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How MHC class II molecules reach the endocytic pathway

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We have examined trafficking of major histocompatibility complex (MHC) class II molecules in human B cells exposed to concanamycin B, a highly specific inhibitor of the vacuolar H^+-ATPases required for acidification of the vacuolar system and for early to late endosomal transport. Neutralization of vacuolar compartments prevents breakdown of the invariant chain (Ii) and blocks conversion of MHC class II molecules to peptide-loaded, SDS-stable αβ dimers. Ii remains associated with αβ and this complex accumulates internally, as ascertained biochemically and by morphological methods. In concanamycin B-treated cells, a slow increase (>20-fold) in surface expression of Ii, mostly complexed with αβ, is detected. This surface-disposed fraction of αβIi is nevertheless a minor population that reaches the cell surface directly, or is routed via early endosomes as intermediary stations. In inhibitor-treated cells, the bulk of newly synthesized αβIi is no longer accessible to fluid phase endocytic markers. It is concluded that the majority of αβIi is targeted directly from the *trans*-Golgi network to the compartment for peptide loading, bypassing the cell surface and early endosomes *en route* to the endocytic pathway.

Key words: acidification/endocytic pathway/MHC class II/ trafficking

Introduction

Major histocompatibility complex (MHC) class II molecules present peptides derived from proteins degraded in the endocytic pathway to $CD4^+$ T lymphocytes (Unanue, 1992). Class II molecules are synthesized in the endoplasmic reticulum (ER) (Cresswell, 1994), where their α and β subunits associate with a trimer of the class II associated invariant chain (Ii) (Roche *et al.*, 1991; Lamb and Cresswell, 1992). Following transfer to the Golgi and *trans*-Golgi network (TGN), the class II αβIi complex is sorted to a compartment which has late endosomal/ lysosomal characteristics, yet is apparently a distinct entity, the MHC class II compartment, or MIIC (Peters *et al.*, 1991; Amigorena *et al.*, 1994; Qiu *et al.*, 1994; Tulp *et al.*, 1994; West *et al.*, 1994). The ultrastructural evidence has been interpreted as supporting a direct transfer of class II molecules from the TGN to this specialized compartment (Peters *et al.*, 1991). However, the detection of αβIi complexes that appear transiently at the cell surface has suggested that delivery of class II molecules to these endocytic compartments could involve the cell surface as a necessary intermediate (Lotteau *et al.*, 1990; Roche *et al.*, 1993), as has been proposed for other constituents of the endocytic pathway (Braun *et al.*, 1989; Peters *et al.*, 1990; Nabi *et al.*, 1991; Mathews *et al.*, 1992; Trowbridge *et al.*, 1993). In MIIC (Peters *et al.*, 1991), class II molecules dissociate from Ii (Teyton *et al.*, 1990; Roche and Cresswell, 1991), bind peptide and thence reach the cell surface (Davidson *et al.*, 1991), where they present the bound peptides to $CD4^+$ T cells (Unanue, 1984, 1992). The Ii, primarily its short cytoplasmic tail, plays a critical role in the sorting of class II molecules (Bakke and Dobberstein, 1990; Lotteau *et al.*, 1990; Lamb and Cresswell, 1992; Pieters *et al.*, 1993; Odorizzi *et al.*, 1994): mice deficient in Ii synthesis express reduced amounts of class II at the cell surface, and have profound deficiencies in their ability to present antigen via class II (Bikoff *et al.*, 1993; Viville *et al.*, 1993; Elliott *et al.*, 1994).

Sorting of membrane glycoproteins along the endocytic pathway is largely determined in the TGN (Griffiths and Simons, 1986; Mellman and Simons, 1992), but it is unlikely to be the only station at which selective targeting events occur in the course of biosynthesis. Selective retrieval from the cell surface and sorting events in the endocytic pathway itself are likely to play a role. There is mounting evidence that acidification of key aspects of the secretory and endocytic pathways may be important for protein trafficking (Klionsky and Emr, 1986; Banta *et al.*, 1988; Chapman and Munro, 1994). Acidotropic agents such as chloroquine and ammonium chloride which neutralize acidic compartments are often used to study the functional role of low pH in the vacuolar system, not only in cell biology, but also in immunology (Nowell and Quaranta, 1985; Blum and Cresswell, 1988; Loss and Sant, 1993). They can discriminate between the two fundamentally different modes of antigen presentation used by MHC class I and class II molecules. Due to the routing of the latter through endosomes, it is the class II pathway that is generally found to be sensitive to lysosomotropic drugs (Nowell and Quaranta, 1985; Blum and Cresswell, 1988; Loss and Sant, 1993; Newcomb and Cresswell, 1993).

Genetic evidence indicates that the vacuolar H^+-ATPase, responsible for acidification of the vacuolar system, is required for the delivery of proteins to the yeast equivalent of a lysosome, the vacuole (Banta *et al.*, 1988; Klionsky *et al.*, 1990, 1992; Nelson and Nelson, 1990). More recently, the formation *in vitro* of carrier vesicles that mediate the delivery of early endosomal contents to late endosomes in mammalian cells was shown to require an active vacuolar H^+-ATPase (Clague *et al.*, 1994). Macrolide antibiotics such as bafilomycin A_1 and concanamycin B are highly selective inhibitors of vacuolar H^+-ATPases (as distinct from plasma membrane and mitochondrial H^+-ATPases) (Kinashi *et al.*, 1984; Bowman *et al.*, 1988; Nelson, 1991). They are active at low concentrations (nM range), and—again in yeast—have been shown to affect targeting of vacuolar contents. Bafilomycin A_1 induces secretion of the vacuolar hydrolases carboxypeptidase Y (CPY) and proteinase A (Klionsky and Emr, 1989). However, there is no effect on targeting of the proenzyme proALP to the vacuole where it is converted to mature ALP by cleavage. Such studies reveal that vacuolar targeting can occur by at least two different mechanisms and demonstrate the usefulness of these compounds as tools in cell biology.

In mammalian cells, the vacuolar H^+-ATPase is located in the membranes of intracellular organelles, including the Golgi apparatus where it creates an acidic milieu in the lumen (Moriyama and Nelson, 1989). We explored the effects of concanamycin B on the breakdown of the class II-associated Ii, and on αβIi trafficking. The efficacy of sorting signals borne by cytoplasmic tails of transmembrane proteins in the endocytic pathway appears to be affected by elevation in lumenal pH (Zeuzem *et al.*, 1992; Chapman and Munro, 1994). We reasoned that if the sorting mechanisms that target αβIi complexes to the endocytic pathway were perturbed by an elevation of pH due to the action of concanamycin B, a block at any stage of transfer should result in accumulation of transport intermediates upstream of the block. Our observations on class II trafficking in cells exposed to concanamycin B establish the existence of two pathways for class II molecules to the endocytic pathway: a minor route via the cell surface, and a major pathway directly from the TGN to MIIC.

Results

Concanamycin B inhibits the appearance of class II SDS-stable dimers and prevents dissociation of Ii from class II molecules

The Epstein–Barr virus (EBV)-transformed B cell line HOM 2 (DR1 homozygous) was used throughout this study. Pulse–chase analysis of HOM 2 was carried out in the presence or absence of 20 nM concanamycin B (Figure 1A). MHC class II molecules were analyzed by immunoprecipitation with Tü36, a monoclonal antibody (mAb) specific for DRαβ and αβIi complexes but not reactive with free α, β or Ii subunits (Shaw *et al.*, 1985; Bijlmakers *et al.*, 1994a). A variable proportion of class II αβ heterodimers loaded with peptide adopts a conformation that allows them to resist dissociation by SDS at room temperature but not at 95°C (Springer *et al.*, 1977; Germain and Hendrix, 1991; Neefjes and Ploegh, 1992; Stern and Wiley, 1992). In control cells, αβ heterodimers resistant to SDS accumulate with time, whereas treatment with concanamycin B inhibits their emergence (Figure 1A). In control cells, a transient increase in class II-associated Ii is observed, attributable to the association of pulse-labeled Ii, present in molar excess over unlabeled class II molecules which continue to be synthesized during the chase (Kvist *et al.*, 1982; Machamer and Cresswell, 1982). At later chase points, the amount of Ii decreases, reflecting its destruction and dissociation from class II αβ heterodimers. In contrast, in concanamycin B-treated cells the amount of Ii recovered by Tü36 increases with time, and Ii remains associated with class II even at later chase points, suggesting that dissociation of Ii is impaired, if not blocked completely. A polypeptide of ~12 kDa derived from Ii is recovered in association with αβ complexes after 4 h of chase in concanamycin B-treated cells, suggesting that some proteolysis of Ii does occur (Figure 1A). [Under certain experimental conditions (cells pretreated and labeled in 20 nM concanamycin B for 60 min then chased for 6 h) the effects of concanamycin B can be reversed following extensive washes. Both intact Ii and the 12 kDa derived polypeptide are degraded and class II SDS-stable dimers can be detected; Villadangos and H.L.Ploegh, unpublished observations.] Failure to convert class II α and β chains to SDS-stable dimers in the presence of concanamycin B most likely results from a prolonged association of class II molecules with Ii. Acquisition of complex type N-linked glycans by DR α and β chains is observed both in the presence and absence of concanamycin B, as judged by their reduced mobility in SDS–PAGE (Figure 1A), conversion to endoglycosidase H (endo H) resistance (data not shown), and the presence of sialic acids (see Figure 5). A titration of concanamycin B revealed nearly complete inhibition of the emergence of SDS-stable dimers at 2 nM (data not shown). For our subsequent experiments 20 nM of concanamycin B was used.

