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Isolation and characterization of the intracellular MHC class II compartment

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An intracellular compartment has been isolated to which MHC class II molecules are transported on their way to the plasma membrane. They arrive with an associated invariant chain which is then proteolytically processed while MHC class II molecules acquire antigenic peptide. These loaded class II molecules then leave the compartment devoid of invariant chain and bound for the plasma membrane. This compartment represents a new stage in the endocytic/lysosomal pathway.

MAJOR histocompatibility complex (MHC) class II molecules present antigenic peptides to CD4-positive T cells[1]. The peptides bound to MHC class II are derived mainly from proteins that have entered the endocytic pathway, whereas MHC class I molecules present peptides derived from proteins synthesized in the cytoplasm[2,3].

At the plasma membrane, MHC class II molecules consist of two non-identical glycoproteins, the α- and β-chains[4]. Early in biosynthesis, class II molecules associate with the invariant chain (Ii)[5], which is thought to perform three functions: (1) it guides folding of class II molecules to facilitate their exit from the endoplasmic reticulum (ER)[6]; (2) it prevents premature binding of antigenic peptides to class II molecules[7,8]; and (3) it functions as a targeting signal for delivery of class II molecules to endocytic compartments[9,10]. Following deposition in the endocytic pathway, the class II-associated Ii is proteolytically processed[11,12].

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The endocytic compartment exists as a complex network of tubulo-vesicular structures[13,14], the precise structure and composition of which is still unknown. The endosomal/lysosomal system is thought to consist of several subcompartments, including early endosomes, late endosomes, and dense lysosomes[15–18]. In addition, multivesicular bodies are found in a number of cell types and may mediate traffic of endocytosed material between early and late endosomes[17].

Using immunocytochemical methods, MHC class II molecules are found in post-Golgi, endosomal and lysosomal-related compartments[12,19,20]. To characterize the intracellular site within the endosomal/lysosomal system where MHC class II molecules and Ii reside, we have analysed their location in the human melanoma cell line Mel JuSo by subcellular fractionation and density-gradient electrophoresis (DGE). Mel JuSo cells and related melanoma and melanocyte lines that are class II-positive[21] can induce a strong MHC class II-dependent T-cell response (A. Bakker, G. Adema and J.P., unpublished results). We report here that in Mel JuSo cells complexes of MHC class II molecules and Ii fragments are present in an intracellular

compartment distinct from early and late endosomes and different from dense lysosomes. Here MHC class II dimers acquire their stability to exposure to SDS at ambient temperature, a feature associated with the peptide-liganded state of class II molecules[22,33]. From this compartment, which may be identical to the morphologically characterized MHC class II compartment[20], MHC class II molecules exit to the plasma membrane.

Density gradient electrophoresis

Endosomes are identified by cytochemistry and biochemically after labelling with endocytic tracers[17]. The surface charge of lysosomal and endosomal compartments is more negative than that of most other subcellular organelles[18], so free-flow electrophoresis can be used to isolate lysosomal and endosomal compartments[24,25].

A density gradient was combined with electrophoresis to separate endosomal/lysosomal compartments from crude microsomes in a single step. Early and late endosomes of Mel JuSo cells were labelled with horseradish peroxidase (HRP)[12,17], membranes were prepared (Fig. 1 legend), subjected to DGE, and the distribution of markers for the various subcellular compartments determined. Two endosomal populations, consisting of early and late endosomes, were resolved (Fig. 1*a*).

Lysosomal compartments were identified by β-hexosaminidase activity and migrated towards the anode, well separated from both early and late endosomes. To analyse the migration of plasma membrane components, Mel JuSo cells were surface-labelled on ice with ^{125}I. The plasma membrane was well resolved from endosomal and lysosomal organelles by DGE (Fig. 1*b*). The position of the ER was established by pulse-labelling Mel JuSo cells for 2 min with ^{35}S-methionine/cysteine before homogenization and DGE. The ER migrated at a position distinct from that of endosomes and lysosomes but overlapped partially with the plasma membrane (Fig. 1*c*). Golgi membranes, identified by enriched galactosyltransferase activity, were slightly shifted anodically and overlapped with early endosomal membranes, in agreement with other subcellular electrophoretic separations[24]. The intracellular distribution of MHC class II molecules, see below, is shown in Fig. 1 (shaded).

Early and late endosomes can thus be separated from a crude microsomal preparation in a single step by density gradient electrophoresis. Lysosomes overlap but do not comigrate with the late endosomal fraction.

Detection of MHC class II compartments

To analyse the subcellular location of MHC class II molecules, Mel JuSo cells incubated with HRP were homogenized and the microsomal fraction subjected to DGE. The distribution of MHC class II molecules at steady state was determined by immunoblotting (Fig. 2*a*). MHC class II molecules were present in two distinct populations; the first comigrated with the plasma membrane (Fig. 1*b*), and the second migrated between early and late endosomes. A small fraction of intracellular class II molecules overlapped with both early and late endosomes (Fig. 1*a*).

