Direct Vesicular Transport of MHC Class II Molecules from Lysosomal Structures to the Cell Surface

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Abstract. Newly synthesized MHC class II molecules are sorted to lysosomal structures where peptide loading can occur. Beyond this point in biosynthesis, no MHC class II molecules have been detected at locations other than the cell surface. We studied this step in intracellular transport by visualizing MHC class II molecules in living cells. For this purpose we stably expressed a modified HLA-DR1 β chain with the Green Fluorescent Protein (GFP) coupled to its cytoplasmic tail (β-GFP) in class II–expressing Mel JuSo cells. This modification of the class II β chain does not affect assembly, intracellular distribution, and peptide loading of the MHC class II complex. Transport of the class II/β-GFP chimera was studied in living cells at 37°C. We visualize rapid movement of acidic class II/β-GFP containing vesicles from lysosomal compartments to the plasma membrane and show that fusion of these vesicles with the plasma membrane occurs. Furthermore, we show that this transport route does not intersect the earlier endosomal pathway.

MHC class II molecules present peptides to $CD4^+$ T cells. Most bound peptides are derived from antigens degraded in the endosomal pathway. To allow association with these peptides, class II molecules are targeted to endosomal compartments by the invariant chain (or Ii)[1] (7, 8). Here, Ii is degraded and Ii-degradation products are exchanged with antigenic peptides, a process catalyzed by HLA-DM (11, 41, 47, 48). The endosomal compartments where class II molecules are loaded with peptide may be considered "special" lysosomes with a multilamellar and/or multivesicular appearance and were originally termed MIIC for MHC class II–containing compartments (31). This "unique" morphology appears to be induced by the expression of class II molecules (4). Although earlier endosomal compartments have been noted as "specialized class II loading compartments" as well (1, 50), HLA-DM and class II molecules are generally located in compartments with lysosomal proteins like CD63, lamp-1, and cathepsin D (13, 24, 32, 42).

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1. *Abbreviations used in this paper*: DGE, density gradient electrophoresis; GFP, Green Fluorescent Protein; Ii, invariant chain; MIIC, MHC class II-containing compartment; NRS, normal rabbit serum; PNS, postnuclear supernatant; TfR, transferrin receptor.

How class II molecules are subsequently transported from MIIC to the plasma membrane is unclear, but they may use the existing endosomal pathway in a retrograde direction. In this case, class II molecules would be able to associate with antigenic fragments in early endocytic structures before cell surface appearance. Alternatively, MHC class II molecules could be transported to the plasma membrane without employing the early endosomal recycling machinery. Class II molecules would then be transported to the plasma membrane as if they were contained in secretory granules. Although secretion of such granules is tightly regulated, no such mechanisms have been described for class II molecules. Transport intermediates between MIIC and the cell surface have never been visualized. These may be below detection levels of the techniques used either due to continuous transport of small quanta of class II molecules from MIIC, or to the very transient existence of intermediate structures. Recently, Raposo et al. (37) reported the association of MIIC-derived vesicles with the plasma membrane. This is the first indication of direct secretion of MIIC-compartments.

To directly visualize these transport processes, we have constructed a chimeric molecule containing the Green Fluorescent Protein (GFP) attached to the cytoplasmic tail of the class II β chain of HLA-DR1 (β-GFP) and stably transfected the human melanoma cell line Mel JuSo. Mel JuSo cells express functional class II HLA-DR3 molecules and all components required for efficient peptide loading of class II molecules (33, 42). The distribution and trans-

port of the class II/β-GFP molecules were studied in living cells by confocal microscopy. We show that MHC class II/β-GFP concentrate in acidic MIIC vesicles in the perinuclear region. These vesicles rapidly migrate towards the plasma membrane, without intersection of earlier endosomes, and ultimately fuse with the plasma membrane. We thus visualize a direct vesicular transport route from lysosomal MIIC structures to the plasma membrane.

Materials and Methods

Antibodies

mAb anti-human CD63 (53), or 435 (21), mAb anti-human lamp-1: BB6 (5), mAb anti-human DMA: 5C1 (43), mAb anti-Ii luminal portion: Bu45 (55), mAb anti-human transferrin receptor: 66Ig10 (52), rabbit anti-rab7 serum (a kind gift from Dr. P. van der Sluys, University of Utrecht, Utrecht, The Netherlands), rabbit anti-human cathepsin D serum (a kind gift from Dr. J.M. Tager and H. Aerts, University of Amsterdam, Amsterdam, The Netherlands), rabbit anti-class II DR-β chain serum (26), mAb anti-human HLA class I complex: W6/32 (29), mAb anti-human HLA class I heavy chain: HC-10 (49), mAb anti-human HLA class II complex: Tu36 (46), rabbit anti-rGFP serum (Clontech, Palo Alto, CA) and Texas red (TxR)-conjugated secondary antibodies (Molecular Probes, Leiden, The Netherlands). For immunofluorescence detection the antibodies were diluted in phosphate-buffered saline containing 0.5% BSA.

Construction of the HLA-DR1 β-GFP and Transfection

The cDNA of GFPS65T (9, 17) was kindly obtained from R. Tsien (University of California, San Diego, CA) and subcloned into pcDNA3 (Invitrogen, San Diego, CA). A fusion construct was generated by PCR creating immediate upstream HindIII–ClaI sites in the GFP cDNA (using oligo 5′ TTCAAGAAGAGGAAATGATAGCTATTCGAACCG and SP6 (Invitrogen) and a downstream ClaI site in DRβ1 eliminating its stop codon (using 5′ GGTTGTCCTAAGGACTCGCGTTAGCTAATATC and T7 (Invitrogen). The GFP portion was cloned into pcDNA3 by HindIII–BamHI digest and the DRβ1 portion was fused upstream in frame using the ClaI site. The coding region of the HLA-DRβ1 portion was checked by sequence analysis.

Mel JuSo cells were transfected using the Calcium Phosphate precipitation procedure and selected in DMEM (GIBCO-BRL, Gaithersburg, MD) supplemented with G418 (1 μg/ml, GIBCO). Positive clones were selected for fluorescent appearance and maintained in Iscoves medium (GIBCO) supplemented with G418 (500 μg/l).

Confocal Analysis

For immunofluorescence labeling, cells were fixed in ice-cold methanol and incubated with various antibodies and Texas red–conjugated secondary antibodies.

Living cells were analyzed in a tissue-culture device at 37°C. Cells were cultured in Hepes-buffered Iscoves medium supplemented with 10% FCS for at least 18 h before and during the confocal analysis. The temperature of the medium was continuously checked during analysis.

Cells were cultured for 4.5 h in the presence of 5 μg/ml Brefeldin A (BFA; Sigma Chemical. Co., St. Louis, MO) or for 3 h in the presence of 100 μM chloroquine (Sigma). Alternatively, cells were incubated for 1 min before analysis with LysoTracker Red DND99 at a concentration of 300 nM. Endosomes were labeled by internalization of Texas red (TxR; 25 μg/ml) or sulforhodamine 101 (SR101; 25 μg/ml).

Confocal analyses were performed using a 600MRC equipped with an Argon/Krypton laser (BioRad Labs, Hercules, CA). Green fluorescence was detected at $\lambda > 515$ nm after excitation at $\lambda = 488$ nm. For dual analyses, green fluorescence was detected at λ 520–560 nm. Red fluorochromes (Sulforhodamine 101, Texas red and LysoTracker Red DND99), purchased from Molecular Probes (Leiden, The Netherlands) were excited at $\lambda = 568$ nm and were detected at $\lambda > 585$ nm. Vesicular transport was visualized by first collecting images at short intervals. Then, images were superimposed and the first and last image of the series were subtracted from the resulting picture, using the program Comos (BioRad). The resulting subtraction is shown in yellow in conjunction with the first and last image in green or red.