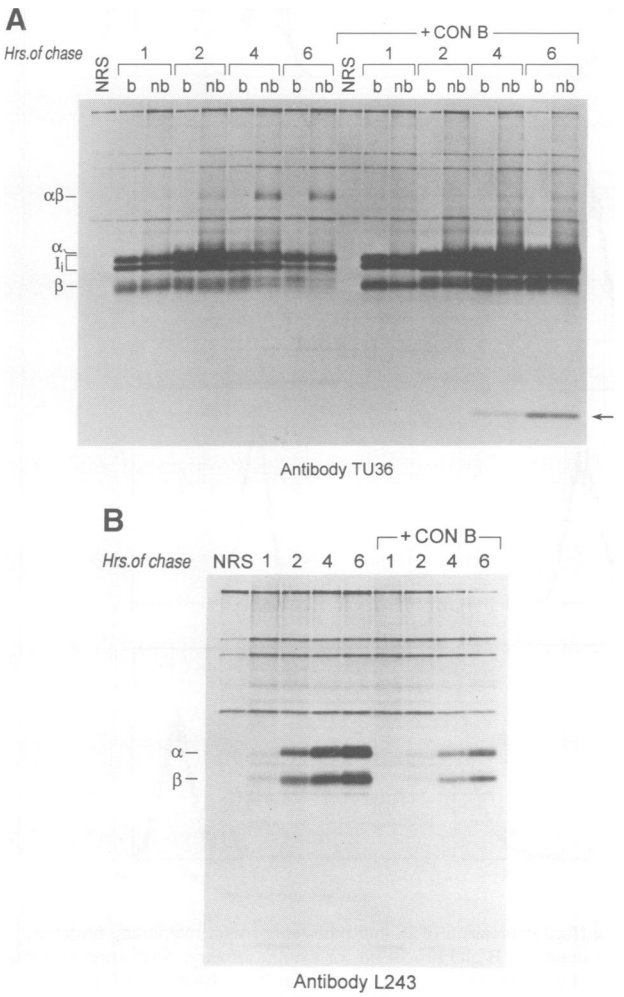
Concanamycin B delays the appearance and reduces the expression of the LB3.1/L243 epitope on class II molecules

As there is considerable variation in the recovery of SDS-resistant dimers between experiments (Neefjes and Ploegh, 1992), we applied additional criteria to score for class II maturation. LB3.1 and L243 are two mAbs specific for similar epitopes expressed by DRαβ complexes devoid of intact Ii (Bijlmakers *et al.*, 1994a). Analysis of L243 immunoprecipitates reveals that concanamycin B induces a reduction of, and a delay in, the expression of this epitope (Figure 1B) (similar results were obtained when the LB3.1 mAb was used, data not shown). At 2 h of chase, recovery of class II polypeptides by L243 is minimal in concanamycin B-treated cells, with a concomitant increase in Tü36-reactive complexes (see Figure 1A). The reciprocal situation applies to control cells. These results, too, indicate a prolonged association of class II molecules with Ii in concanamycin B-treated cells, thus preventing both the expression of the LB3.1/L243 epitope and the emergence of SDS-stable αβ dimers.

Differential proteolytic processing of Ii in concanamycin B- and leupeptin-treated cells

Leupeptin is a thiol protease inhibitor that interferes with Ii breakdown (Blum and Cresswell, 1988), with peptide

loading of class II molecules, and with egress of class II molecules to the cell surface (Neefjes and Ploegh, 1992; Zachgo *et al.*, 1992). We compared the effects induced by concanamycin B and by leupeptin on Tü36-reactive material (Figure 1c). Tü36 immunoprecipitates were boiled in SDS and reimmunoprecipitated with ViC-Y1 (anti-Ii cytoplasmic tail) or with polyclonal sera against α and β chains to allow unambiguous identification of the relevant subunits and possible breakdown products (Figure 1C). In concanamycin B-treated cells, breakdown of Ii is strongly inhibited, but a small Ii-derived fragment of ~12 kDa is consistently observed (Figure 1A and C). Ii polypeptides, free of class II molecules and reactive with either Bü45 or ViC-Y1 were also detected (data not shown). In cells treated with leupeptin, inhibition of conversion to SDS-stable dimers and co-immunoprecipitation of Ii-derived polypeptides or LIP (leupeptin induced polypeptide) (Blum and Cresswell, 1988) is seen (Neefjes and Ploegh, 1992). The main LIP fragment is ~22 kDa, while a smaller fragment of ~11 kDa (migrating just ahead of that induced by concanamycin B) is also present, but in lesser amounts.



Prolonged exposure to concanamycin B induces cell surface expression of the invariant chain and other endosomal constituents

Human EBV-transformed B cell lines constitutively express low levels of Ii at their cell surface (Lotteau *et al.*, 1990; Wraight *et al.*, 1990; Koch *et al.*, 1991). Surface expression of Ii was confirmed by cytofluorimetric analysis of HOM 2 cells (Figure 2A) and a variety of other human B cell lines (data not shown). In cells treated with increasing concentrations of concanamycin B for 20 h before FACS analysis, exposure to the inhibitor strongly induced expression of the Bü45 epitope which is located in the luminal portion of Ii (Figure 2A). This induction was dose dependent and already maximal at 2 nM. These concentrations parallel those found to be inhibitory for the formation of SDS-stable dimers and the appearance of the 12 kDa Ii polypeptide (data not shown). The effect of concanamycin B on expression of the Bü45 epitope as assayed cytofluorimetrically was slow: it was apparent at 5 h of exposure, further increased at 24 h and was only slightly stronger at 44 h (data not shown). In contrast, leupeptin even after 24 h did not induce any cell surface expression of Ii as determined by staining with Bü45 (Figure 2B). Similar effects were observed when three other B cell lines expressing different class II alleles (DR7, DR6 and DR1) were exposed to either drug (data not shown). The use of mAb LN-2 (another mAb also directed against the luminal region of Ii; Epstein *et al.*, 1984), gave the same result as seen for Bü45 (data not shown). As expected, staining with ViC-Y1 (anti-Ii

Fig. 1. Concanamycin B blocks Ii breakdown and inhibits the appearance of class II SDS-stable dimers. (**A**) HOM 2 cells were pulse-labeled with [^{35}S]methionine for 15 min and chased in the presence or absence of 20 nM concanamycin B (A–C), or in the presence of 1 mM leupeptin (C), for the times indicated. Class II molecules were immunoprecipitated with the mAb Tü36 (reactive with αβ dimers and αβIi complexes) and analyzed on SDS–PAGE after incubation in SDS at 95°C (b, boiled), or at room temperature (nb, non-boiled), to visualize αβ heterodimers. NRS, normal rabbit antiserum control. (←), the arrow indicates the 12 kDa product derived from Ii in the presence of concanamycin B. (**B**) Class II molecules were immunoprecipitated with mAb L243, specific for αβ heterodimers not associated with Ii, and analyzed on SDS–PAGE after incubation in SDS at 95°C. (**C**) Tü36 reactive material was boiled in 1% SDS and reimmunoprecipitated with ViC-Y1 or with rabbit sera raised against denatured α (anti-α) and β (anti-β) chains. Longer exposures of the reimmunoprecipitations are shown. LIP, leupeptin induced polypeptides; (*), concanamycin B-induced polypeptides.