Can the intracellular compartments containing MHC class II molecules (as defined by their migration in fractions 11–16 after DGE) be reached from the outside by endocytosis? Mel JuSo cells were allowed to internalize HRP for 4 min and then chased for the times indicated (Fig. 2*b*). After a 10-min chase, HRP cofractionates with the intracellular MHC class II molecules (Fig. 2*b*). After further purification by sucrose-gradient sedimentation of the DGE-defined intracellular MHC class II compartments, the bulk of the internalized marker again cofractionates with the MHC class II-positive fractions (Fig. 5*a*).

Marker proteins for the endosomal/lysosomal pathway include the lysosomal-associated membrane protein LAMP[26] and the cation-independent mannose 6-phosphate receptor (CI-MPR)[15]. The location of both marker proteins was analysed after DGE. Most of the LAMP immunoreactivity was found at

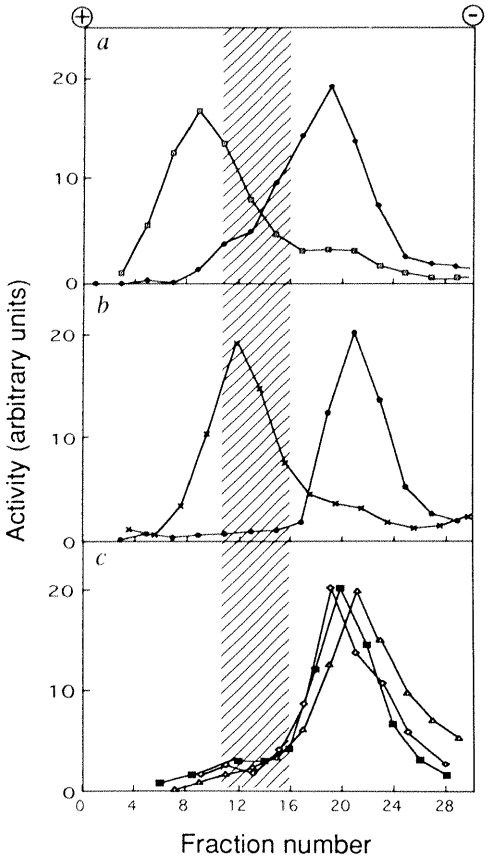


FIG. 1 Subcellular fractionation of Mel JuSo organelles by density-gradient electrophoresis (DGE). Mel JuSo cells were allowed to internalize horseradish peroxidase (HRP) at 37 °C for 4 min to label early endosomes, or for 4 min followed by a 30-min chase to label late endosomes in normal medium. Microsomes prepared from Mel JuSo cells were electrophoresed for 35 min at 10 mA and 0.3-ml fractions collected. *a*, Detection of early (filled diamonds) and late (open squares) endosomes by HRP activity as described[17]. *b*, Detection of lysosomes by β-hexosaminidase activity[39] (cross) and of plasma membrane (filled circles) by ^{125}I label. *c*, Golgi membranes assayed by galactosyl transferase activity[40] (diamonds), endoplasmic reticulum, as assayed by the presence of ^{35}S after pulsing the cells for 2 min with ^{35}S-methionine/cysteine (filled squares), and total protein determined according to ref. 41 (triangles). The shaded region represents fractions containing the intracellular MHC class II molecules (Fig. 2).

METHODS. Monolayers of Mel JuSo cells were scraped in homogenization buffer (10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, 0.25 M sucrose, pH 7.4) and collected by centrifugation at 136*g* for 7 min. The cell pellet was resuspended in 1 ml homogenization buffer and homogenized at 4 °C by passing 20 times through a 22G1 1/4 needle fitted on a 1-ml plastic syringe[42]. After centrifugation for 15 min at 840*g* at 4 °C, the postnuclear supernatant (PNS) was treated with trypsin for 5 min at 37 °C at a concentration of 25 μg per mg protein[16]. Digestion was stopped by cooling on ice and addition of 100 μg soybean trypsin inhibitor per mg. The PNS was subsequently centrifuged at 100,000*g* for 60 min at 4 °C and resuspended in 0.5 ml 5% Ficoll-70 in homogenization buffer. The electrophoretic device consisted of a Perspex cylinder separated from the bottom buffer reservoir by a cationic-permeable membrane at the bottom of the tube[43]. The sample was layered in the middle of a linear gradient from 10 to 0% Ficoll-70 in electrophoresis buffer. Plasma membrane labelling was achieved by radioiodination of cells on ice by the lactoperoxidase–glucose oxidase method[44].

the position of the intracellularly located MHC class II molecules (Fig. 2*c*). The small amount of LAMP immunoreactivity detected in fractions 22–24 may represent ER-resident and/or LAMP molecules present at the plasma membrane[26].

The bulk of the CI-MPR was present in fractions containing early endosomes/Golgi compartments (Fig. 2*d*), probably reflecting the extensive recycling of the CI-MPR through the *trans*-Golgi network[27]. But fractions containing MHC class II-positive intracellular compartments (fractions 11–16) also contain considerable amounts of the CI-MPR.

We conclude that the MHC class II molecules are present in an intracellular compartment that is distinct from early and late endosomes. Internalized proteins have access to these compartments and comigrate partially with the CI-MPR, the lysosomal marker β-hexosaminidase (see later), and the lysosomal-associated protein LAMP.