Electron Microscopy

Mel JuSo cells were fixed in either a mixture of 4% formaldehyde (wt/vol) and 0.1% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) (Figs. 4 *A* and 6 *A*) or only 4% formaldehyde (Fig. 4 *B*). Ultrathin cryosections were subsequently incubated with anti-CD63 (mAb 435) and either the rabbit anti-class II β chain or the rabbit anti-GFP sera followed by either goat anti–rabbit IgG or goat anti–mouse IgG linked to 10- or 5-nm gold particles, respectively (Amersham, 's-Hertogenbosch, The Netherlands). The sections were subsequently embedded in a mixture of methylcellulose and uranyl acetate and examined with a CM10 electron microscope (Philips Electronic Instruments, Eindhoven, The Netherlands).

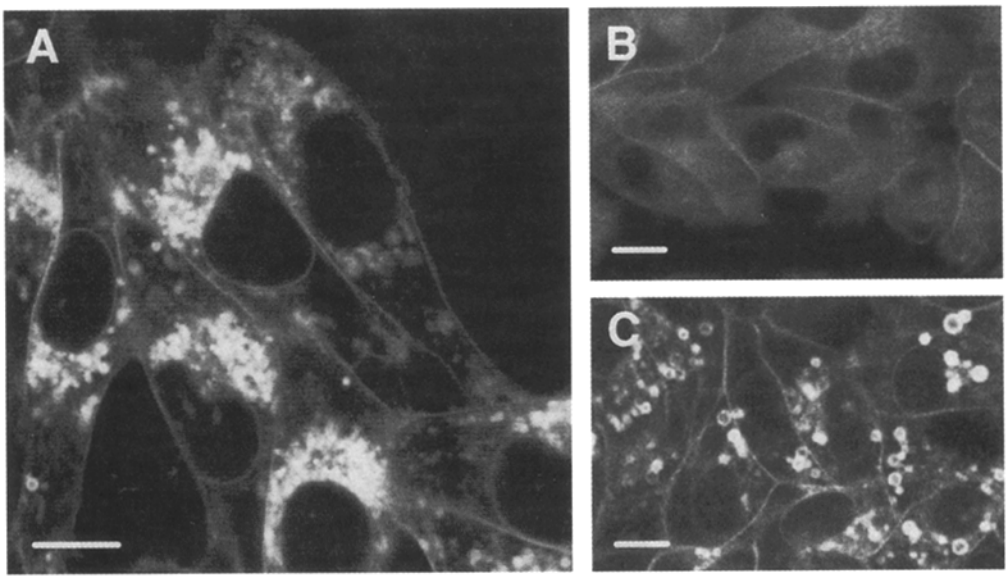


Figure 1. Distribution of class II/β-GFP in living cells. The melanoma cell line Mel JuSo was stably transfected with a construct containing GFP coupled to the cytoplasmic tail of the class II DR1 β-chain and analyzed by confocal microscopy in a tissue culture chamber at 37°C. (*A*) Distribution of class II/β-GFP. Fluorescence is observed at the plasma membrane and is found accumulated in multiple vesicles in the perinuclear region. The nucleus is devoid of fluorescence. Note the presence of vesicles in the periphery of the cell. (*B*) Distribution of class II/β-GFP after 4.5 h incubation with BFA to inhibit transport from the ER. Class II/β-GFP containing vesicles disappear and fine-reticulate (*ER*), as well as cell surface fluorescence is observed. (*C*) Chloroquine causes swelling of the β-GFP containing vesicles. The transfectants were incubated with chloroquine for 3 h. As a result, the fluorescent vesicles appear as large swollen structures while class II molecules remain present at the plasma membrane. Bar, 10 μm.

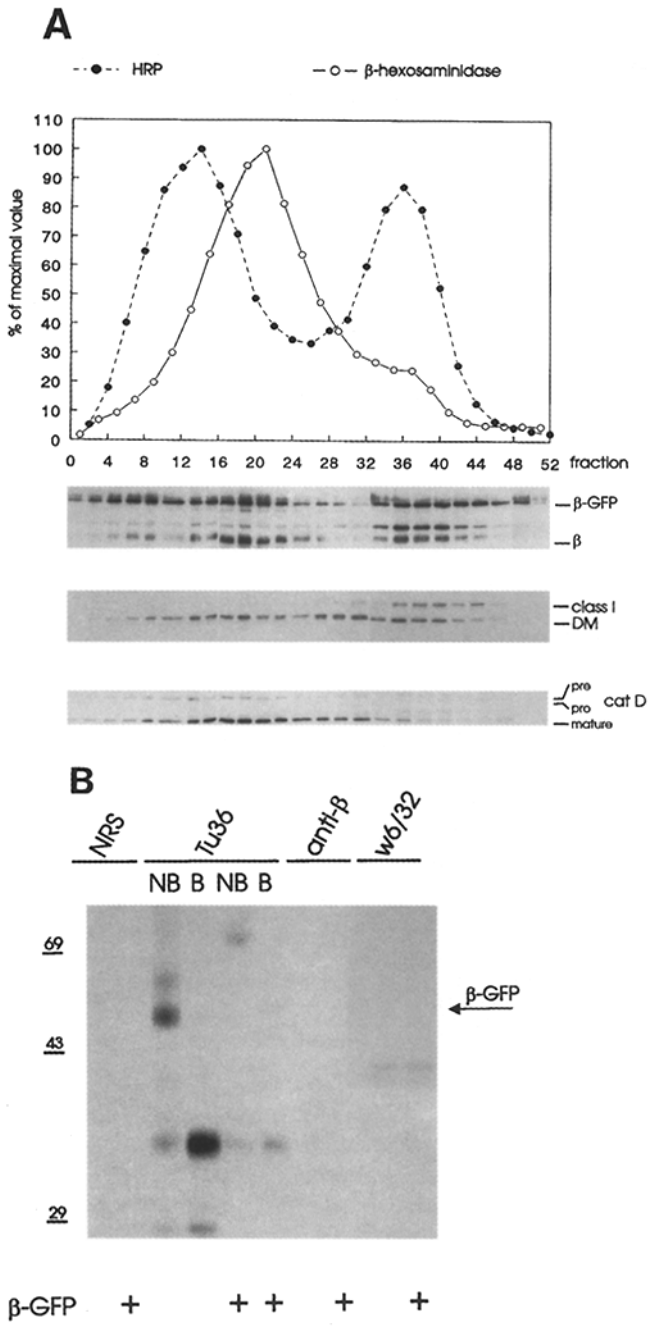


Figure 2. Biochemical analysis of endogenous and β-GFP containing class II molecules. (*A*) Subcellular distribution of β-GFP in transfected Mel JuSo cells. Cells were labeled by fluid phase uptake of HRP for 30 min, and postnuclear supernatant was obtained and fractionated in an electric field by density gradient electrophoresis. The enzymatic activity of HRP (*closed circles*) and of β-hexosaminidase (*open circles*) were determined in alternate fractions. Proteins in the even fractions were collected by TCA-precipitation and analyzed by SDS-PAGE and subsequent immunoblotting. The distribution of β-GFP and endogenous β-chain were determined by probing the immunoblots with an anti-class II β-chain serum. The position of HLA class I molecules, DM, and cathepsin D (cat D) was determined by incubating the same immunoblot with different antibodies. The position of the three forms of cathepsin D (pre-, pro-, and mature form) are indicated. (*B*) Biochemical analysis of cell surface class II molecules. Radioiodinated Mel JuSo cells or transfectants expressing β-GFP (*bottom lanes* +) were lysed followed by immunoprecipitation with either normal rabbit serum (NRS), anti-class II mAb Tu36, an antiserum that recognizes free class II β-chains (lanes β), or anti-class I mAb W6/32, respectively. The Tu36 isolate was split and analyzed after incubation with SDS at either room temperature (lanes *NB*) or after boiling (lanes *B*). Immunoprecipitates were analyzed by 10% SDS-PAGE and the positions of the markers and β-GFP are indicated. Surface class II molecules are isolated from control cells while the β-GFP expressing cells mainly contained surface class II/β-GFP. Both control class II and class II/β-GFP are observed in an SDS-stable compact conformation migrating at 54 kD and 75 kD, respectively (lanes *NB*). These complexes dissociate upon boiling (lanes *B*). Note that free class II β- or β-GFP chains cannot be recovered from the cell surface (lanes β).