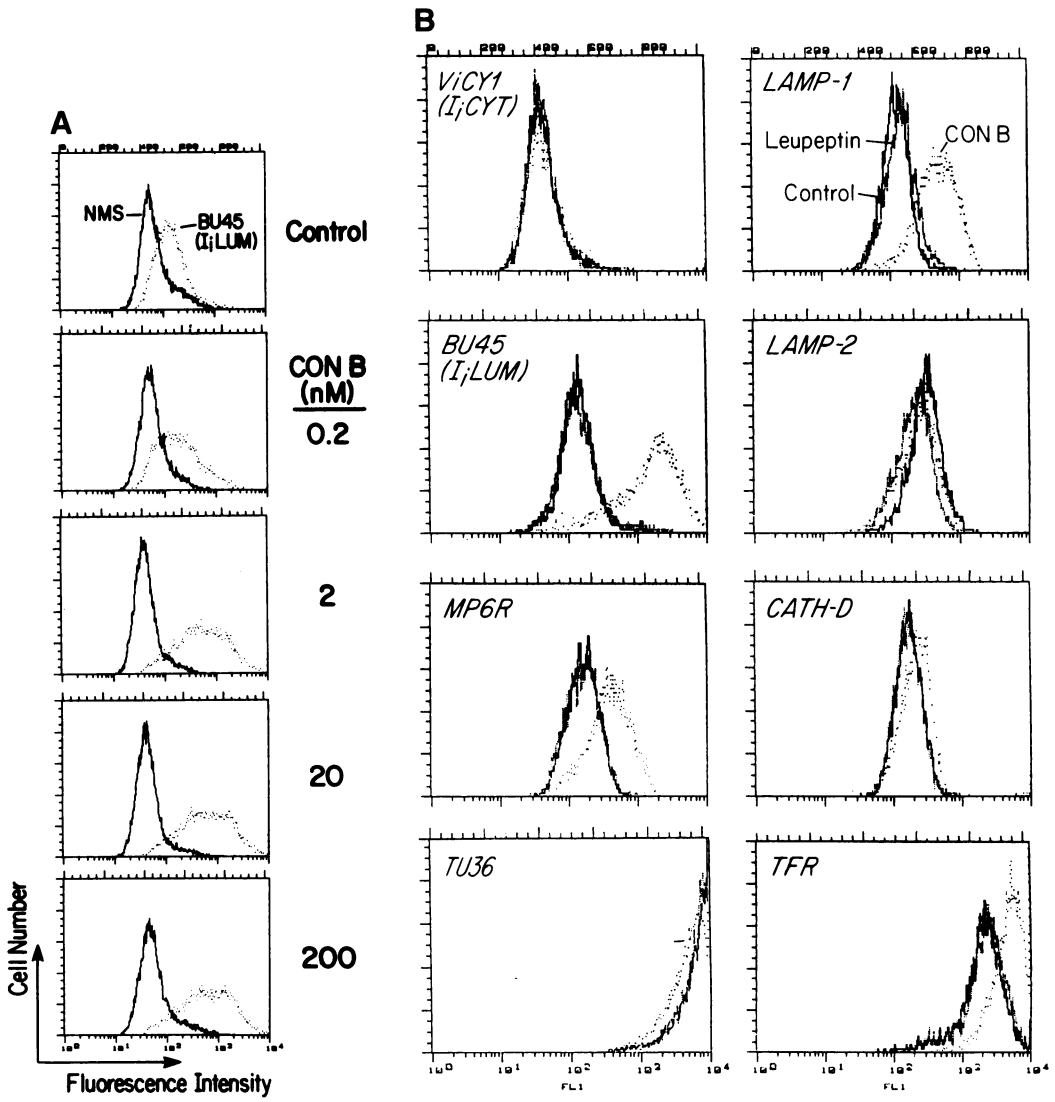


Fig. 2. Prolonged exposure to concanamycin B, but not leupeptin, induces cell surface expression of Ii, and other endosomal membrane proteins. (**A**) HOM 2 cells were treated for 20 h with the indicated concentrations of concanamycin B and processed for cytofluorimetry. NMS (normal mouse serum); Bü45 (anti-Ii luminal domain). (**B**) Cells were exposed to concanamycin B (20 nM) or leupeptin (1 mM) for 24 h. Bü45 (anti-Ii luminal domain); ViC-Y1(anti-Ii cytoplasmic tail); M6PR (anti-mannose-6-phosphate receptor); Tü36 (anti-class II); LAMP-1 and LAMP-2 (anti-lysosomal associated membrane proteins 1 and 2); CATH-D (anti-cathepsin D); Tfr (anti-transferrin receptor).

cytoplasmic tail) performed as a control gave a negative result for all conditions tested (Figure 2B).

We also examined by cytofluorimetry the effects of concanamycin B and leupeptin on other proteins in the endocytic pathway (Figure 2B). We found that control, concanamycin B-treated, or leupeptin-treated cells were stained to similar intensities for lysosomal-associated membrane protein 2 (LAMP-2) and for cathepsin D, the surface expression of which could be due to some adsorption of secreted enzyme. In contrast, we observed a strong increase in surface expression of the mannose-6-phosphate receptor (M6PR), the transferrin receptor (Tfr) and the lysosomal-associated membrane protein 1 (LAMP-1) in cells exposed to concanamycin B. Tü36-reactive class II molecules were reduced somewhat in the presence of concanamycin B (Figure 2B). Leupeptin-treated cells did not differ from control cells for any of the antibodies tested. We conclude that a number of membrane proteins usually associated with the endocytic pathway are represented at the cell surface at increased levels in the presence of concanamycin B.

αβ*Ii complexes reside at the surface of concanamycin B-treated cells*

Because expression of Ii in untreated cells was minimal as assessed by cytofluorimetry (Figure 2, control), we could use surface iodination to verify the expression of Ii on the cell surface in concanamycin B-treated cells. When intact cells were surface-labeled by iodination, abundant class II expression in control, concanamycin B-treated and leupeptin-treated cells was observed (Figure 3A, antibody Tü36). This is the result of contributions of pre-existing class II molecules (which have a long half-life; Davis and Cresswell, 1990) as well as any insertion of new molecules following addition of inhibitors. When Tü36 immunoprecipitates were analyzed under mildly denaturing conditions, SDS-stable dimers which must consist of pre-existing class II–peptide complexes (Figure 1) were

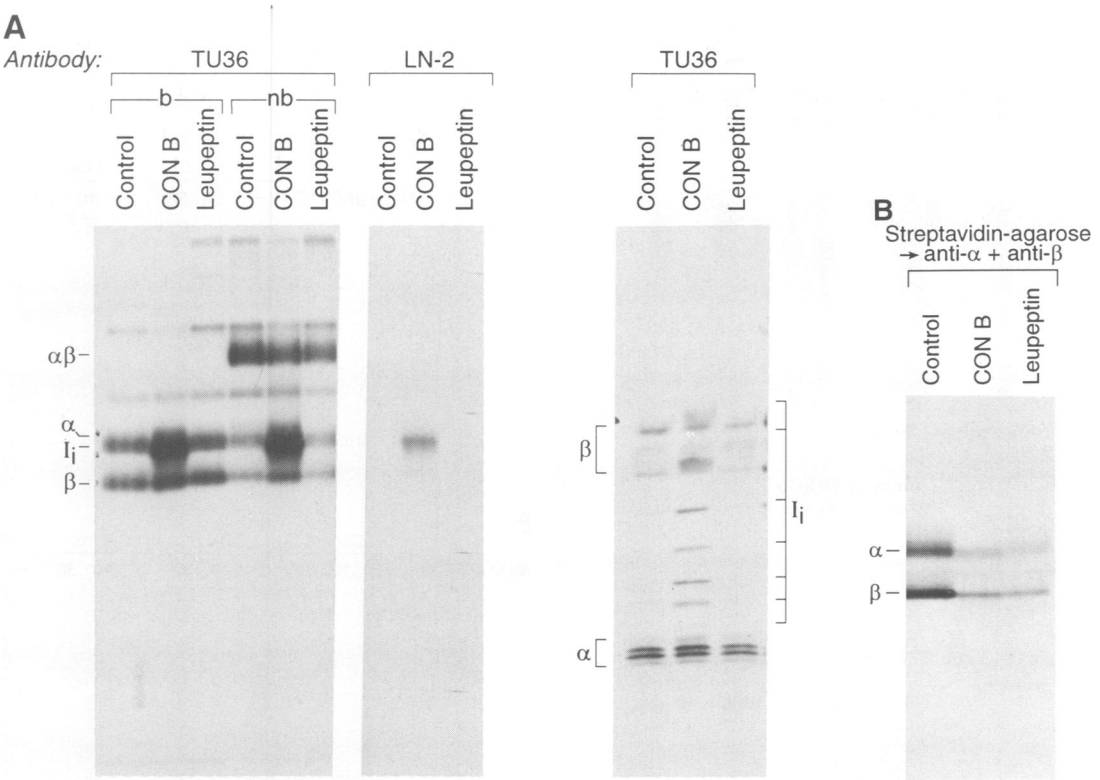


Fig. 3. Concanamycin B induces Ii at the cell surface in association with αβ: after prolonged exposure to the inhibitor (**A**) and reduces surface expression of newly synthesized αβ complexes (**B**). (A) HOM 2 cells treated for 24 h with or without inhibitors were surface iodinated as described in Materials and methods. Total cell lysates were immunoprecipitated in parallel with mAbs Tü36 or with LN-2 (anti-Ii luminal domain). Tü36 reactive material was analyzed on SDS−PAGE after incubation in SDS at 95°C (b, boiled), or at room temperature (nb, non-boiled). One-dimensional IEF analysis of the Tü36 precipitates is shown in the right panel. (B) Cells were pretreated with or without inhibitors for 2 h, pulse labeled with [^{35}S]methionine for 20 min, and chased for 24 h as in the pretreatment. Surface biotinylated products from total cell lysates were recovered on streptavidin−agarose, boiled in 1% SDS and immunoprecipitated with anti-α and anti-β sera.

observed for all three conditions. Mild sample denaturation allows us to remove the SDS-stable αβ dimers from the region of the gel where Ii migrates. The unique presence of the latter at the surface of concanamycin B-treated cells is easily visualized, as verified also by one-dimensional IEF (Figure 3A). Some free Ii was detected at the surface of concanamycin B-treated cells, both by immunoprecipitation with ViC-Y1 (data not shown), and the anti-Ii luminal antibody, LN-2 (Figure 3A). It may derive from the small fraction of Ii homotrimers that successfully escape the endoplasmic reticulum (Cresswell, 1994).