Transport of MHC class II/Ii complexes

To investigate the nature of the intracellular target compartment of newly synthesized class II molecules and Ii, we either pulsed Mel JuSo cells with ^{35}S-methionine/cysteine for 10 min or labelled them for 20 min and chased for the times indicated in Fig. 3. After DGE of the homogenates, MHC class II molecules were immunoprecipitated from the different fractions. For longer chase times, transferrin receptor and MHC class I molecules can be used as markers for early endosomes and plasma membrane, respectively. As expected, after a short pulse most of the class II molecules, as well as MHC class I and transferrin receptor molecules, migrated with the ER marker (Fig. 3*a*). A proteolytic fragment of the class II-associated Ii (P25) that was generated during or directly after biosynthesis[12], and P41, an alternatively spliced form of Ii (ref. 28), were both present in the class II immune complexes. With increasing chase times, the class II/Ii positive compartments migrated further towards the anode (Fig. 3). After 2 and 4 hours of chase, MHC class II molecules associated with the Ii fragments P22, P18 and P12 migrated between early and late endosomes, at the same position as the cohort of intracellularly located class II molecules determined by immunoblotting (Fig. 2). After 2 and 4 h chase, biosynthetically labelled transferrin receptors and MHC class I molecules migrated at the positions of early endosomal compartments and plasma membrane, respectively.

MHC class II molecules have a long half-life (15–50 h)[29]. In Mel JuSo cells, class II molecules remain associated with N-terminal Ii fragments within the endocytic pathway[12]. Later, these Ii fragments are further degraded and dissociated from class II molecules. The N-terminal fragments of Ii contain the sorting and retention signals for endosomal compartments[9,10,30], and removal of these Ii fragments by proteolysis can lead to the appearance at the plasma membrane of MHC class II molecules[12,23]. After 48 h chase, biosynthetically labelled MHC class II complexes devoid of Ii fragments migrated predominantly at the position of the plasma membrane (Fig. 3*e*). The small intracellular fraction may represent MHC class II molecules internalized from the plasma membrane[31]. MHC class I molecules were no longer detected after 48 h chase; the position of the transferrin receptor was shifted slightly towards the anode compared to the 2- or 4-h chase points, and may be due to the transition of small amounts of transferrin receptor from early endosomes to later stages of the endocytic pathway[32]. Thus, newly synthesized class II/Ii complexes are transported to an intracellular compartment that is distinct from early and late