Biochemical Analysis

Subcellular fractionation by density gradient electrophoresis (DGE). Mel JuSo cells were incubated for 30 min with horseradish peroxidase (HRP, type VIA; Sigma) at a concentration of 2 mg/ml at 37°C, as described (51). Cells were washed three times with PBS (0°C), removed from the culture dish by scraping in homogenization buffer (10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, 0.25 M sucrose, pH 7.4) and collected by centrifugation (139 *g*, 7 min). Cells were homogenized by passing 10–15 times through a 22-G × 1.5 needle and postnuclear supernatant (PNS) was collected by centrifugation at 840 *g* for 15 min. The PNS was treated with trypsin (25 μg/mg total protein) for 5 min at 37°C, trypsin inactivated (100 μg soybean trypsin inhibitor/mg total protein and 1 mM PMSF) and vesicles collected (100,000 *g* for 1 h at 4°C). Vesicles were separated by a DGE device with an extended electrophoretic pathway of 7 cm, as described (51). Electrophoresis was performed at 10.30 mA constant current for 80 min. Fractions of 250 μl were collected starting at the anodic site. Proteins were collected by TCA-precipitation and analyzed by Western blotting after separation by 10% SDS-PAGE. HRP activity was determined spectrophotometrically at 455 nm using O-dianisidine as a substrate (16). β-Hexosaminidase activity was determined spectrophotometrically at 405 nm using *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma) as a substrate, (22).

Biochemical analysis of surface class II molecules. A 10-cm dish of Mel JuSo cells or Mel JuSo cells expressing β-GFP was labeled at 4°C using lactoperoxidase-catalyzed iodination with 400 μCi $Na^{125}I$. Cells were washed four times with PBS (0°C) and lysed in NP-40 containing lysis mixture. Nuclei were removed (5 min, 12,000 *g*) and the lysate precleared with normal rabbit serum (NRS). The lysate was split and immunoprecipitated with NRS, Tu36, anti-class II β serum or W6/32, respectively. The Tu36 isolate was further split and incubated in nonreducing sample buffer at room temperature or at 95°C before separation by 10% SDS-PAGE. The other isolates were analyzed by SDS-PAGE after incubation in reducing sample buffer at 95°C.

Results

Distribution of GFP-tagged Class II Molecules in Living Cells

Mel JuSo cells express endogenous class II molecules and all components to efficiently load class II molecules with peptides (including HLA-DM; [42, 51]). In addition, these cells are adherent which is instrumental in our real-time confocal analyses. Mel JuSo cells were transfected with a chimera of the HLA-DR1 β-chain with GFP attached to the cytoplasmic tail (β-GFP). Since GFP is a bulky fluorescent protein, it may interfere with correct assembly and intracellular distribution of the chimeric class II/β-GFP molecules. However, the distribution of green fluorescence in the transfectants cultured and analyzed at 37°C was as expected for endogenous class II molecules (Fig. 1). Green fluorescence is observed at the plasma membrane and in numerous vesicles that are concentrated in the perinuclear

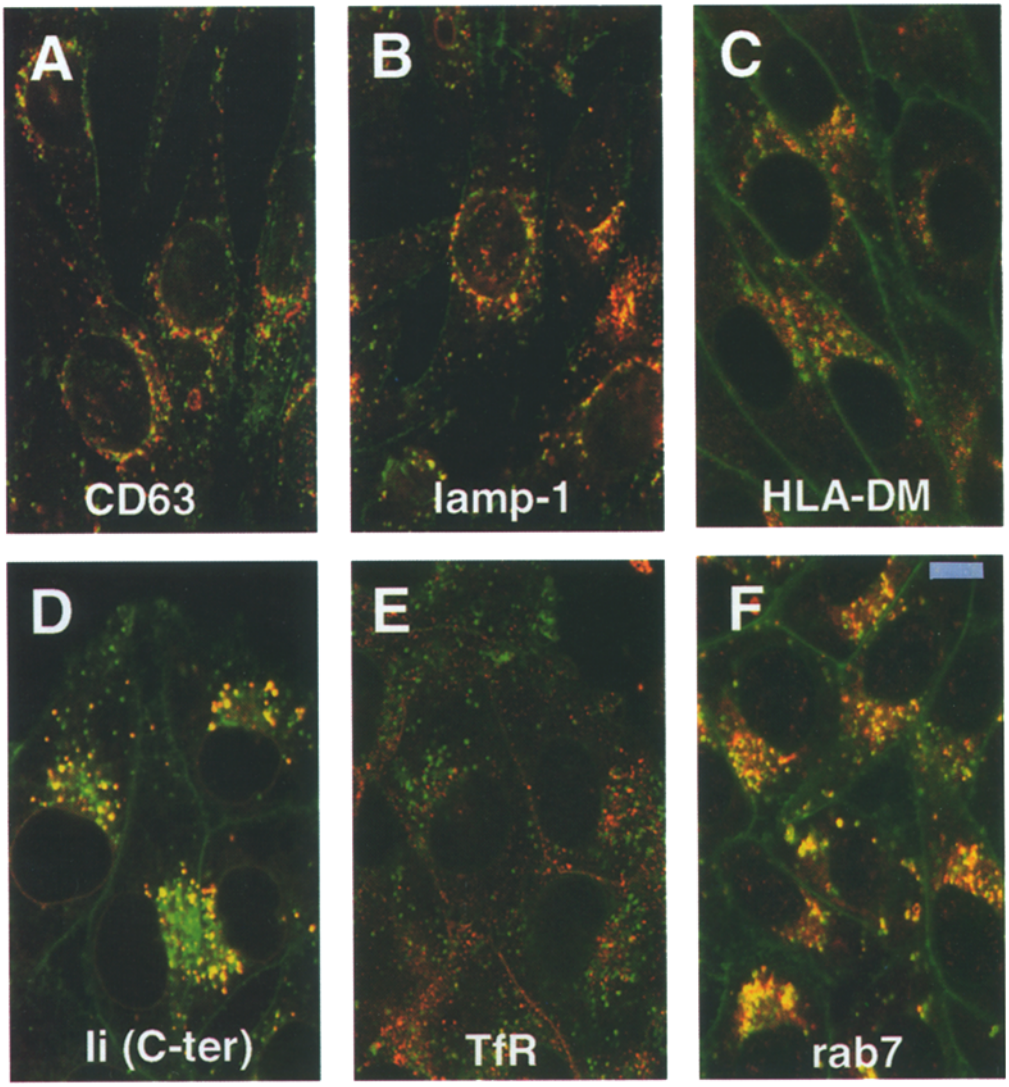


Figure 3. Double labeling of fixed β-GFP–transfected cells. Mel JuSo cells fixed in ice-cold methanol and stained with antibodies against the indicated marker proteins and Texas red–conjugated secondary antibodies, were analyzed by confocal microscopy. The images shown were obtained by merging the green image, corresponding to β-GFP, and the red image, corresponding to immunodetected markers. Colocalization is seen in yellow due to the combination of the green and the red signal. Perinuclear β-GFP containing vesicles were positive for the MIIC markers CD63 (*A*), lamp-1 (*B*), and HLA-DM (*C*). Ii (*D*) was detected with an antibody directed against the luminal portion of the Ii. The antibody only stained a fraction of the perinuclear β-GFP containing vesicles. (*E*) Immunodetection of the transferrin receptor (TfR) to label plasma membrane and early endosomes showed no colocalization between β-GFP and the TfR in intracellular vesicles. (*F*) Most of the β-GFP containing vesicles were positive for the late endosome-associated GTPase rab7. (Optical sections: 1 μm; magnification, 1200; Bar, 10 μm.)

region where the nucleus is indented (Fig. 1 *A*). No fluorescence was observed with nontransfected Mel JuSo cells (not shown). The perinuclear location of β-GFP in vesicles is consistent with the distribution of MIIC as observed by immuno-electron microscopy (31, 32) (see Fig. 4).