Concanamycin B reduces surface expression of newly synthesized αβ complexes

Because iodination does not discriminate between pre-existing class II molecules, and those inserted after imposition of an inhibitor block, we used surface biotinylation on [^{35}S]methionine labeled cells to explore accessibility at the cell surface of newly synthesized class II molecules. Cells were pulse-labeled with [^{35}S]methionine, and chased in the presence of leupeptin or concanamycin B for 24 h. Following surface biotinylation of intact cells, the biotinylated proteins were recovered on streptavidin−agarose (equivalent amounts of trichloroacetic acid-precipitable radioactivity were used). Proteins were eluted from streptavidin−agarose beads by boiling in SDS and, following dilution, immunoprecipitated with rabbit anti-α and anti-β antibodies (Figure 3B).

We found that unlike class II molecules (Figure 3B), biotinylation of Ii was inefficient (data not shown). Both leupeptin and concanamycin B strongly inhibited surface deposition of class II molecules compared with untreated cells, but did not completely block it, as assessed by this method. We therefore conclude that the bulk of newly synthesized αβ heterodimers and associated Ii are routed to an intracellular compartment, and do not accumulate at the surface of leupeptin-treated cells, in agreement with an earlier report (Neefjes and Ploegh, 1992), nor as shown here, in concanamycin B-treated cells. This leads us to conclude that the emergence of αβIi complexes at the cell surface of concanamycin B-treated cells as detected by surface iodination must be a slow process, representing a minor pathway, and confirms our cytofluorimetric observations.

Access of newly synthesized molecules in concanamycin B-treated cells to neuraminidase at 0 and 37°C

Neuraminidase digestion experiments at 0°C (Neefjes *et al.*, 1990) were also carried out to score for newly synthesized molecules at the cell surface of drug-treated cells. Cells were chased for 5 or 24 h in the presence or absence of concanamycin B or leupeptin. Sequential immunoprecipitations of Tfr, MHC class I and MHC class II molecules were carried out and analyzed by one-dimensional IEF. Those molecules that reside at the cell

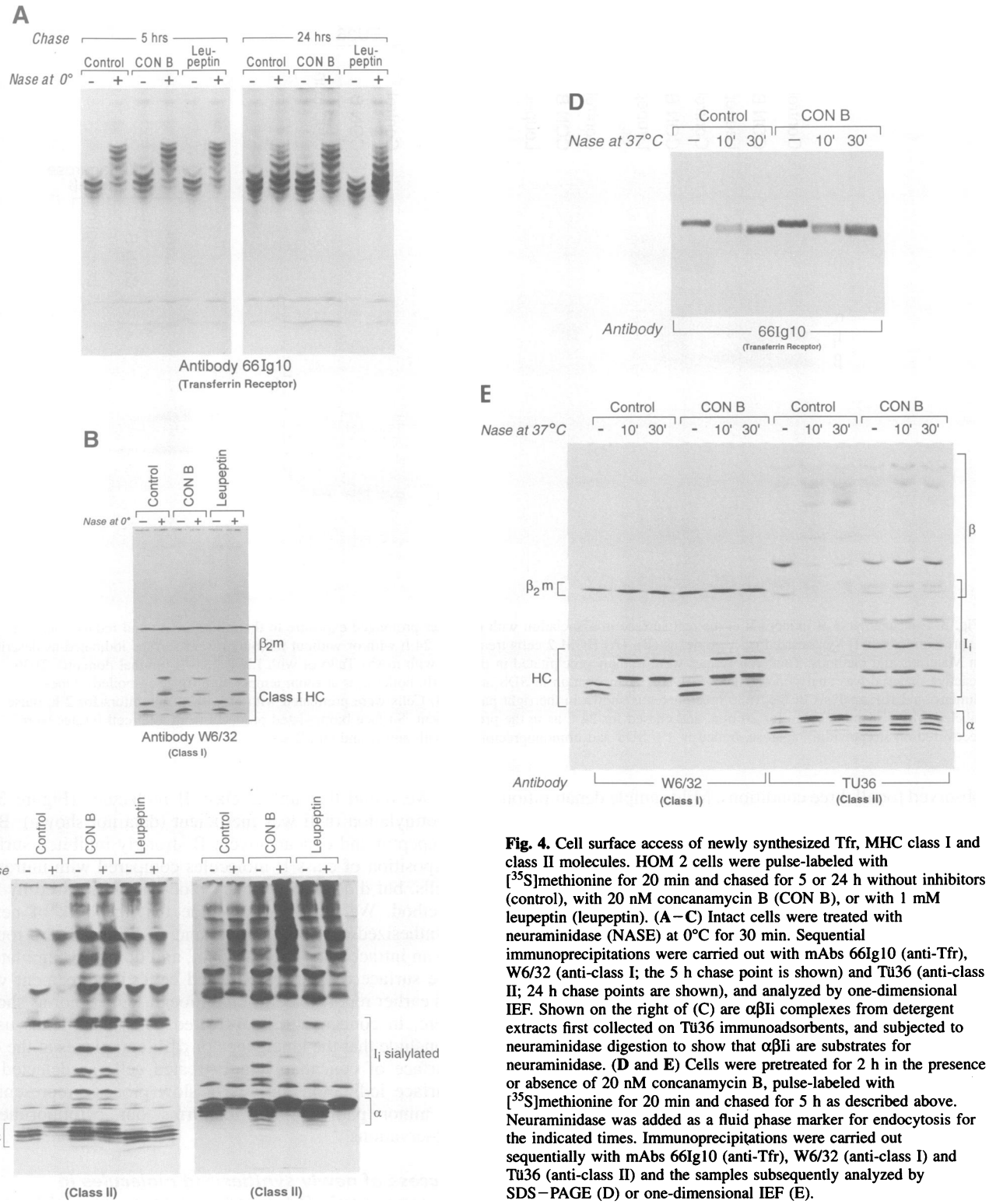


Fig. 4. Cell surface access of newly synthesized Tfr, MHC class I and class II molecules. HOM 2 cells were pulse-labeled with [^{35}S]methionine for 20 min and chased for 5 or 24 h without inhibitors (control), with 20 nM concanamycin B (CON B), or with 1 mM leupeptin (leupeptin). (**A**–**C**) Intact cells were treated with neuraminidase (NASE) at 0°C for 30 min. Sequential immunoprecipitations were carried out with mAbs 66Ig10 (anti-Tfr), W6/32 (anti-class I; the 5 h chase point is shown) and Tü36 (anti-class II; 24 h chase points are shown), and analyzed by one-dimensional IEF. Shown on the right of (C) are αβIi complexes from detergent extracts first collected on Tü36 immunoadsorbents, and subjected to neuraminidase digestion to show that αβIi are substrates for neuraminidase. (**D** and **E**) Cells were pretreated for 2 h in the presence or absence of 20 nM concanamycin B, pulse-labeled with [^{35}S]methionine for 20 min and chased for 5 h as described above. Neuraminidase was added as a fluid phase marker for endocytosis for the indicated times. Immunoprecipitations were carried out sequentially with mAbs 66Ig10 (anti-Tfr), W6/32 (anti-class I) and Tü36 (anti-class II) and the samples subsequently analyzed by SDS–PAGE (D) or one-dimensional IEF (E).

surface will be sensitive to neuraminidase at 0°C, and undergo a shift in isoelectric point. Sialic acid-bearing glycoproteins that are cell-internal will be refractory to such treatment, and do not undergo a shift in isoelectric point. At 5 h of chase, there was no change in the amount of Tfr exposed at the surface (neuraminidase-sensitive) for all conditions tested (Figure 4A, antibody 66Ig10). Because the distribution of Tfr over the surface (neuraminidase-sensitive) and cell-internal (neuraminidase-resistant) fractions is controlled by recycling (Neefjes *et al.*, 1990), we conclude that concanamycin B does not inhibit the constitutive recycling of Tfr. By 24 h of chase, a shift in favor of surface-disposed Tfr in concanamycin B-treated cells was observed, as determined by the fraction of molecules now accessible to neuraminidase at the cell surface. This increase in Tfr at the plasma membrane