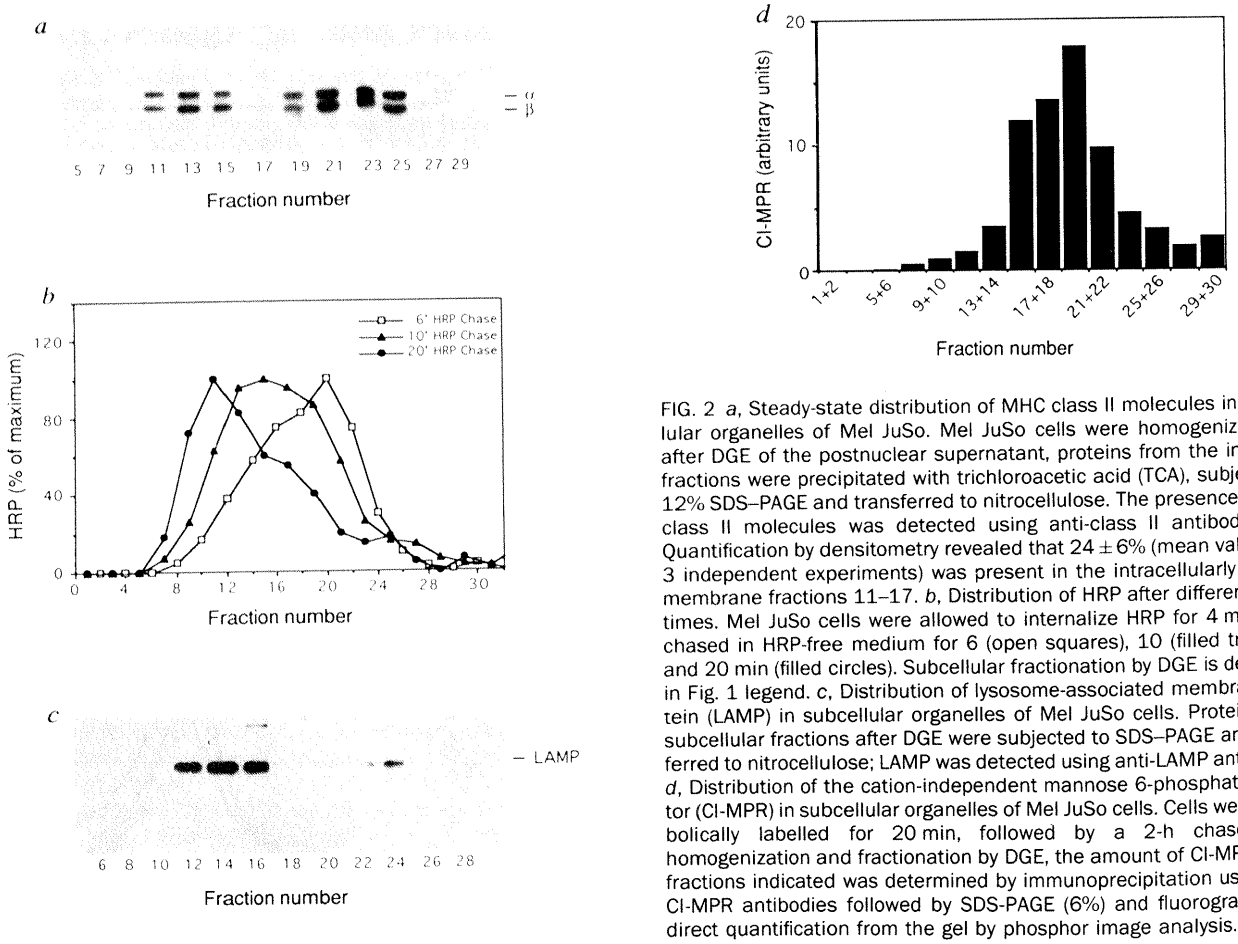


FIG. 2 *a*, Steady-state distribution of MHC class II molecules in subcellular organelles of Mel JuSo. Mel JuSo cells were homogenized, and after DGE of the postnuclear supernatant, proteins from the indicated fractions were precipitated with trichloroacetic acid (TCA), subjected to 12% SDS–PAGE and transferred to nitrocellulose. The presence of MHC class II molecules was detected using anti-class II antibodies[45,46]. Quantification by densitometry revealed that 24 ± 6% (mean value from 3 independent experiments) was present in the intracellularly located membrane fractions 11–17. *b*, Distribution of HRP after different chase times. Mel JuSo cells were allowed to internalize HRP for 4 min, then chased in HRP-free medium for 6 (open squares), 10 (filled triangles) and 20 min (filled circles). Subcellular fractionation by DGE is described in Fig. 1 legend. *c*, Distribution of lysosome-associated membrane protein (LAMP) in subcellular organelles of Mel JuSo cells. Proteins from subcellular fractions after DGE were subjected to SDS–PAGE and transferred to nitrocellulose; LAMP was detected using anti-LAMP antibodies. *d*, Distribution of the cation-independent mannose 6-phosphate receptor (CI-MPR) in subcellular organelles of Mel JuSo cells. Cells were metabolically labelled for 20 min, followed by a 2-h chase. After homogenization and fractionation by DGE, the amount of CI-MPR in the fractions indicated was determined by immunoprecipitation using anti-CI-MPR antibodies followed by SDS-PAGE (6%) and fluorography and direct quantification from the gel by phosphor image analysis.