To show that the fluorescent vesicles contain newly synthesized class II/β-GFP in transit to the plasma membrane, transport from the ER was inhibited by culture in the presence of BFA (20) (Fig. 1 *B*). Peripheral vesicles were not derived from the constitutive secretory pathway since no effect on the intracellular distribution of class II/β-GFP was observed after 1.5 h (not shown). However, fluorescent vesicles had disappeared after a 4.5-h BFA treatment and intracellular fluorescence appeared dispersed over the cytoplasm (Fig. 1 *B*). This fluorescence pattern suggests that class II/β-GFP is retained in the ER by BFA treatment and the perinuclear vesicles obtained class II/β-GFP from the biosynthetic pathway. Class II molecules in Mel JuSo cells (data not shown), but also in B cells (31, 26) require about 4 h for quantitative plasma membrane exposure.

To test whether the β-GFP containing compartments are acidic, as expected for MIIC compartments (31), cells were treated with the weak base chloroquine. Chloroquine raises the lysosomal pH and causes swelling of the acidic compartments (10, 45), which is indeed observed for the β-GFP containing vesicles (Fig. 1 *C*).

Intracellular Distribution and Peptide Loading of Class II/β-GFP

We next performed subcellular fractionation in an electric field by DGE (13, 42, 51) in order to determine whether the intracellular distribution of class II/β-GFP resembled that of endogenous class II molecules (Fig. 2 *A*). Transfected Mel JuSo cells were cultured in the presence of the fluid phase endocytosis marker HRP for 30 min to label the endosomes. After fractionation, the HRP and β-hexosaminidase activities were determined to position the endosomes and lysosomes, respectively (*upper panel*). Two endosomal populations are visualized by the HRP activity; early endosomes located around fraction 36, and late endosomes around fraction 14, as described (51). Proteins in the even fractions were collected by TCA-precipitation

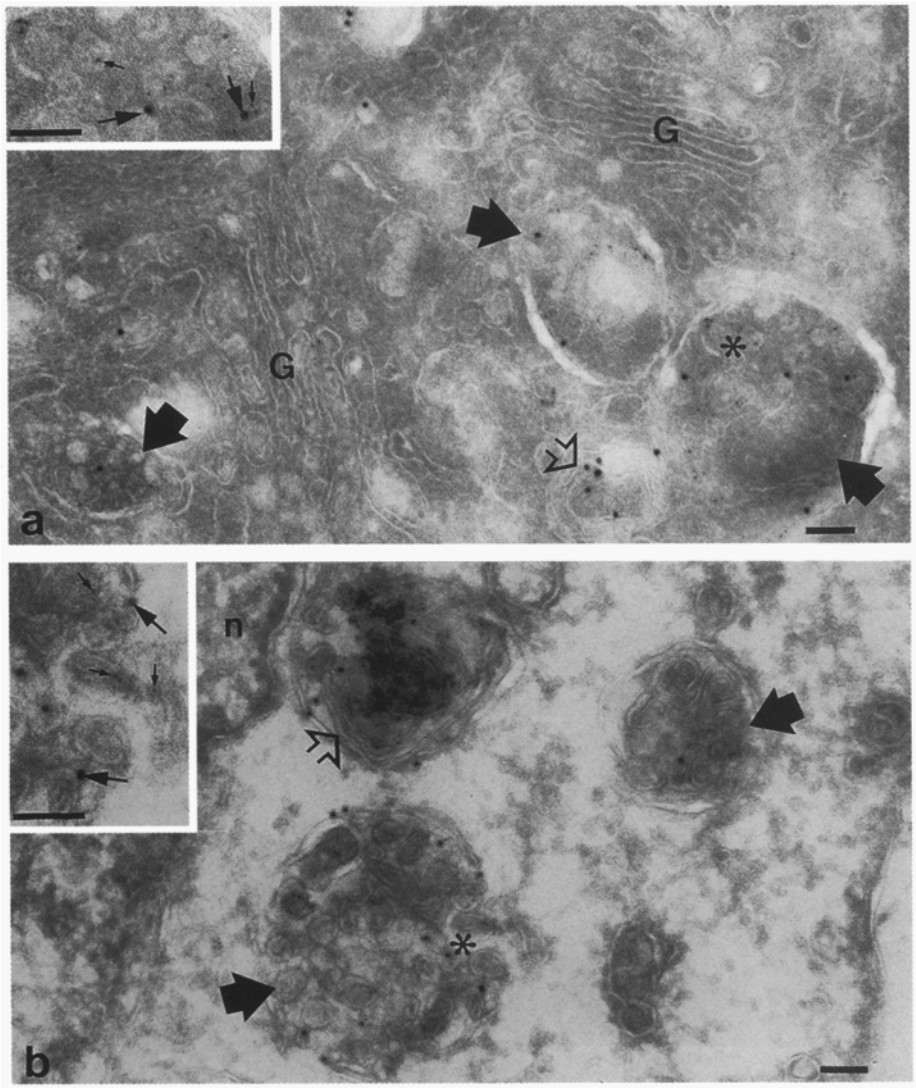


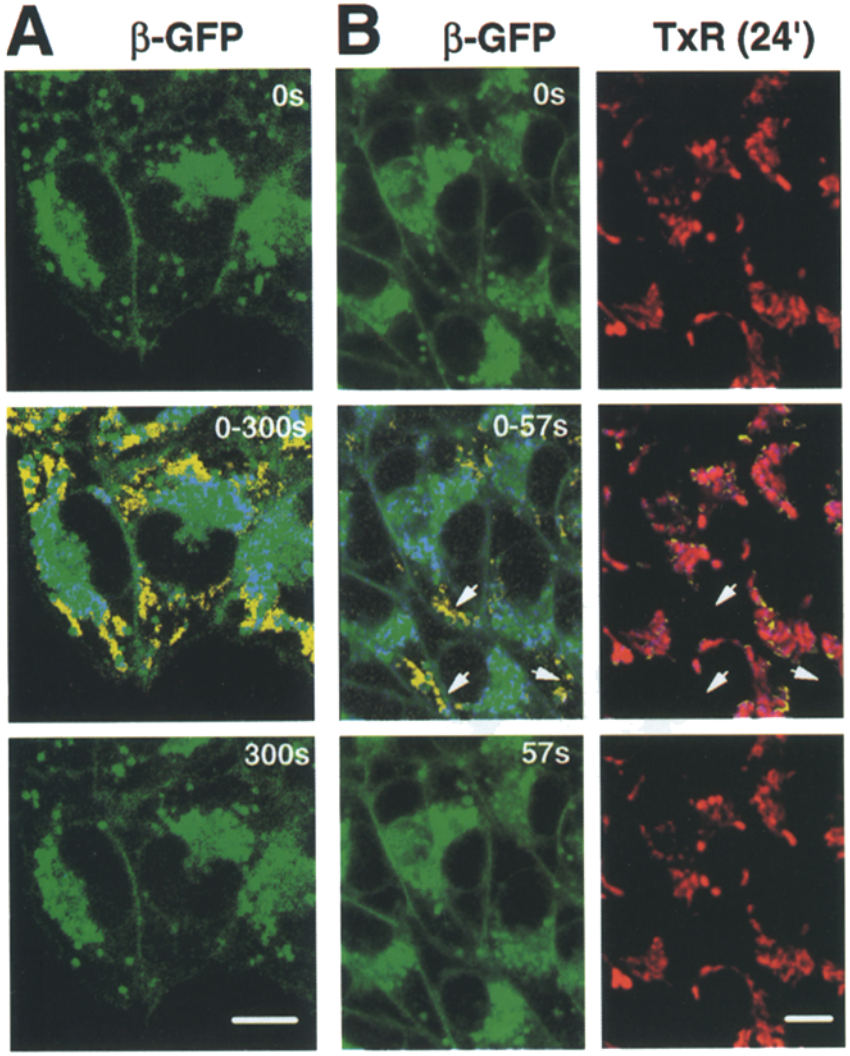
Figure 4. Class II/β-GFP location in CD63-containing multivesicular and multilamellar compartments. Mel JuSo cells transfected with β-GFP were fixed and sections were labeled with anti-CD63 (5 nm gold) and anti-class II β chain antibodies (10 nm gold, *A*) or anti-GFP antibodies (10 nm gold, *B*). The inset shows a higher magnification of the multivesicular compartments shown by an asterisk. *G*, Golgi; *n*, nucleus. (*Filled arrow*) Multivesicular compartment; (*open arrow*) multilamellar compartment. Inset: (*large arrows*) anti-β chain or anti-GFP; (*small arrow*) anti-CD63. The class II β chain and GFP are observed in CD63-positive multivesicular and multilamellar structures. Bar, 100 nm.