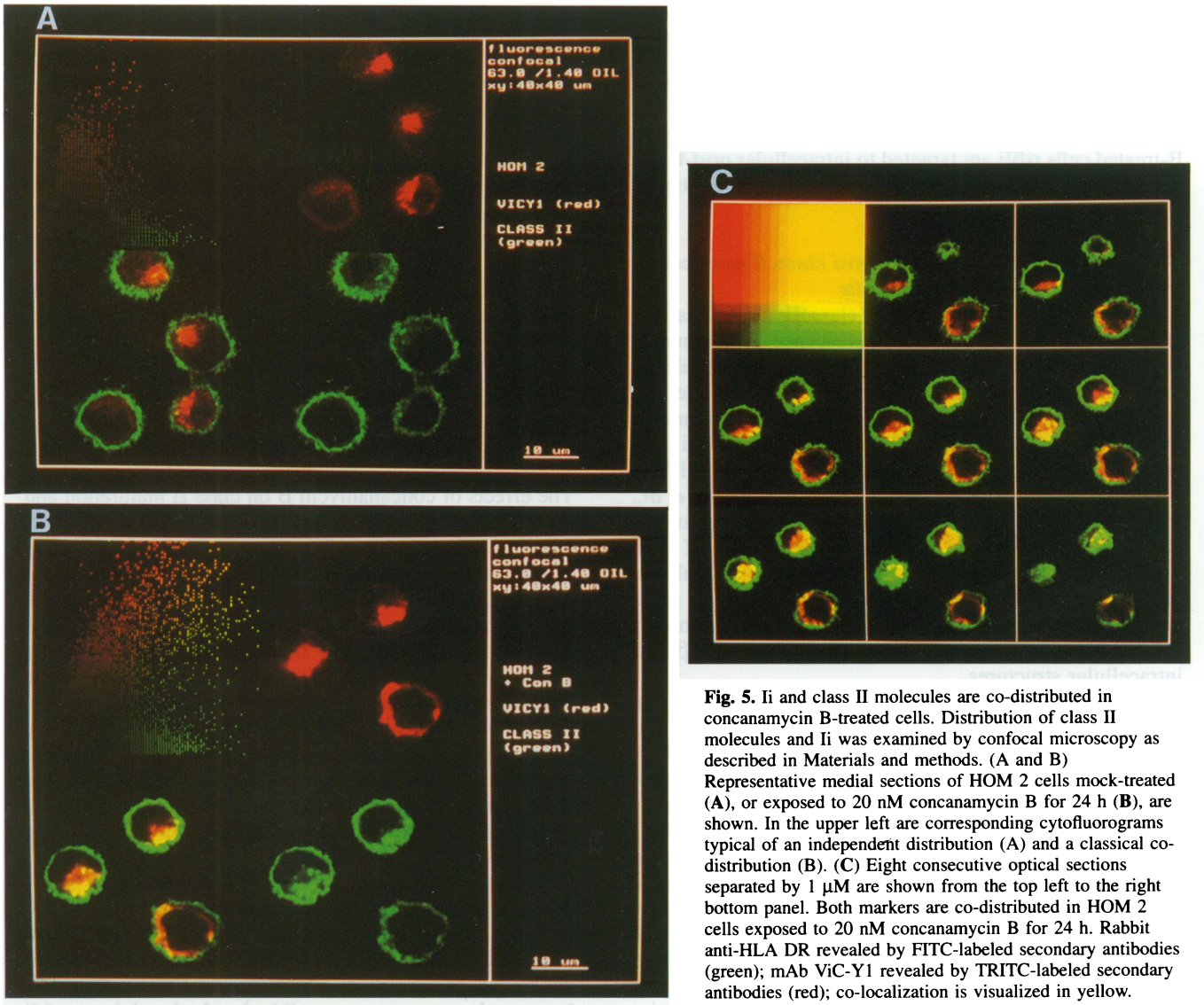


Fig. 5. Ii and class II molecules are co-distributed in concanamycin B-treated cells. Distribution of class II molecules and Ii was examined by confocal microscopy as described in Materials and methods. (A and B) Representative medial sections of HOM 2 cells mock-treated (**A**), or exposed to 20 nM concanamycin B for 24 h (**B**), are shown. In the upper left are corresponding cytofluorograms typical of an independent distribution (A) and a classical co-distribution (B). (**C**) Eight consecutive optical sections separated by 1 μM are shown from the top left to the right bottom panel. Both markers are co-distributed in HOM 2 cells exposed to 20 nM concanamycin B for 24 h. Rabbit anti-HLA DR revealed by FITC-labeled secondary antibodies (green); mAb ViC-Y1 revealed by TRITC-labeled secondary antibodies (red); co-localization is visualized in yellow.

agreed with the cytofluorimetric data (Figure 2B). Deposition of class I heavy chain at the surface was observed, as expected for a protein traversing the secretory pathway (Figure 4B, antibody W6/32), and was not affected by the presence of leupeptin or concanamycin B. However, the rate with which class I molecules reached the cell surface was somewhat slower in concanamycin B-treated cells (data not shown; Yilla *et al.*, 1993).

Neither Tfr nor class I molecules show any obvious alterations in their surface deposition in cells exposed to concanamycin B for 5 h. In contrast biosynthetically labeled class II molecules were largely resistant to neuraminidase treatment at 0°C in concanamycin B- and leupeptin-treated cells (Figure 4C, antibody Tü36), in accord with an earlier study using leupeptin (Neefjes and Ploegh, 1992). This is best seen by comparing the migration of the class II α chains, and for concanamycin B-treated cells, Ii, because the class II β chains do not focus as sharp bands in our equilibrium pH gradient. This result must be due to the cell-internal disposition of biosynthetically labeled class II molecules, and not to an intrinsic resistance of αβIi complexes to neuraminidase digestion: purified αβIi complexes are readily digested by neuraminidase (Figure 4C, antibody Tü36).

When newly synthesized molecules were exposed to neuraminidase at 37°C in concanamycin B-treated cells, accessibility of Tfr (Figure 4D, antibody 66Ig10) and surface-disposed class I heavy chain (Figure 4E, antibody W6/32) was essentially complete (we often observe poor focusing of Tfr, so SDS−PAGE analysis is shown). We detected trace amounts of neuraminidase-resistant class I molecules in concanamycin B-treated cells (compare with control) which we attribute to their presence in late endocytic/lysosomal compartments, where their breakdown is inhibited, owing to the elevated pH imposed by the action of concanamycin B. However, access of the majority of class II molecules to the endocytic tracer was strongly impaired (Figure 4E, antibody Tü36), and only a small fraction of αβIi was digested. The cell-internal fraction of αβIi molecules could not be accessed even after 60 min of neuraminidase treatment (data not shown; see also Figure 6ii, C). Note that the addition of sialic acids to newly synthesized Tfr, class I and class II molecules is essentially complete in concanamycin B-treated cells. We have observed drastic effects of concanamycin B on sialic acid addition to α1-antitrypsin in the hepatoma cell line HepG2 (Yilla *et al.*, 1993). While we usually observe a slight reduction in sialylation of MHC

products and Tfr in concanamycin B-treated B cells, we attribute this difference to the relative abundance of α1-antitrypsin and to the fact that it is a secretory protein, as contrasted with the less abundant and surface-disposed Tfr and MHC products. We conclude that in concanamycin B-treated cells αβIi are targeted to intracellular post-Golgi compartments which have restricted access to endocytic markers.

Subcellular distribution of Ii and class II molecules in concanamycin B-treated cells

The intracellular distribution of class II molecules was analyzed by double immunofluorescence and confocal microscopy (Figure 5). As expected, class II molecules in untreated HOM 2 cells were found mainly at the cell surface, while the Ii cytoplasmic epitope was found in intracellular compartments (Figure 5A). Some Ii was detectable at the cell surface, in agreement with our FACS data (Figure 2) and with previous reports (Lotteau *et al.*, 1990; Wraight *et al.*, 1990). Examination of concanamycin B-treated cells revealed a major redistribution of both markers: co-distribution of Ii and class II molecules is greatly enhanced, found mostly in intracellular small vesicles (Figure 5B and C). We conclude that concanamycin B treatment leads to an accumulation of αβIi in intracellular structures.

Examination by immuno-electron microscopy on ultrathin frozen sections provided further support for our biochemical observations (Figure 6). In control cells Ii could be detected only in the Golgi area when using the anti-Ii luminal antibody ICC5 (a rabbit polyclonal anti-peptide serum), and was virtually absent from MIIC (Figure 6i). However, in concanamycin B-treated cells, abundant staining with the ICC5 antibody was observed not only in the Golgi area, but also in MIIC, easily recognized by their high class II content and characteristic multi-vesicular appearance (Figure 6ii, A; Table I), and at the cell surface (data not shown). BSA–gold conjugates were used to examine access of the class II compartment to this endocytic tracer. In accordance with our observation that αβIi in concanamycin B-treated cells is largely resistant to neuraminidase as a fluid phase marker, no co-localization of BSA–gold with class II was observed after a 10 min pulse, followed by a 20 min chase of the conjugate (Figure 6ii, A). Some co-localization could already be observed in control cells after 10 min (Figure 6ii, B). After 80 min of chase, co-localization of BSA–gold with class II and Ii was seen in concanamycin B-treated cells (Figure 6ii, C), but this was significantly less than that detected in control cells (Figure 6ii, D). The number of BSA–gold particles after a 10 min pulse, followed by a 50 min chase, was quantified, and it was found that 65% of all MIIC were positive for BSA–gold in untreated cells, whereas in concanamycin B-treated cells only 5% of the MIIC contained the endocytic tracer, in good agreement with the biochemical experiment shown in Figure 4E (data not shown). The increase in staining intensity observed with the ICC5 antibody in concanamycin B-treated cells is entirely consistent with the near complete inhibition of Ii breakdown, as seen biochemically. In general, the appearance of MIIC in concanamycin B-treated cells was more electron-lucent, and the multivesicular structures present themselves in a less compact manner than seen in control cells (Neefjes *et al.*, 1990; Peters *et al.*, 1991). It has been argued that the presence of low molecular weight protein breakdown fragments is responsible for the electron-dense appearance of lysosomes (Peters *et al.*, 1991). Because concanamycin B impairs lysosomal function, less of these breakdown products are expected to be present, and could contribute to a more translucent appearance. In addition, a number of lysosomal membrane constituents have a strong net negative charge at neutral pH (Lippincott-Schwartz and Fambrough, 1986). In the absence of an active vacuolar H^+-ATPase, electrostatic repulsion could prevent close membrane apposition, as would be possible at the low pH under normal conditions, when the negative charge of lysosomal membrane proteins would be counteracted at acidic pH.