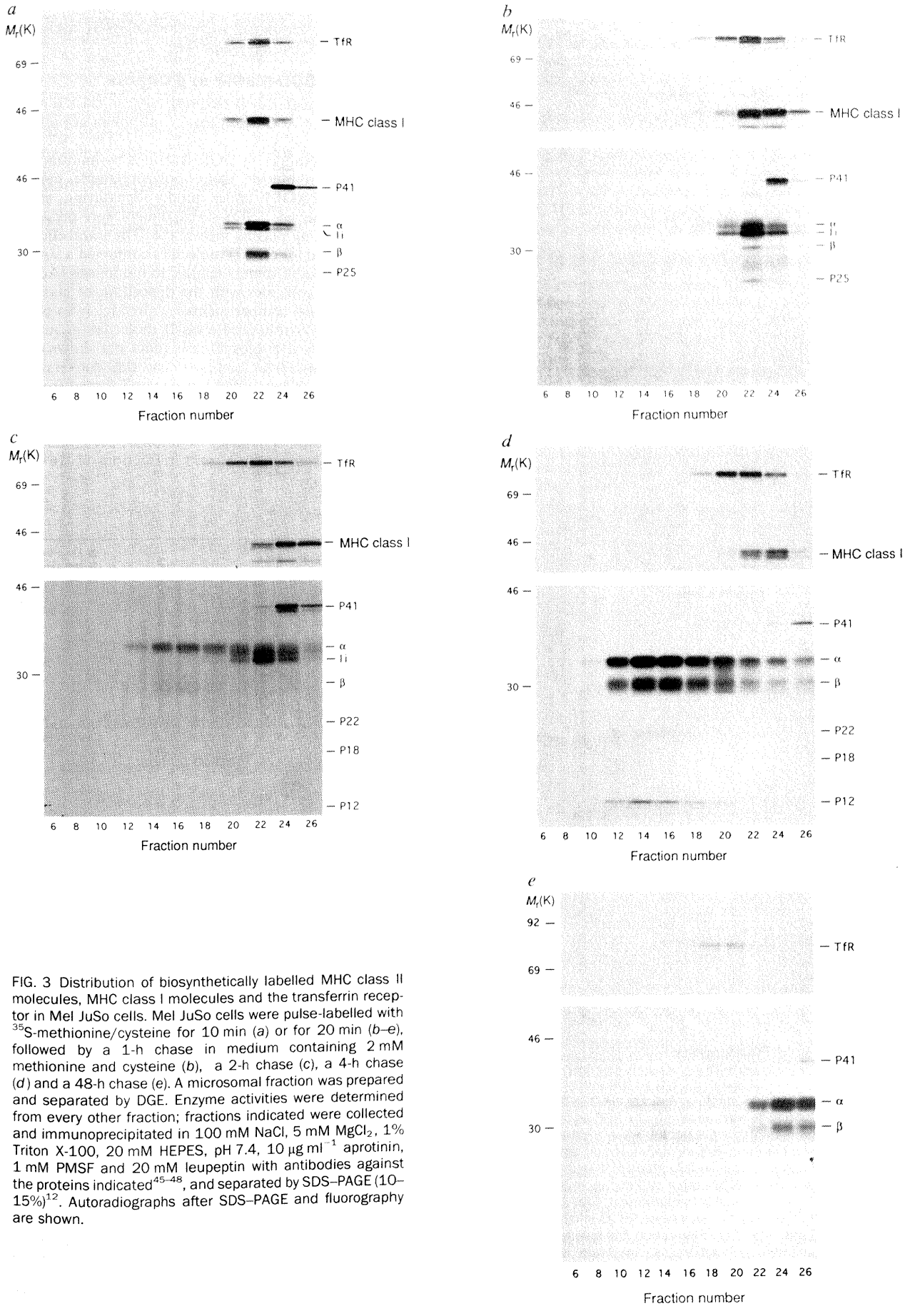


FIG. 3 Distribution of biosynthetically labelled MHC class II molecules, MHC class I molecules and the transferrin receptor in Mel JuSo cells. Mel JuSo cells were pulse-labelled with ^{35}S-methionine/cysteine for 10 min (*a*) or for 20 min (*b*–*e*), followed by a 1-h chase in medium containing 2 mM methionine and cysteine (*b*), a 2-h chase (*c*), a 4-h chase (*d*) and a 48-h chase (*e*). A microsomal fraction was prepared and separated by DGE. Enzyme activities were determined from every other fraction; fractions indicated were collected and immunoprecipitated in 100 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 20 mM HEPES, pH 7.4, 10 μg ml^{-1} aprotinin, 1 mM PMSF and 20 mM leupeptin with antibodies against the proteins indicated[45–48], and separated by SDS–PAGE (10–15%)[12]. Autoradiographs after SDS–PAGE and fluorography are shown.