and the distribution of various proteins was determined by Western blot analysis (*lower panels*). Class I molecules were observed in fractions 36-48, thereby positioning the ER and the plasma membrane. Immature HLA-DMA is found at the position of the ER (fraction 34-44) (42) and mature HLA-DM at the position of late endosomes and lysosomes (fractions 6-24). Mature cathepsin D is distributed in a similar fashion as the β-hexosaminidase activity. Most importantly, endogenous class II β (~30 kD), as well as the β-GFP chain (~55 kD; GFP adds 25 kD), codistributed at the position of class I molecules (the ER and plasma membrane, fractions 34-46) and at the position of late endosomal/lysosomal fractions that also contained mature HLA-DM (fractions 6-24).

To exclude that addition of GFP to the cytoplasmic tail of the class II β chain has any effect on the ability to assemble and mature into a proper peptide-binding class II complex, control and β-GFP transfected Mel JuSo cells were surface iodinated and analyzed for the presence of plasma membrane embedded SDS-stable class II dimers (Fig. 2 *B*). Class II complexes (lanes *Tu36*), free class II β chains (lanes β) or class I molecules (lanes *W6/32*) were immunoprecipitated. The class II isolates were split and analyzed by SDS-PAGE after incubation in SDS followed either by boiling (lanes *B*) or by incubation at room temperature (lanes *NB*). A portion of peptide-loaded class II molecules will not dissociate under the latter conditions and remain in a so-called compact form (14, 38, 39) (Fig. 2 *B*). In control cells, a large fraction of class II molecules is in this compact form (migrating at 54 kD). In the β-GFP transfectants, class II is largely a class II/β-GFP heterodimer at the plasma membrane and for a major part in a compact configuration (now migrating at 75 kD).

Characterization of Class II/β-GFP Containing Vesicles by Confocal and Immuno-Electron Microscopy

To further establish that the class II/β-GFP containing vesicles represent MIIC compartments, the transfectants were fixed and stained with antibodies against markers for MIIC (lamp-1, CD63, cathepsin D, HLA-DMA) (31, 42) and for the plasma membrane and early endosomes (transferrin receptor [TfR]). In addition, we localized rab7, a small GTPase cosedimenting with class II–containing structures (36) and late endosomes (12). Lamp-1, CD63, HLA-DM, and rab7 all showed colocalization with



most perinuclear vesicles but only few peripheral vesicles (Fig. 3, *A–C*, and *F*). A similar picture was seen for cathepsin D (not shown). As expected, vesicles are found that are exclusively labeled for the marker proteins, since class II molecules are only observed in a subfraction of lysosomes (13, 31, 36). The MIIC marker proteins were not detected at the plasma membrane. Finally, the invariant chain (Ii) was detected with an antibody against the luminal portion of Ii. Ii colocalized with class II/β-GFP in the nuclear envelope due to the retention of a relative large fraction of Ii in the ER and the continous synthesis of novel β-GFP. Furthermore, the antibody only labeled some β-GFP containing peripheral vesicles (Fig. 3 *D*), since the luminal portion of Ii is rapidly degraded during transport in endosomal compartments (32, 33). Ii was not observed at the plasma membrane.

To analyze whether (part of) the β-GFP containing vesicles correspond to early endosomes, the fixed cells were labeled with anti-transferrin receptor (TfR) antibodies. Class II/β-GFP and TfR colocalized at the plasma membrane, although TfR mainly in patches. Intracellularly, TfR and class II/β-GFP labeled distinct vesicles (Fig. 3 *E*), in agreement with subcellular fractionation experiments (13).

Thus, the perinuclear class II/β-GFP containing vesicles are acidic, contain various late endosomal/lysosomal markers, and are distinct from early endosomes, suggesting that these vesicles are actual MIIC structures.

To confirm the colocalization with MIIC markers and to resolve the ultrastructure of the β-GFP containing vesicles, transfectants were analyzed by immuno-electron microscopy (Fig. 4). Sections were labeled with antibodies directed against CD63 (5 nm gold) and either against the class II β chain (Fig. 4 *A*) or GFP (Fig. 4 *B*) (both 10 nm gold). Class II molecules colocalized with CD63 in multivesicular (*closed arrow*) and multilamellar structures (*open arrow*) near the Golgi (Fig. 4 *A*) and were present at the cell surface (not shown). Anti-GFP antibodies stained identical intracellular structures as anti-class II β chain antibodies (Fig. 4 *B*).

In summary, the β-GFP chimera efficiently assembles into class II–Ii complexes (not shown), obtains a peptide-loaded configuration, and has the normal intracellular distribution as read biochemically and immunohistochemically. In addition, the kinetics of compact or SDS-stable class II complex formation is unaltered (not shown). Thus, the addition of GFP to the cytoplasmic tail of the class II β chain does not affect assembly, intracellular transport, distribution, and peptide loading of class II molecules.