Discussion

The effects of concanamycin B on class II maturation and trafficking of Ii are profound. Breakdown of Ii is inhibited, and the vast majority of Ii remains associated with class II αβ heterodimers, thus preventing the emergence of peptide-bound αβ dimers. There is abundant surface expression of intact Ii in association with class II in cells exposed to concanamycin B, as assessed biochemically and cytofluorimetrically, but prolonged exposure to the inhibitor is required to observe this effect. Nevertheless, pulse-labeled class II αβIi are resistant to attack by neuraminidase as a fluid phase marker. We therefore conclude that the bulk of class II molecules is delivered directly from the secretory pathway to the MHC class II compartment, MIIC, and that direct transfer of αβIi to the cell surface is a minor pathway.

Vacuolar H^+-ATPase inhibitors abrogate proton translocation into the membrane compartments in which the enzyme resides, impair lumenal acidification and, consequently, the activity of enzyme systems that require an acidic environment. These include the endosomal/lysosomal proteases, responsible for the breakdown of Ii and the generation of antigenic peptides. The inhibition of class II maturation (Figure 1) is related to these indirect effects of concanamycin B on proteolysis. Not all proteolysis would cease at neutral pH nor is inhibition of acidification necessarily complete. Indeed, pulse–chase experiments revealed that a class II-associated short polypeptide (~12 kDa) derived from the N-terminus of Ii accumulates in concanamycin B-treated cells. The size of this fragment is close to that of the minimal Ii segment (residues 1–104) capable of interacting with class II molecules translated *in vitro* (Bijlmakers *et al.*, 1994b), and is likely to contain the CLIP region (Chicz *et al.*, 1992; Riberdy *et al.*, 1992). This short fragment of Ii (residues 1–28) carries the ViC-Y1 epitope (Quaranta *et al.*, 1984) and the transmembrane region; both regions contain signals required for targeting (Bakke and Dobberstein, 1990; Lotteau *et al.*, 1990; Pieters *et al.*, 1993; Odorizzi *et al.*, 1994). In concanamycin B-treated cells, the 12 kDa fragment also carries the ViC-Y1 epitope (Figure 1C) and must therefore contain the targeting signal.

Of considerable interest, though less well understood, is the effect of vacuolar H^+-ATPase inhibitors on membrane trafficking. In yeast, disruption of the vacuolar H^+-ATPase gene causes missorting of some, but not all hydrolases to

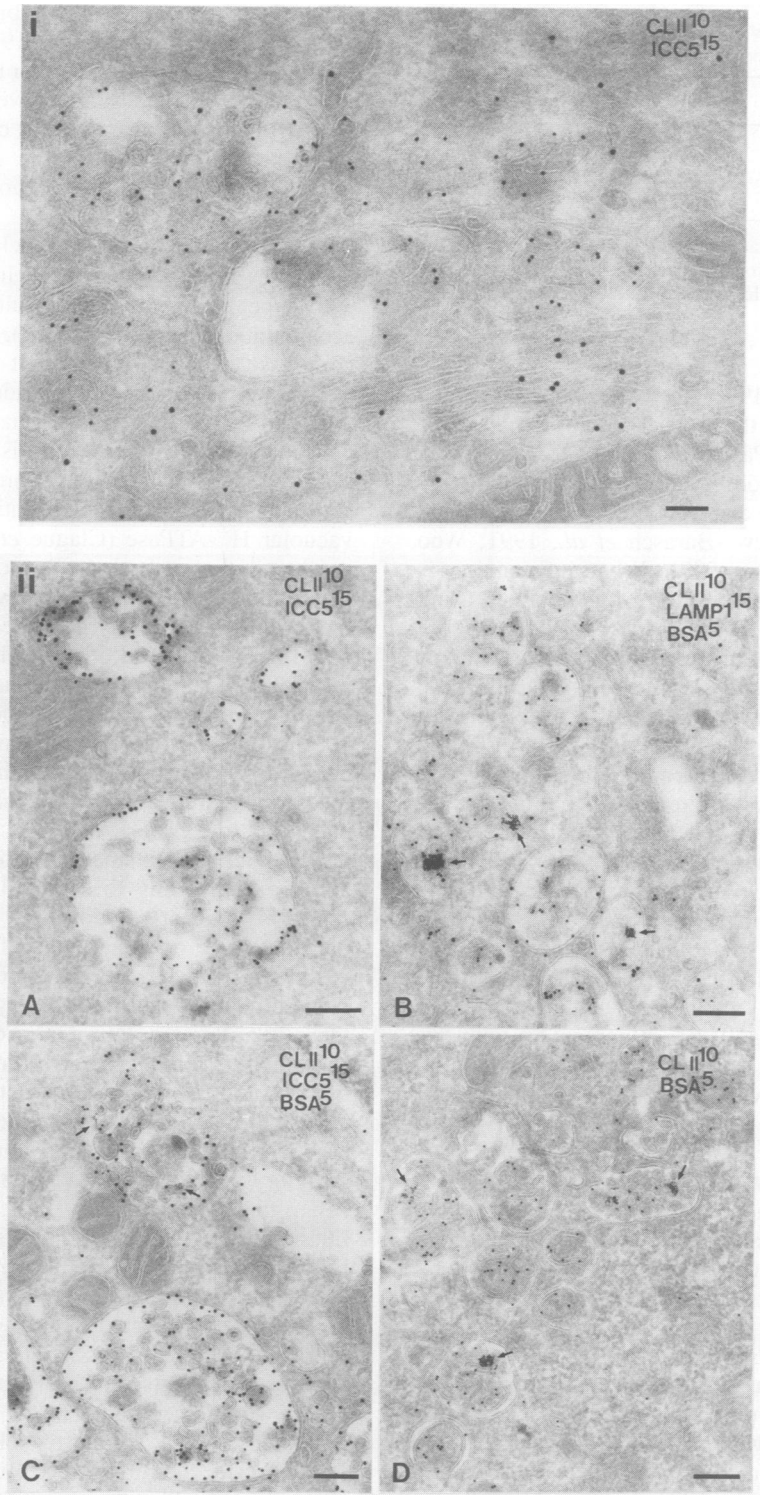


Fig. 6. Immuno-electron microscopical analysis of concanamycin B-treated cells. HOM 2 cells were treated with or without concanamycin B for 15 h, subsequently fixed and ultrathin cryosections prepared for immuno-gold labeling as described. Sections were labeled with ICC5 (anti-Ii luminal domain), Cl II (anti-class II) or LAMP-1 (anti-LAMP-1). (**i**) Control cells showing Ii staining of Golgi (15 nm gold particles) and class II staining of MIIC (10 nm gold particles). (**ii**) Cells were pulsed for 10 min with 5 nm BSA–gold particles and chased for either 20 min (A and B) or for 80 min (C and D). (A) Concanamycin B-treated cells showing co-localization of Ii (15 nm gold particles) and class II (10 nm gold particles) in the MIIC. No BSA–gold staining is detected. (B) Control cells showing that MIIC, revealed by class II (10 nm gold particles) is LAMP-1 (15 nm gold particles) positive, and that endosomes are accessed by 5 nm BSA–gold particles (see arrows). (C) Concanamycin B-treated cells showing slight co-localization of 5 nm BSA–gold particles in MIIC, revealed by class II (10 nm gold particles) and Ii (15 nm gold particles). (D) Control cells showing abundant staining by 5 nm BSA–gold particles of the MIIC, revealed by class II (10 nm gold particles).

Table I. Class II-positive profiles in untreated and concanamycin B-treated cells; Ii (luminal epitope) staining

Concanamycin B	−	+
Endoplasmic reticulum	382	393
Golgi	230	560
MIIC	78	2227

Quantitive analysis of intracellular Ii labeling with ICC5 (anti-Ii luminal domain) from the experiment shown in Figure 6 is given. Absolute numbers of gold particles counted from 26 cells are shown.

the vacuole (Banta *et al.*, 1988; Klionsky *et al.*, 1990, 1992; Nelson and Nelson, 1990), the yeast equivalent of the lysosome. In keeping with these observations, inhibition of the yeast vacuolar H^+-ATPase by bafilomycin A_1 produces effects similar to those observed in the vacuolar H^+-ATPase mutants (Klionsky and Emr, 1989). Data on higher eukaryotic systems are few (Barasch *et al.*, 1991; Woo *et al.*, 1992; Yilla *et al.*, 1993). What information is available should be compared with results obtained using chloroquine, ammonium chloride and the ionophore monensin, all of which require much higher concentrations for inhibitory activity and are less selective. Application of chloroquine has been shown to affect delivery of the lysosomal membrane glycoprotein, lEP100 (lgpA) in MDCK cells (Nabi *et al.*, 1991), and delivery of TGN38 and furin to the TGN following retrieval from the surface (Chapman and Munroe, 1994). Similarly, cell-free assays employing the vacuolar H^+-ATPase inhibitor bafilomycin A_1, have revealed a block in transfer of contents from early to late endosomes, by inhibiting the formation of carrier vesicles (Clague *et al.*, 1994). Bafilomycin A_1 does not inhibit Tfr internalization and recycling (W.Stoorvogel, personal communication) and our own experiments (Figure 4A, antibody 66Ig10) are in agreement with this observation.

