this region of class II molecules induces the multilaminar morphology of the MIIC-like structures.

How does expression of class II molecules result in the generation of MIIC? Induction of compartmentalization by a single protein has been observed in a few other cases. Expression of von Willebrand factor (vWf) in a number of different cell types results in the generation of characteristic Weibel-Palade-like bodies (Wagner et al., 1991; Voorberg et al., 1993). It is believed that these structures are the result of aggregation of vWf, as induced by the mildly acidic pH in the *trans*-Golgi reticulum (Wagner et al., 1991). The resulting Weibel-Palade bodies are generated after budding from the *trans*-Golgi reticulum. Other compartments can be induced by overexpression of ER-retained proteins. The resulting autophagosomes are generated by invagination of a sheet of RER resulting in a vesicle surrounded by two membranes (Dunn, 1990*a*). The intermembrane space contains the lumen of the RER (Dunn, 1990*a*). Multilaminar autophagosomes have also been observed (Dunn, 1990*a*). However, when the autophagosome fuses with endocytic compartments, the multiple membranes are gradually lost (Dunn, 1990*b*), and lysosomal transmembrane proteins are introduced into the outer membrane of autophagosomes during maturation into lysosomes (Dunn, 1990*b*). Although MIIC has some morphological characteristics of an autophagosome, it certainly does not fit the description because the transmembrane lysosomal markers are distributed in both inner and outer membranes, and because class II molecules are not retained in the ER, but they are quantitatively transported from the ER in our transfectants and in B cells (Neefjes et al., 1990). Furthermore, single subunits of class II molecules in 293 cells are retained in the ER (Nijenhuis and Neefjes, 1994), which results in the formation of autophagosome-like structures that are morphologically different from the induced MIIC-like structures (not shown). Induction of endosomal multivesicular structures has been observed in melanoma cells cultured in the presence of the protease inhibitor leupeptin (Zachgo et al., 1992). Leupeptin not only inhibits a number of endosomal proteases, but it also inhibits the degradation of li (Blum and Cresswell, 1988; Nguyen et al., 1989), resulting in the retention of class II molecules in the endocytic route (Neefjes and Ploegh, 1992*b*; Zachgo et al., 1992). The induction of endocytic compartments by leupeptin (Zachgo et al., 1992) might be explained by the increased number of class II molecules present in endosomes.

The mechanism by which class II molecules induce the formation of characteristic endosomal compartments is still not established. Introduction of proteins that should be degraded but are stably expressed in compartments (be it ER or endosomes) may result in the formation of autophagosome-like structures. Another possibility is compartment formation through aggregation of class II molecules in endocytic structures, as is observed for unoccupied (peptide-free)

class II molecules (Germain and Rinkler, 1993). This then would resemble the induction of Weibel-Palade bodies by vWf (Wagner et al., 1991; Voorberg et al., 1993). Class II molecules themselves do contain information for the induction of the formation of MIIC-like compartments because transmembrane and cytoplasmic region of the class II molecule appears to be critical for the formation of multilaminar structures. It is currently investigated how this region of the class II molecule is inducing multilaminar endocytic structures.

Taken together, we conclude that MIIC not only contains MHC class II molecules but that these structures are induced by the very presence of MHC class II molecules.

We thank D. Verwoerd for experimental support, Drs. I. van de Pavert and M. van Herk for the calculation of the three-dimensional structure of the MIIC, and N. Ong for the preparation of the micrographs. We thank Dr. W. Moolenaar and J. Borst for critically reading the manuscript and J. Overwater for typing the manuscript.

This research was supported by grant NWO 900-509-155 and NKB 93-525 from the Dutch Cancer Foundation.

Received for publication 11 January 1994 and in revised form 5 May 1994.

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