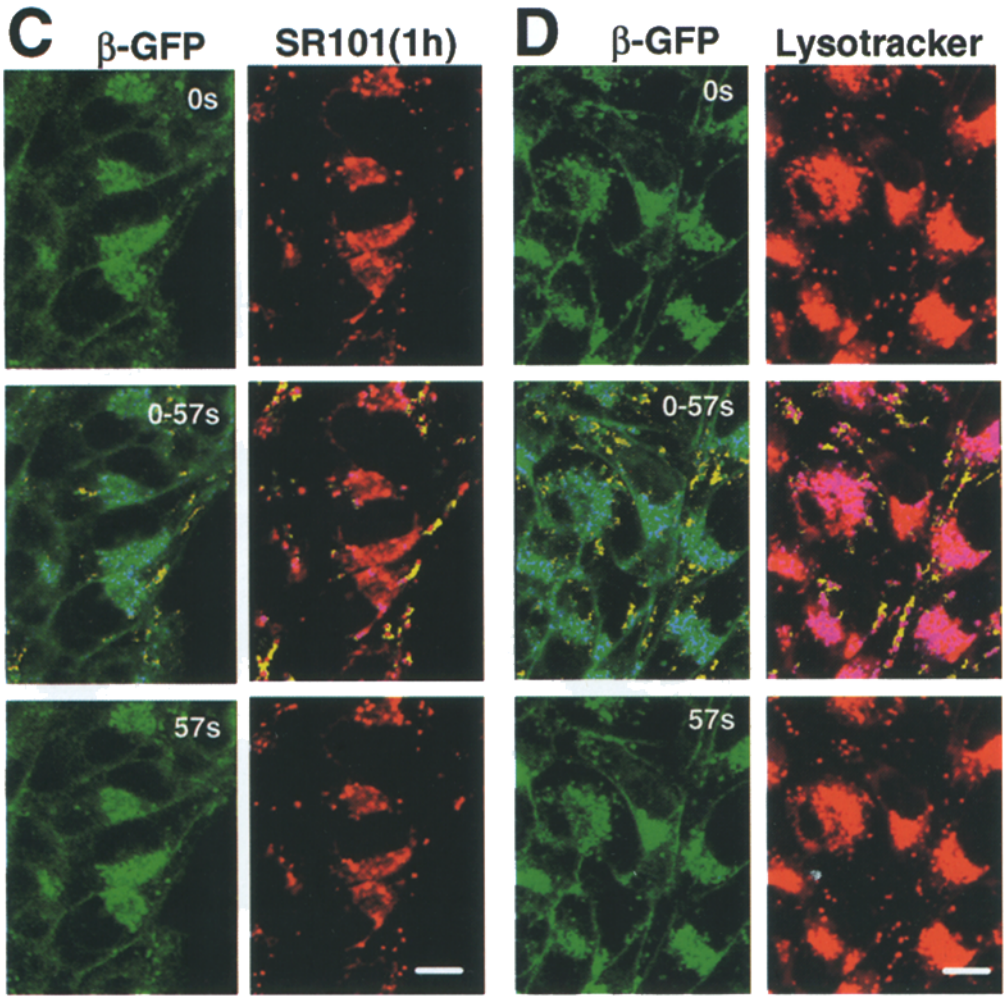


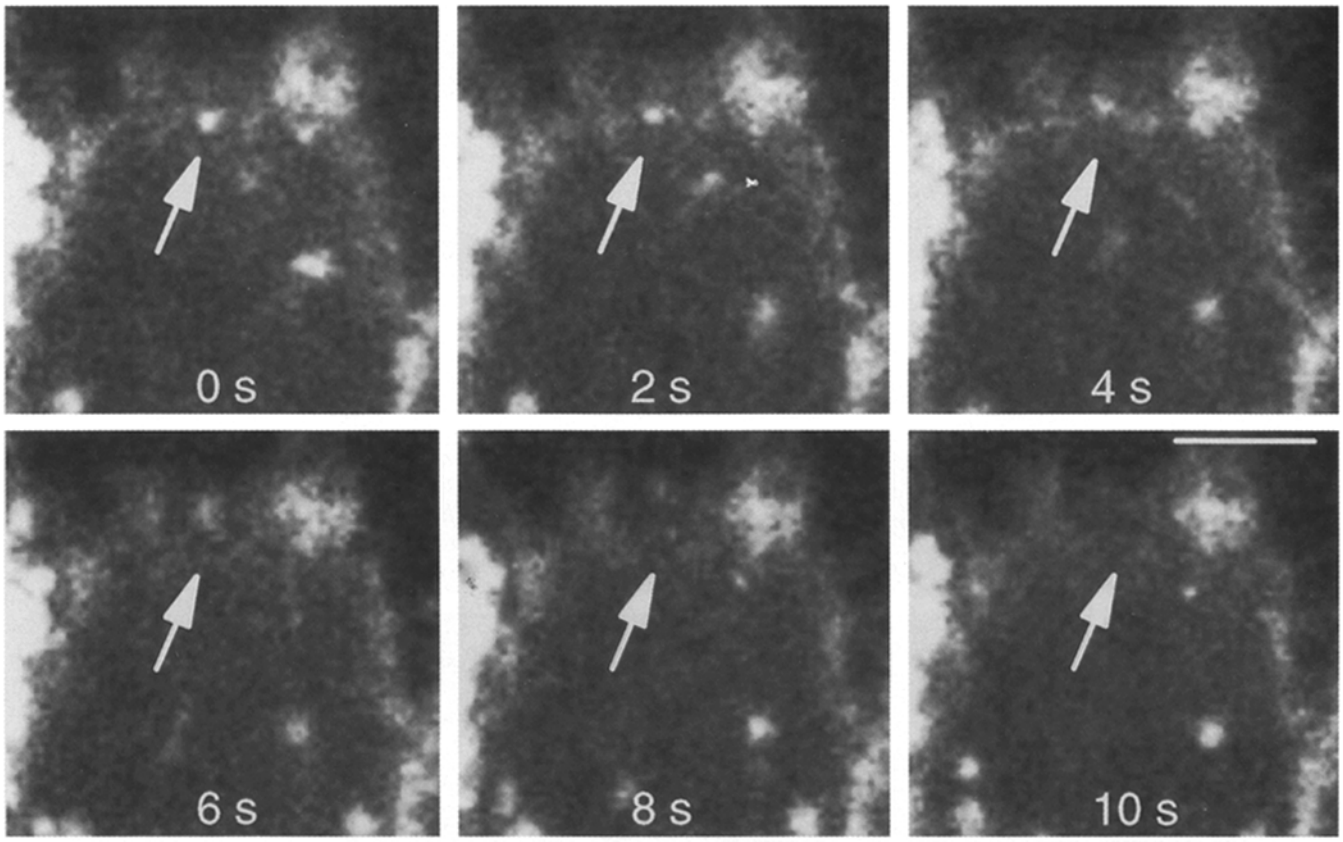
Figure 5. Acidic vesicles migrate from the perinuclear region to the plasma membrane without intersecting endosomal structures. Living β-GFP transfected cells were observed by confocal microscopy at 37°C and images were collected every 5 (*A*) or 3 (*B*, *C*, and *D*) s for a period of 5 or 1 min, respectively. Upper and lower panels correspond to the first and the last image of the series. The middle panel shows merged images of the first and last image (*green* or *red*) and movement within the imaging period is shown in yellow. (*A*) β-GFP migration within a 5-min window. Yellow trails are formed by migrating vesicles. (*B*) Migration recording after Texas red (TxR) uptake. Cells were incubated with TxR for 10 min at 37°C, washed, and chased for an additional 14 min. Images were collected of both the GFP (*green*) and the TxR signal (*red*). Identical positions in the cells are indicated by arrows. Note that the TxR-labeled endosomal structures hardly alter during the 1-min image collection. They are structurally different from the β-GFP containing vesicles and (*yellow*) trails. (*C*) Migrating class II/β-GFP containing vesicles are part of the endocytic pathway. The transfectants were cultured with the red fluorochrome sulforhodamine 101 (SR101) for 30 min, followed by washing and subsequent chasing for another 30 min. Endocytosed material (*red*) and β-GFP (*green*) showed a similar pattern. Rapidly moving vesicles are observed in the overlays that contain both β-GFP and SR101. (*D*) Both perinuclear and peripheral moving β-GFP labeled vesicles are acidic. Transfectants were incubated with the acidophilic fluorochrome LysoTracker Red (*red*) for 1 min and migration patterns were obtained as above. Arrows denote moving (*yellow*) acidic β-GFP containing vesicles. (Optical sections: 2 μm; Bar, 10 μm.)

Transport of Class II/β-GFP Containing Vesicles and Endosomes in Living Cells

To visualize transport of class II molecules from the perinuclear region to the plasma membrane, the β-GFP transfectants were cultured at 37°C and confocal images were made every 5 s for 5 min (Fig. 5 *A*). The first and last image is shown in the upper and lower panel, respectively. To directly visualize vesicle motion, the 60 images were superimposed and the first and last were subtracted (*middle panel*). The image shown is a merge of the subtracted image (*yellow*) and a combined first and last image (*green*). The subtraction procedure selectively visualizes movement. Clearly, within the 5-min period, a number of vesicles were released from the perinuclear region and moved towards the plasma membrane (Fig. 5 *A*). The position of the perinuclear vesicles and the plasma membrane hardly alters within this timespan.