The mechanisms by which class II molecules reach endocytic compartments are now beginning to be unraveled (Peters *et al.*, 1991; Amigorena *et al.*, 1994; Qiu *et al.*, 1994; Tulp *et al.*, 1994; West *et al.*, 1994). Morphological studies show the presence of αβIi complexes in endocytic compartments, but the static nature of immunocytochemical methods does not allow measurement of rates of transfer between compartments on the timescale at which these transfers are likely to occur in living cells. However, based on immuno-electron microscopical evidence, a direct transfer of class II molecules from the TGN to endocytic compartments was proposed (Peters *et al.*, 1991), as was put forward for M6PR, lgp molecules and for Ii−Tfr chimeric molecules (Kornfeld and Mellman, 1989; Fukuda, 1991; Harter and Mellman, 1992; Odorozzi *et al.*, 1994). The results from recent subcellular fractionation experiments are consistent with this proposition (Amigorena *et al.*, 1994; Qiu *et al.*, 1994; Tulp *et al.*, 1994; West *et al.*, 1994). An alternate pathway by which αβIi, or at least some fraction of it, makes a brief appearance at the cell surface and is then rapidly internalized to the location where class II molecules are loaded with peptide, has been postulated (Lotteau *et al.*, 1990; Roche *et al.*, 1993; Odorozzi *et al.*, 1994). Such targeting via the cell surface has been shown for other protein constituents of the endocytic pathway (Braun *et al.*, 1989; Peters *et al.*, 1990; Nabi *et al.*, 1991; Mathews *et al.*, 1992; Trowbridge *et al.*, 1993; Sandoval and Bakke, 1994). We show that it is a rather minor pathway compared with the amount of class II delivered directly to endocytic compartments. These latter compartments must be positioned downstream of the early to late endosomal transition inhibited by the vacuolar H^+-ATPase inhibitor concanamycin B.

Surface deposition of newly synthesized class II molecules is severely reduced in both concanamycin B- and leupeptin-treated cells resulting in their intracellular accumulation (see Figure 3B). The inability of neuraminidase (it was verified that the enzyme is active at neutral pH; data not shown), added in the fluid phase at 37°C, to attack newly synthesized class II molecules in concanamycin B-treated cells is entirely consistent with the suggestion that delivery from early endosomes to the subsequent (late) endocytic station(s) requires an active vacuolar H^+-ATPase (Clague *et al.*, 1994). The bulk of newly synthesized class II molecules is refractory to neuraminidase treatment, from which we infer that this is the fraction most likely delivered directly from the TGN to MIIC. Note that in contrast class II molecules retained intracellularly in leupeptin-treated cells do retain sensitivity to endocytosed neuraminidase (Neefjes and Ploegh, 1992). The immuno-electron microscopical observations are in agreement with the biochemical data and confirm the inaccessibility of MHC class II and Ii to early endocytic tracers.

It is likely that a small fraction of αβIi exits the TGN, and escapes sorting to MIIC: it reaches the cell surface directly or via early endosomes. We do not know whether the fraction of αβIi that would follow this alternate route is itself affected by neutralization of the TGN and other compartments along its pathway. We have observed that there is a delay in intra-Golgi or TGN-to-surface traffic for bona fide soluble secretory proteins, and the possibility of an acidic pH being important for proper sorting at the exit from the Golgi was raised (Yilla *et al.*, 1993). Small amounts of αβIi and Ii may thus enter the constitutive secretory pathway and reach the cell surface. In untreated cells, these αβIi and Ii molecules would be rapidly internalized by virtue of the internalization signals borne by Ii (Roche *et al.*, 1993; Odorizzi *et al.*, 1994).

In our ultrathin frozen sections, class II molecules or Ii have not been detected in early endosomes (Peters *et al.*, 1991). If vacuolar acidification is indeed required to allow transfer of contents from early to late endosomes, the αβIi and Ii would accumulate at the surface of concanamycin B-treated cells by simple recycling, as described for Tfr. If the residence time of αβIi in early endosomes is short, we would not expect to see their accumulation immunocytochemically at that location in inhibitor-treated cells. The extended times of exposure to concanamycin B required to obtain maximal Ii expression at the surface are consistent with this proposal. The increases seen in the other late endosomal constituents, LAMP-1 and M6PR could also be explained by this mechanism. Tfr recycles predominantly between early endosomes and the cell surface (Goldstein *et al.*, 1985). The increase in surface expression, and the slightly altered balance between surface-disposed and cell-internal fraction seen in long term inhibitor-treated cells, must be the consequence of

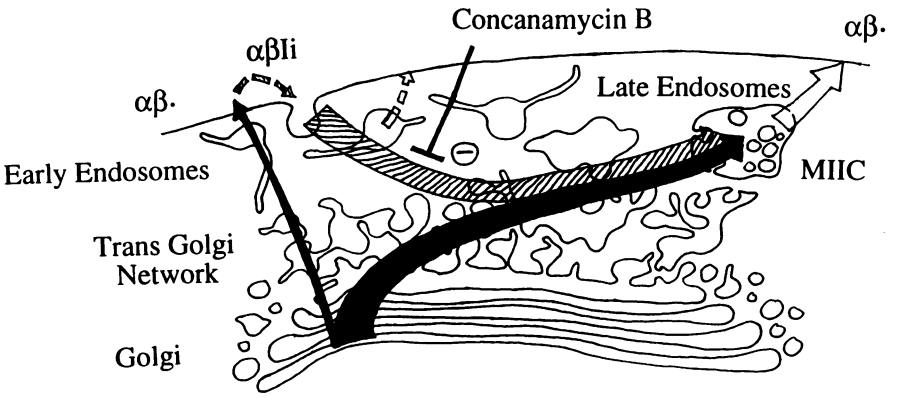


Fig. 7. Trafficking of class II molecules to the endocytic pathway. The bulk of newly synthesized αβIi is targeted directly to the compartment for peptide loading of class II molecules (MIIC), possibly via late endosomes, from the *trans*-Golgi network (large solid arrow). A small fraction of αβIi is delivered to the cell surface (small solid arrow) from which it rapidly enters the endocytic pathway (large hatched arrow). The concanamycin B-imposed block between early and late endosomes accounts for the persistence of this fraction at the cell surface of inhibitor-treated cells. A small fraction of αβ heterodimers may be loaded with peptide in the endoplasmic reticulum (αβ.), and is deposited at the cell surface directly (small solid arrow). The majority of peptide-loaded class II molecules (αβ.) reaches the surface from the MIIC (open arrow).

failure of a fraction of Tfr to be delivered to late endosomes and subsequent compartments, where in untreated cells they would be destroyed. Instead, in concanamycin B-treated cells they remain in the Tfr recycling circuit. By increasing total Tfr content in this loop, the balance in cell-surface versus cell-internal Tfr is altered.

Under normal conditions, Tfr, cation-independent M6PR and LAMP-1 are reasonably stable and survive endosomal conditions. Not so Ii: its destruction is a prerequisite for allowing class II αβ heterodimers to bind peptide. The combined effects of neutralization of endosomal compartments on protease activity and trafficking of αβIi thus contribute to the increase in Ii observed at the surface of concanamycin B-treated cells.

We propose the following scheme for class II trafficking in human B cells, based on the present work and earlier data (Figure 7). The majority of αβIi complexes that exit the endoplasmic reticulum is delivered to MIIC directly via the Golgi and TGN, and possibly late endosomes. A small fraction of αβIi and Ii escape this sorting and reach the cell surface, from where they are rapidly internalized and delivered to endocytic compartments. Inhibition of endosomal acidification blocks only this latter aspect of class II trafficking in preventing access of material in early endosomes to later endocytic structures. Formally, we cannot distinguish between delivery of surface-disposed αβIi from the TGN to the surface, or from endocytic compartments, in particular MIIC downstream of the concanamycin B block, to explain accumulation of αβIi at the cell surface (see Figure 7). Any contribution of the latter mode of insertion of αβIi at the cell surface would obviously be at the expense of the TGN-to-surface route, and shift the balance even further in favor of direct trafficking of αβIi to the endocytic pathway. Class II αβ heterodimers may also associate with peptide in the ER, fail to assemble with Ii as a consequence (Bijlmakers *et al.*, 1994a), and be transported to the cell surface directly. Because SDS-stable αβ complexes have not been observed at the Endo H-sensitive, pre-Golgi stage, this fraction is likely to be extremely small. The effects of concanamycin B on class II trafficking are likely to extend to other proteins in the endocytic pathway (Figure 2B) and suggest an important role for maintenance of low endosomal pH in preserving compositional integrity of the endocytic pathway.

Materials and methods

Materials

Concanamycin B was obtained from Ajinomoto Co. (Kanagawa, Japan). A 10 μM stock solution of concanamycin B was prepared in ethanol. Leupeptin, neuraminidase type VIII, poly-L-lysine, and DABCO were obtained from Sigma Chemical Co. (St Louis, MO). Sulpho-NHS-biotin and streptavidin–agarose were from Pierce (Rockford, IL). Leupeptin was added to the medium at a final concentration of 1 mM. [^{35}S]-methionine (specific radioactivity, >1000 Ci/mmol) and Na^{125}I (specific radioactivity, 1 Ci = 37 GBq) were obtained from Du Pont New England Nuclear (Boston, MA); fetal calf serum, Dulbecco's modified Eagle's medium (DMEM), RPMI, penicillin/streptomycin (100×) and methionine-free media were from Gibco BRL (Gaithersburg, MD).