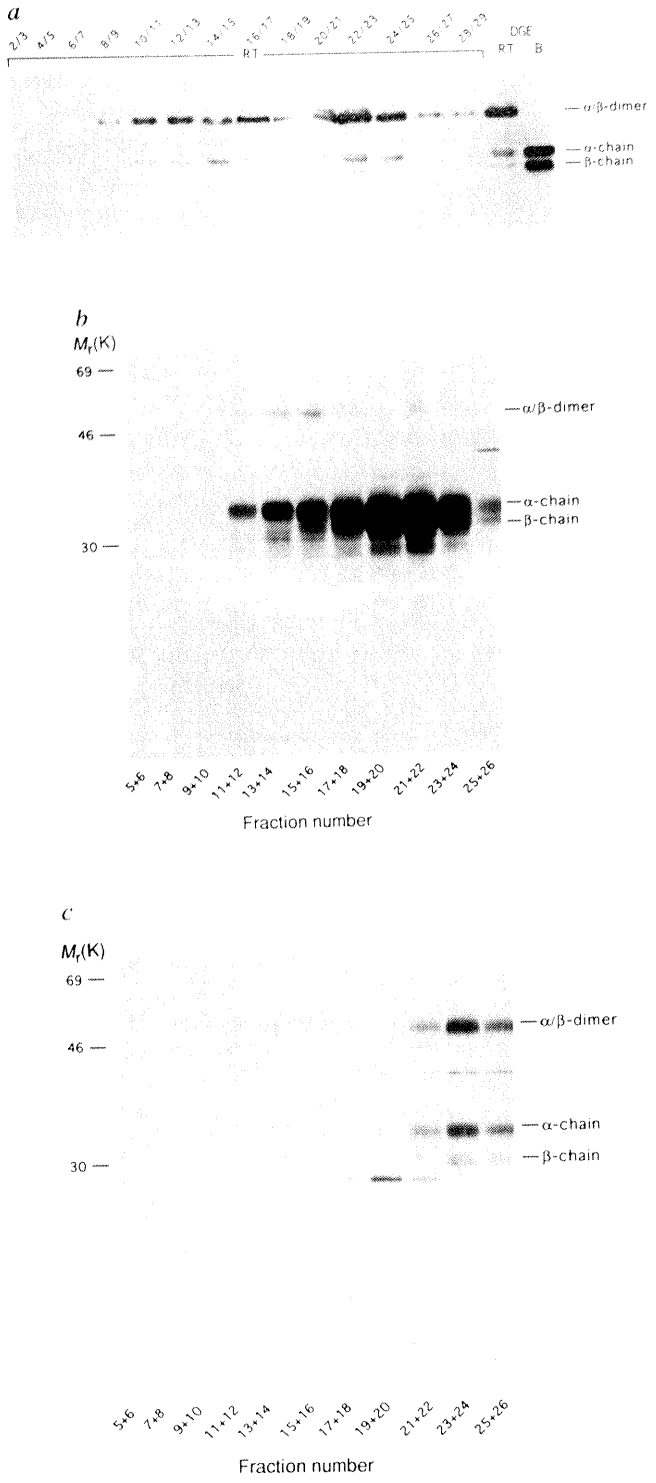


FIG. 4 *a*, Distribution of SDS stable α/β dimers in DGE fractions. Microsomes from Mel JuSo cells were separated by DGE as for Fig. 2. Before SDS–PAGE and immunoblotting, samples were allowed to stand for 30 min at room temperature (RT), or denatured at 95 °C (B, boiled). DGE, membrane fraction before density gradient electrophoresis. *b* and *c*, Presence of newly formed SDS-stable α/β dimers in the DGE-defined MHC class II-positive intracellular compartment. Mel JuSo cells were pulse-labelled with ^{35}S-methionine/cysteine for 20 min followed by a 2-h (*b*) or 48-h (*c*) chase. After homogenization and subcellular fractionation by DGE, proteins from the fractions indicated were immunoprecipitated with anti-class II antibodies and separated by SDS–PAGE after denaturation at ambient temperature. Shown are autoradiographs after SDS–PAGE and fluorography.

endosomes. In this compartment Ii is proteolytically processed from its carboxy terminus, after which class II molecules are transported to the plasma membrane.

Detection of SDS-stable α/β dimers

Peptide loading confers increased resistance of class II molecules to denaturation by SDS detergent[22,33], although not all peptide-occupied class II molecules are resistant to SDS[34]. We analysed MHC class II molecules in DGE fractions by immunoblotting, following their resolution using polyacrylamide gel electrophoresis (SDS–PAGE) under mildly denaturing conditions (Fig. 4*a*). As expected[33], SDS-stable dimers were present in the fractions containing plasma membrane. The intracellular compartment enriched for class II molecules contained a similar proportion of SDS-stable dimers relative to monomers. Acquisition of SDS stability coincides with the deposition of class II molecules in post-Golgi compartments[22,23]. In Mel JuSo cells, as in other cells, newly synthesized class II molecules acquire resistance to SDS ~2 h after biosynthesis (data not shown). To find out in which subcellular compartment this conversion takes place, cells that had been pulse-labelled and chased for 2 h were fractionated by DGE and the presence of SDS-stable MHC class II dimers was tested by immunoprecipitation followed by SDS–PAGE under mildly denaturing conditions. SDS-stable class II dimers were predominantly present in fractions 11–16 (Fig. 4*b*).

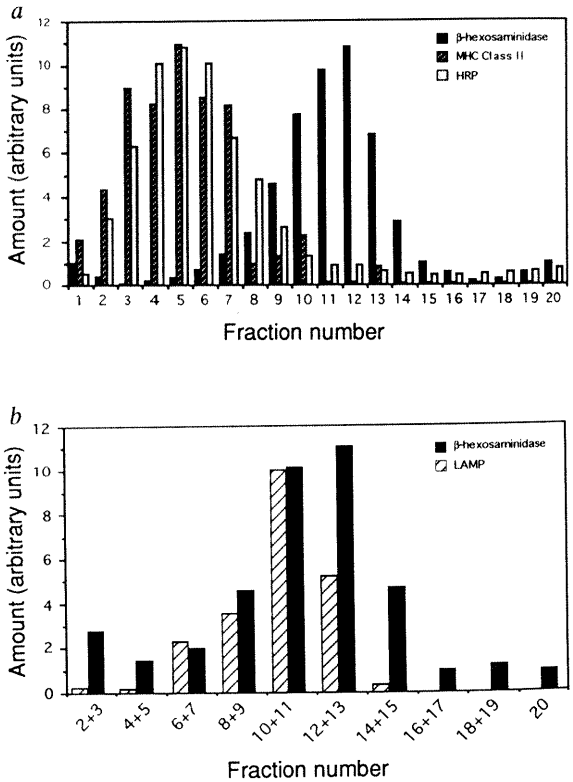


FIG. 5 Separation of MHC class II-positive compartments from lysosomes. Mel JuSo cells were allowed to internalize HRP for 4 min, followed by a 10-min chase. After homogenization and DGE, fractions containing MHC class II-positive intracellular compartments resolved by DGE (fractions 11–16; Fig. 2) were pooled and layered onto a linear sucrose gradient (0.6–1.6 M sucrose in DGE buffer). After centrifugation (5 h at 27,000 r.p.m. in a Beckman SW-41 rotor), 0.5-ml fractions were collected from the top; β-hexosaminidase (*a*, *b*) and HRP (*a*) activity was determined in each fraction. Proteins from each fraction were precipitated in 10% TCA/acetone, and separated by SDS–PAGE. Following transfer to nitrocellulose, class II molecules (*a*) and LAMP (*b*) were quantified in each fraction by immunolabelling and densitometry.