To determine whether class II molecules are transported to the cell surface using the endocytic route in a retrograde fashion or in a manner independent of the endosomal route, cells were allowed to internalize the red fluorochromes Texas red (TxR) or Sulforhodamine 101 (SR101) for various periods to label the endocytic pathway (Fig. 5, *B* and *C*). Transport of β-GFP and fluid phase marker-containing vesicles was followed by generating confocal images every 3 s for a total of 60 s. Again, the first and last images are shown in the upper and lower panel and the middle panel visualizes movement as described for Fig. 5 *A*. Both the green β-GFP signal (*left panel*, GFP) and the red signal (*right panel*, TxR or SR101) are shown.

First, endosomes were labeled by culturing the cells for 10 min with TxR followed by a chase period of 14 min, and intracellular transport of β-GFP containing vesicles vs TxR-labeled endosomes was visualized in a 60-s window (Fig. 5 *B*). By DGE, fluid phase markers have been shown to enter early and late endosomes of Mel JuSo cells within this timespan (13, 51). Endosomal structures were labeled by TxR in a similar region in the cell where β-GFP accumulated but the structures clearly have a different mor-



phology. Both TxR-containing endosomes as well as the perinuclear class II/β-GFP vesicles hardly move within the 1-min time span (*middle panel*). However, some β-GFP containing vesicles are rapidly moving in the periphery of the cell. Importantly, these vesicles migrate in an area devoid of TxR-labeled endosomal structures (arrows point at trails of moving β-GFP containing vesicles and identical positions in the TxR panel). Identical results were obtained when endosomes were loaded by shorter periods of culture with TxR or SR101 (not shown).

Class II–containing compartments are situated late in the endocytic pathway (31, 32). To analyze whether the rapidly moving β-GFP containing vesicles were able to transport endocytosed material as well, cells were allowed to internalize the red fluorochrome SR101 for 30 min and then cultured for an additional 30 min in the absence of SR101 (Fig. 5 *C*); cells were analyzed as in Fig. 5 *B*. SR101-labeled late endocytic structures displayed a different morphology from those labeled by shorter incubations (see Fig. 5 *B*), and now resemble the intracellular distribution of class II/β-GFP containing vesicles. When the identical analysis, as in Fig 5 *A*, was performed, rapidly moving vesicles (*yellow*) were observed that contained both class II/β-GFP and SR101. Apparently, (a fraction of) endocytosed material can be regurgitated after long periods of internalization. Since β-GFP does not colocalize with early endosomal structures (Fig. 5 *B*), this transport route is different from recycling early endosomes.

To analyze whether the migrating β-GFP containing vesicles remain acidic, cells were incubated with LysoTracker Red, a membrane diffusable red fluorochrome that accumulates in acidic compartments, and the cells were analyzed as in Fig. 5 *B*. All class II/β-GFP containing vesicles, including those moving (shown in yellow), accumulated LysoTracker indicating that they are acidic (Fig. 5 *D*). Note the presence of vesicles exclusively labeled with LysoTracker, consistent with earlier observations that late lysosomes are devoid of class II molecules (13, 31).

Fusion of Class II/β-GFP Containing Vesicles with the Plasma Membrane

To visualize fusion of the fluorescent vesicles with the plasma membrane, images of the transfectants cultured at 37°C were made at 2-s intervals. Fig. 6 *A* shows part of a cell with plasma membrane fluorescence and some rapidly migrating peripheral vesicles. Arrows denote a vesicle moving towards the plasma membrane (at $t = 0$ and 2 s), incorporating in the membrane fluorescence (at $t = 4$, 6 and 8 s) and finally disappearing (at $t = 10$ s).

However, the resolution of confocal microscopy is insufficient to visualize the details of fusion of the class II/β-GFP containing compartments. Electron microscopy may give sufficient details of this process but requires distinction between fusion of secretory class II–containing vesicles and internalization. Hence, sections were stained with

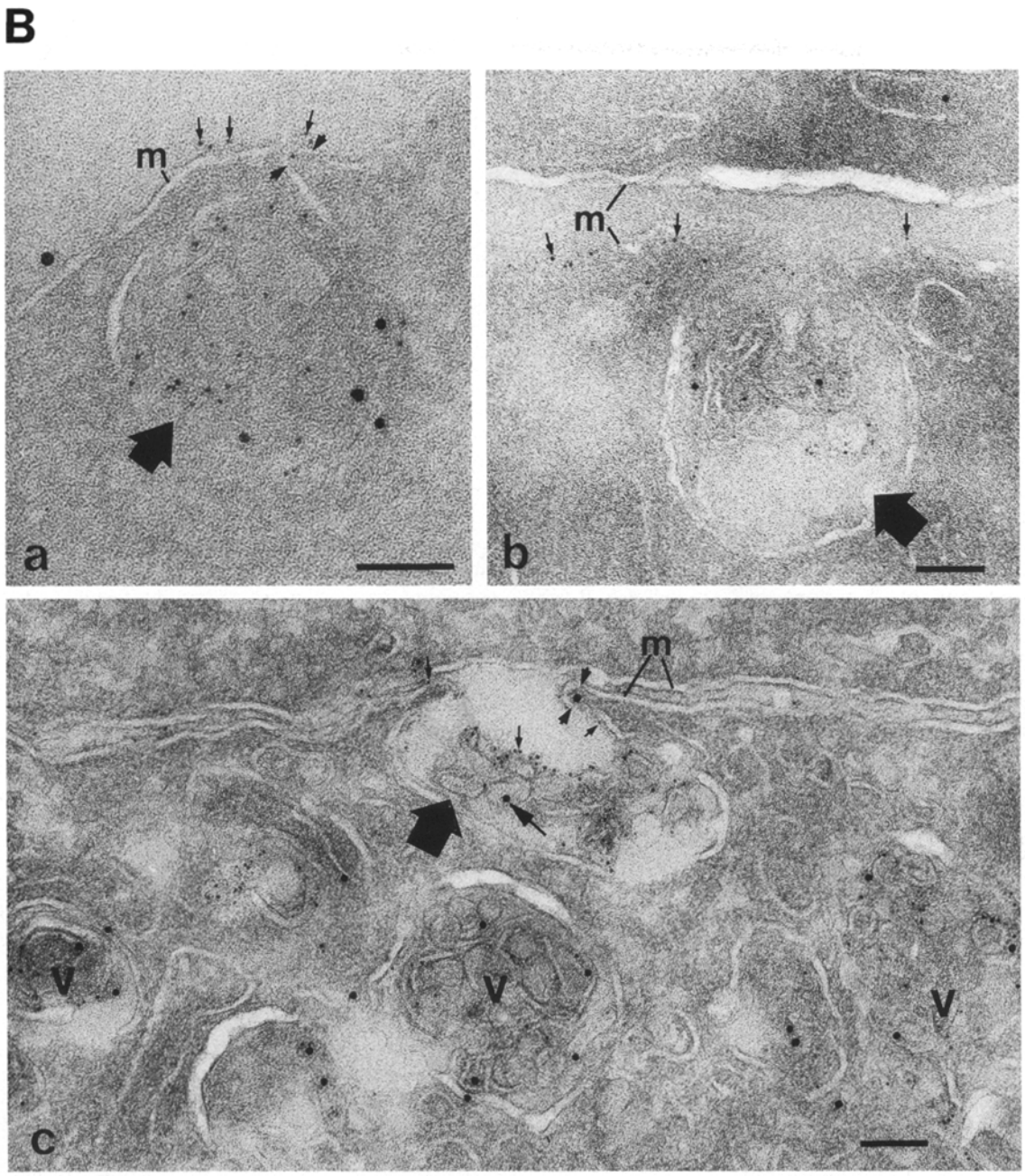


Figure 6. Fusion of class II–containing vesicles with the plasma membrane. (*A*) Living β-GFP–transfected cells were analyzed at 37°C by confocal microscopy and images were collected every 2 s. Only part of a cell is shown with some plasma membrane labeling and a number of peripheral vesicles. Part of a perinuclear region of a neighboring cell is visible at the left side of the figure. The arrow denotes a vesicle that is moving to the plasma membrane (t = 0 and 2 s), associating with the membrane fluorescence (t = 4, 6, and 8 s) and finally disappearing (t = 10 s). (*B*) β-GFP–transfected cells were fixed and cryosections were labeled with anti-CD63 (5 nm gold) and anti-class II β chain (10 nm gold). Multivesicular compartments (*large thick arrows*) containing class II β chain and CD63 are shown that have fused with the plasma membrane. In *A* and *C*, arrowheads point at the continuity of the plasma membrane with the vesicular membrane. The surface membrane (*m*) close to the fused vesicles is labeled for CD63 (*small arrows*). In *C*, several multivesicular compartments containing class II β chain and CD63 are shown (*v*) near the cell surface. The marked multivesicular compartment contains class II (*middle size arrow*) and CD63 (*small arrows*). It has fused with the plasma membrane and its lumen is clearly open to the intercellular space. (Optical sections: 2 μm; Bar, [*A*] 5 μm.) Bars, 100 nm.

antibodies against the class II β chain and CD63. Since CD63 is abundantly present in class II–containing compartments, but hardly expressed at the plasma membrane, a fusing vesicle should contain CD63, although this marker protein should rarely be present in endocytic vesicles. Fig. 6 *B* shows three fusion events of vesicles containing both class II molecules (*large gold*) and CD63 (*small gold*). The upper panel shows some CD63 labeling at the acceptor plasma membrane around the site of fusion. Since CD63 labeling is not observed at the plasma membrane, this sug-

gests that some CD63 has already diffused from the fusing vesicle onto the plasma membrane. All observed fusing vesicles contained both CD63 and class II molecules in agreement with a recent report showing multivesicular compartments fusing with the plasma membrane (37).

Discussion

MHC class II molecules enter the endocytic route to associate with peptide fragments derived from degraded proteins before cell surface appearance. The class II–associated invariant chain (Ii) targets class II molecules to the endocytic pathway either by sorting in the *trans*-Golgi network or by internalization from the plasma membrane. In endocytic vesicles, Ii is degraded which is an essential step that precedes peptide loading of class II molecules and release from the endocytic pathway (7, 8, 44). The exact endocytic location where peptide loading of class II molecules occurs, has been a matter of debate. Immuno-electron microscopy showed class II molecules in so-called MIIC compartments with a multivesicular or multilamellar morphology that contain many lysosomal marker proteins (31, 32, 15). Some, but not all, epitopes of Ii could be observed in these structures suggesting that Ii-degradation occurs in MIIC (32). Subcellular fractionation experiments have observed class II molecules in transferrin-receptor containing endosomes (1), middle endosomes (50), and lysosomes (36, 54), and thus confused an unequivocal determination of the intracellular site of peptide loading of class II molecules. Recently, a molecule, HLA-DM, has been identified that supports proper peptide loading of class II molecules (11, 41, 47, 48.). HLA-DM therefore is a better indicator for the intracellular site of class II–peptide loading and has been observed in MIIC structures in EBV-transformed B cells by immuno-electron microscopy (32, 42). In this study, we have followed transport of MHC class II in the melanoma cell line Mel JuSo because its intracellular distribution and transport of class II molecules is extensively studied (33, 50, 51, 56). Using subcellular fractionation, we recently established for this cell line that class II molecules and HLA-DM are expressed in early or low density lysosomes and that early and middle endosomes are devoid of class II molecules, HLA-DM and cathepsin D (13). The intracellular distribution of class II/β-GFP is identical to endogenous class II molecules and class II/β-GFP accumulates in lysosomal structures with all characteristics of MIIC. We have not identified structures corresponding to CIIV (which should have contained TfR) in these cells (Fig. 3 *E*, see reference 13).

How class II molecules are transported from these early lysosomal MIIC-like compartments to the plasma membrane is unknown and no similar pathway (i.e. from lysosomes to the plasma membrane) has been visualized. MHC class II molecules could be transported in a retrograde endosomal pathway or they may be transported directly from MIIC to the plasma membrane. Experiments combining pulse chase experiments with subcellular fractionations have not been successful in elucidating this problem. Here, we have visualized class II molecules in living cells. The addition of GFP onto the cytoplasmic tail of the class II β chain does not affect normal intracellular distribution, assembly of the class II complex, and peptide loading. In addition, the timing of peptide loading is identical to that of endogenous class II molecules (data not shown) suggesting no major effects of GFP addition on entry into class II–loading compartments. Apparently, addition of the fluorochrome did not interfere with the sorting signals of the invariant chain/MHC class II complex (2, 34, 3, 28, 6, 35).

We have subsequently followed transport of these vesicles in real time. This allowed visualization of a direct rapid transport pathway of acidic class II–containing vesicles towards the plasma membrane. Since these vesicles are accessible to fluid phase markers only after uptake periods longer than 45 min, they should correspond to late endosomal/lysosomal structures. Fusing class II vesicles contain the lysosomal marker CD63 indicating that these are also lysosomal structures that lack detectable Ii (not shown). The transport route from lysosomal structures to the plasma membrane, as used by MHC class II molecules, is distinct from the endocytic route because: (*a*) migrating β-GFP containing vesicles do not fuse with endosomes labeled by 24 min endocytosis of TxR; (*b*) these endosomes have a morphology different from the moving β-GFP-containing vesicles; and (*c*) the rate of transport of β-GFP containing vesicles is considerably faster (estimated at ~5 μm/min) than endosomal transport (endosomal structures hardly alter within the 1-min timespan [see Fig. 5 *B*] and move with ~0.5 μm/min [9]). This transport process involves microtubuli since nocodazole and colcemid block the movement of the fluorescent vesicles (not shown).

Although fusion of class II–containing vesicles with the plasma membrane can be visualized, it remains unclear how the internal membranes observed in these vesicles are incorporated in the plasma membrane. Recently, a mechanism was suggested where part of the vesicles were secreted (so called exosomes, reference 37). The exposed luminal portion of class II was suggested to confer antigen presenting capacities to these vesicles. In addition, a shuttle theory was proposed to allow neighboring cells to take up exosomes. The latter is not observed at a detectable level in our cells since cells that lost expression of the class II/GFP chimeras failed to obtain class II molecules from the surrounding cells.

Since class II molecules are rapidly and directly transported from lysosomal structures to the plasma membrane, they do not interact with antigen generated in earlier endosomal structures. Since early and middle endosomes of Mel JuSo cells lack cathepsin D, HLA-DM, and peptidase activity (13, 42), antigen uptake in earlier endosomes would have been inefficient anyway. Finally, endocytosed material can use the route used by class II molecules for surface appearance to become secreted back to the medium, as suggested here for SR101 (Fig. 5 *C*) and as observed for internalized HRP (not shown). This offers the possibility that a fraction of antigenic peptide fragments generated in the endosomal/lysosomal pathway is able to load plasma membrane-embedded class I and II molecules upon secretion. Because the efficiency of this process is unclear, it may occur only when large quantities of antigen are internalized.

The existence of a transport route from lysosomes to the plasma membrane has been suggested before on the basis of the resistance to chloroquine of surface deposition of

certain lysosomal proteins (23), and recently on the basis of MIIC-derived vesicles associated with the plasma membrane and in the medium (37). Here we directly visualized the existence of such a transport route that is used by MHC class II molecules in the melanoma cell line Mel JuSo. The existence of a corresponding pathway in professional antigen presenting cells remains to be shown. Direct transport from lysosomal structures to the plasma membrane has some remarkable similarities with the process of regulated secretion as observed for example by cytolytic granules in CTLs (18,30). These granules also contain a number of lysosomal markers but the release of their content is tightly regulated. In contrast, transport of class II molecules to the plasma membrane appears to be constitutive although it requires efficient degradation of the Ii chain (25, 56) and may be altered in activated DC (27, 40). The processes that determine the release of class II–containing vesicles from the perinuclear region and their transport to the plasma membrane remain to be established.

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