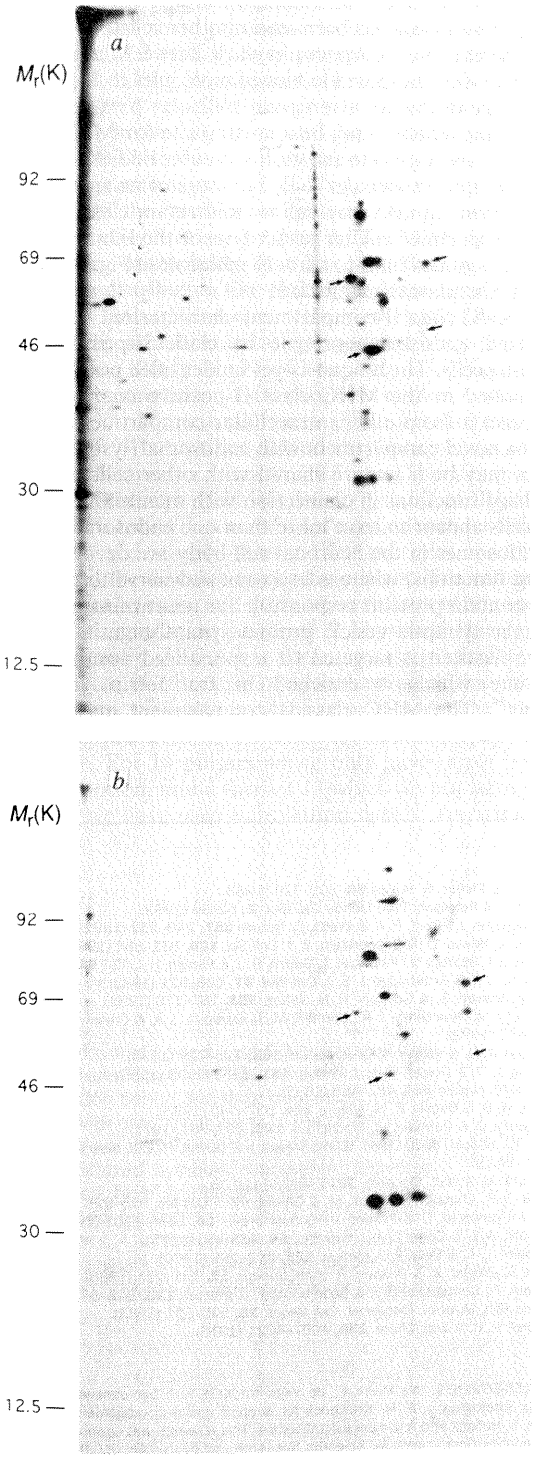
A small percentage of SDS stable dimers were present in fractions 19–22, probably representing the few class II dimers that either recycle through the Golgi or have reached the plasma membrane. After 48 h of chase, SDS-stable $\alpha\beta$ dimers appeared in plasma membrane fractions, as expected (Fig. 4*c*). We conclude that conversion of class II molecules to SDS-stable heterodimers is largely completed in this DGE-defined compartment; some unstable class II molecules may be degraded intracellularly rather than being transported to the plasma membrane[35].

Separation from lysosomes

The intracellular compartment containing MHC class II molecules partially overlaps with lysosomes in DGE (Figs 1 and 2). To resolve further the class II-containing compartments, MHC class II-positive membrane fractions from Mel JuSo cells (fractions 11–16; Fig. 2*a*), were subjected to sucrose density gradient centrifugation (0.6–1.6 M sucrose). The distributions of β-hexosaminidase (representing lysosomes) and class II molecules were determined (Fig. 5*a*). MHC class II-containing fractions (light fractions, 1–7) were well resolved from the denser fractions containing β-hexosaminidase activity (fractions 9–14). The lysosome-associated membrane protein LAMP is present mainly in fractions containing dense lysosomes but also in the lighter MHC class II-positive fractions (Fig. 5*b*). We conclude that the MHC class II-containing intracellular compartment is different from lysosomes, although it is still positive for LAMP.

Proteins in the MHC class II compartment

The physical properties of the MHC class II-containing intracellular compartment suggest that it represents a compartment distinct from endosomes and lysosomes. Proteins in the different subcellular fractions were analysed by two-dimensional isoelectric focusing (IEF)/SDS–PAGE of proteins from total membranes (Fig. 6*a*) and from pooled fractions containing intracellular MHC class II molecules after DGE (Fig. 6*b*). After further purification on a sucrose gradient (Fig. 5), the protein profile shown in Fig. 6*c* is obtained. Four spots of roughly



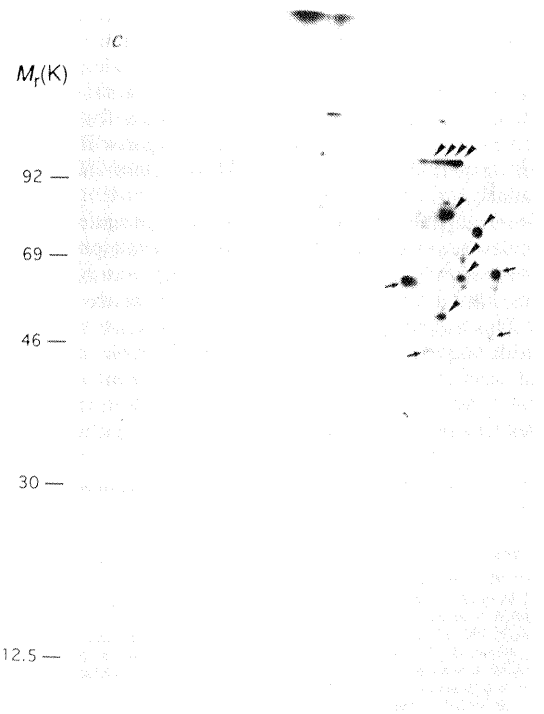


FIG. 6 Analysis of total proteins from the subcellular fractions obtained from Mel JuSo cells after DGE and sucrose-gradient fractionation. Mel JuSo cells were labelled for 15 h with ^{35}S-methionine/cysteine, after which a microsomal fraction was prepared that was fractionated by DGE (*a*, *b*) and sucrose gradient centrifugation (*c*) as described in Fig. 5 legend. Fractions were pooled and TCA-precipitated proteins analysed by two-dimensional (2-D) IEF/SDS–PAGE (*a*, total membranes before DGE; *b*, fractions 11–16 after DGE; *c*, fractions 1–7 after sucrose-density centrifugation). Basic, left; acidic, right. Four spots can be detected in each panel (arrows). In *c*, spots that cannot be detected in the 2-D patterns of the other subcellular fractions are indicated by an arrowhead.

METHODS. Two-dimensional gel electrophoresis was performed as described[49]. Isoelectric focusing was done in 200 µl containing 3.75% acrylamide, 7.2 M urea, 2% NP-40 and 2% ampholytes (LKB; consisting of a mixture of 83% (pH 3.5–10), 8.3% (pH 4–6) and 8.3% (pH 5–8)).

equivalent intensities are present in the $M_r = 40$–60K region (Fig. 6*a*–*c*, arrows). The pattern is strikingly different in the lighter fractions (Fig. 6*c*), which contain polypeptides not evident in Fig. 6*a*, or *b*. Thus, MHC class II molecules are transported to a compartment which houses proteins of a characteristic composition profile distinct from that of other subcellular fractions.

Discussion

We have identified an intracellular compartment in the human melanoma cell line Mel JuSo that contains MHC class II molecules and which may be similar to the morphologically defined MIIC[20].

Two populations of MHC class II-containing membrane fractions are resolved by DGE, one in the plasma-membrane-containing fractions and the other in fractions comprising an intracellular compartment comigrating with the lysosomal-associated membrane protein LAMP and containing protease activity, as deduced from the degradation of the class II-associated Ii. This intracellular pool of class II molecules only partially overlaps with early and late endosomes, is accessible to a fluid-phase marker and is separable from dense lysosomes. Two-dimensional IEF/SDS PAGE analysis indicates that the MHC class II-containing compartment has a protein composition different from that of the other subcellular organelle fractions.

Newly synthesized class II molecules are found in this compartment 2–4 h after biosynthesis and are associated with N-terminal cytoplasmic fragments of the invariant chain; after longer chase times the majority of class II molecules, are present at the plasma membrane without Ii. The N-terminal cytoplasmic tail of Ii is known to contain sorting signals for endosomal compartments[9,10,30]. Our findings are consistent with a function of Ii in targeting/retaining the MHC class II molecules intracellularly.

In this intracellular MHC class II-positive compartment, class II molecules acquire peptide, with only a small proportion of newly synthesized class II molecules being occupied at first, increasing until at steady state the bulk of them are loaded with peptide. This suggests that class II molecules are retained and that peptide acquisition is relatively slow. Unstable, non-peptide-occupied class II molecules might be degraded intracellularly[35].

In Mel JuSo cells there are only small amounts of class II molecules present in lysosomes[12]; class II molecules and Ii are found in endosomes and in multivesicular bodies[12]. These multivesicular bodies were identified by their characteristic morphology, because only a subset of them contained endosomal markers, including the CI-MPR. Our results are in good agreement with these earlier observations; although some class II/Ii complexes comigrated with markers for early and late endosomes, the major portion was present in the DGE-defined intermediate membrane fractions, which may be identical to multivesicular bodies.

This class II-positive intracellular compartment also resembles MIIC, another multivesicular-like structure[20]. These MIIC are not lysosomes, they contain LAMP and can be reached by endocytic tracers. In the cell line JY, endocytic tracers reached the MIIC within 60 min, whereas in Mel JuSo cells the multivesicular bodies were loaded with tracers within 15 min, which agrees with our results and has been seen in other cells[12,36]. The kinetics of internalization, which could vary between cell types, may differ from the transport kinetics of molecules to MIIC from the biosynthetic pathway.

This compartment may be a sorting site for MHC class II/Ii complexes, analogous to the multivesicular bodies for other cell-surface receptor molecules[14]. Ii, by virtue of its endosomal sorting/retention signals, may deliver and retain class II molecules in this compartment. After proteolysis of the Ii luminal domain, antigenic peptides bind to class II molecules (Fig. 4) for presentation at the plasma membrane.

The MHC class II compartment characterized here may be a specialized endosome, present in class II-positive antigen-presenting cells. The unique as-yet unidentified polypeptides that are enriched in the MHC class II-positive compartment also distinguish it from other intracellular compartments. The presence of a novel compartment with endosomal/lysosomal characteristics may be a feature shared with other cells that perform specialized functions in connection with membrane traffic. Neuronal cells appear to have more than one endosomal pathway[37]. The endosomes in the neuronal cell body are devoted to housekeeping functions, whereas in axons and nerve terminals endosomes contain proteins responsible for neurotransmitter release. When the synaptic vesicle protein synaptotagmin is expressed in fibroblasts, it is targeted to a specialized non-housekeeping endosome which is enriched in transferrin receptors for example[38]. The MHC class II compartment may represent a similar specialization for class II-positive antigen-presenting cells. □

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