Antibodies

The following anti-HLA-DR antibodies were used; the mAb Tü36 (Shaw *et al.*, 1985); mAb LB3.1 (Knudsen and Strominger, 1986), kindly provided by Dr J.Strominger, Harvard University, Cambridge, MA; mAb L243 (Lampson and Levy, 1980), obtained from American Type Culture Collection, and polyclonal rabbit sera raised against denatured α (anti-α) and β (anti-β) chains respectively (Neefjes *et al.*, 1990). Three Ii chain-reactive mouse mAbs were used: ViC-Y1 (Quaranta *et al.*, 1984), specific for the N-terminus and was kindly provided by Dr W.Knapp; Bü45 (Wraight *et al.*, 1990) directed against the C-terminus (from The Binding Site, Birmingham, UK) and LN-2 (Epstein *et al.*, 1984), directed against the C-terminus. In addition, mAb W6/32 (Parham *et al.*, 1979), recognizing HLA-A, -B and -C locus products; mAb 66Ig10 (Van de Rijn *et al.*, 1983), recognizing human transferrin receptor; mAb H4A3, raised against human LAMP-1 (from Developmental Studies Hybridoma Bank (DSHB), Iowa City, Iowa); rabbit anti-M6PR (kindly provided by B.Hofflack); rabbit anti-human cathepsin D (Geuze *et al.*, 1988), and mAb CD3, raised against human LAMP-2. Secondary goat antibodies adsorbed on human serum (from Southern Biotech. Co., AL). The antibodies used for immuno-electron microscopy were rabbit anti-human class II (Peters *et al.*, 1991); ICC5, anti-Ii luminal domain (kindly provided by Dr Peter Morton, Monsanto, St Louis, MO) and rabbit anti-human LAMP-1 (Fukuda *et al.*, 1988).

Cell culture

The cell line HOM 2 (homozygous for HLA-DR1) was maintained in RPMI containing 10% fetal calf serum, 2 mM glutamine and 1/1000 dilution units/ml penicillin and 100 μg/ml streptomycin.

Biochemical analysis

Pulse–chase experiments were performed as follows: HOM 2 cells were cultured in methionine-free DMEM medium for 45 min prior to labeling with 0.5 mCi/ml [^{35}S]methionine. Incorporation of label was terminated by the addition of 1 mM cold methionine. Cells (2–4×10^6 cells per chase point) were chased in RPMI + 10% FCS. Concanamycin B or leupeptin was absent from the labeling medium and included in the chase media at the concentrations indicated in the figure legends. Control cells were treated with comparable concentrations of solvent. In some experiments, cells were pretreated for 2 h in the presence or absence of the drugs. Surface appearance of molecules was determined by incubating cells at the end of the chase in 100 µl of a solution of 5 U/ml neuraminidase (Sigma, type VIII) in PBS + 1 mM $CaCl_2$ on ice for 30 min. Neuraminidase was removed by washing the cells three times with PBS + 10% FCS (Neefjes *et al.*, 1990). Neuraminidase as a fluid phase marker was used as previously described (Neefjes *et al.*, 1990). In surface biotinylation experiments, cells were washed four times in ice-cold PBS at the end of the chase and then incubated in 0.5 mg/ml sulfo-NHS-biotin in PBS for 30 min. Biotinylation was stopped by quenching in 25 mM L-lysine in PBS. In surface iodination experiments, cells (~20×10^6) were surface-labeled with 1.0 mCi of $Na^{125}I$ in ice-cold PBS by lactoperoxidase-catalyzed iodination. Following all experiments, cells were lysed in NP40 Lysis mix (0.5% NP-40, 50 mM Tris–HCl and 5 mM $MgCl_2$ pH 7.3), containing 1 mM PMSF (Neefjes *et al.*, 1990).

Gel electrophoresis

All immunoprecipations of total lysates were preceded by pre-clearing the NP-40 supernatant with normal mouse serum or normal rabbit serum, following which immune complexes were removed by adsorption to *Staphylococcus aureus*. Precleared lysates were immunoprecipitated with the different antisera either sequentially, or in parallel, as indicated in the figure legends, and the immune complexes collected by centrifugation. Immune complexes were washed as described and prepared for SDS–PAGE (Burke *et al.*, 1984) or isoelectric focusing (Neefjes *et al.*, 1986).

Cytofluorimetry

Cells were incubated in culture medium containing the indicated drug, as for biochemical analysis. Typically, 7×10^5 cells/ml for 20 h were analyzed. At the end of the incubation period, cells were recovered, washed and then stained with various mouse mAbs or rabbit antibodies revealed by FITC-labeled goat anti-mouse or anti-rabbit IgGs. Stained cells were analyzed for fluorescence intensity on a FACScan system (Becton Dickinson & Co., Mountain View, CA) using propidium iodide to exclude dead cells.

Immunofluorescence and confocal microscopy

Cells were incubated in culture medium in the presence or absence of concanamycin B, as for biochemical analysis. Typically, 7×10^5 cells/ml for 20 h were analyzed. Cells were washed in PBS and then sedimented on glass cover slips previously coated with poly-L-lysine. Cells were fixed in 4% paraformaldehyde followed by quenching in 0.1 M glycine in PBS, and permeabilization in 0.1% Triton X-100 in PBS. Saturation and the two-step staining procedure were performed in PBS containing 0.2% BSA, 0.01% NaN_3. Primary antibodies were allowed to bind for 45 min; ViC-Y1 (5 µg/ml) and rabbit anti-class II, 1:200. After four washes, secondary antibodies (FITC-labeled goat anti-rabbit Ig and TRITC-labeled goat anti-mouse Ig) were incubated at a 1:150 final dilution, for 30 min. Following four washes in PBS, slides were mounted in mowiol medium containing 100 mg/ml of DABCO and examined under a confocal microscope (Leica, Heidelberg, Germany), which uses an argon-krypton laser operating in multi-line mode. Cell preparations were sequentially analyzed at 488 and 567 nm. A series of optical sections was recorded at 0.5 nm intervals with a ×63 lens.

Electron microscopy

HOM 2 cells were treated with concanamycin B for 15 h and then prepared for immuno-electron microscopy as described (Peters *et al.*, 1991; Harding and Geuze, 1992). To study endocytic compartments, cells were pulsed for 10 min with 5 nm BSA–gold particles, washed and chased for the indicated times. Endocytosis was stopped in ice-cold medium and the cells washed twice in cold medium. Cells were fixed with a mixture of 2% paraformaldehyde/0.2% glutaraldehyde in 0.2 M phosphate buffer and processed as described (Harding and Geuze, 1992).

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References

Amigorena,S., Drake,J., Webster,P. and Mellman,I. (1994) *Nature*, **369**, 113–120.
Bakke,O. and Dobberstein,B. (1990) *Cell*, **63**, 707–716.
Banta,L.M., Robinson,J.S., Klionsky,D.J. and Emr,S.D. (1988) *J. Cell Biol.*, **107**, 1369–1383.
Barasch,J., Kiss,B., Prince,A., Saiman,L., Gruenert,D. and Al-Awqati,Q. (1991) *Nature*, **352**, 70–73.
Bijlmakers,M.J.E., Benaroch,P. and Ploegh,H.L. (1994a) *EMBO J.*, **13**, 2699–2707.
Bijlmakers,M.J.E., Benaroch,P. and Ploegh,H.L. (1994b) *J. Exp. Med.*, **180**, 623–629.
Bikoff,E.K., Huang,L.Y., Episkopou,V., van Meerwijk,J., Germain,R.N. and Robertson,E.J. (1993) *J. Exp. Med.*, **177**, 1699–1712.
Blum,J.S. and Cresswell,P. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 3975–3979.
Bowman,E.J., Siebers,A. and Altendorf,K. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 7972–7976.
Braun,M., Waheed,A. and von Figura,K. (1989) *EMBO J.*, **8**, 3633–3640.
Burke,B., Matlin,K., Bause,E., Legler,G., Peyrieras,N. and Ploegh,H.L. (1984) *EMBO J.*, **3**, 551–556.
Chapman,R.E. and Munro,S. (1994) *EMBO J.*, **13**, 2305–2312.
Chicz,R.M., Urban,R.G., Lane,W.S., Gorga,J.C., Stern,L.J., Vignali,D.A.A. and Strominger,J.L. (1992) *Nature*, **358**, 764–768.
Clague,M.J., Urbe,S., Aniento,F. and Grunberg,J. (1994) *J. Biol. Chem.*, **269**, 21–24.
Cresswell,P. (1994) *Annu. Rev. Immunol.*, **12**, 259–293.
Davidson,H.W., Reid,P.A., Lanzavecchia,A. and Watts,C. (1991) *Cell*, **67**, 105–116.
Davies,J.E. and Cresswell,P. (1990) *J. Immunol.*, **144**, 990–997.
Elliott,E.A., Drake,J.R., Amigorena,S., Elsemore,J., Webster,P., Mellman,I. and Flavell,R.A. (1994) *J. Exp. Med.*, **179**, 681–694.
Epstein,A.L., Marder,R.J., Winter,J.N. and Fox,R.J. (1984) *J. Immunol.*, **133**, 1028–1036.
Fukuda,M. (1991) *J. Biol. Chem.*, **266**, 21327–21330.
Fukuda,M., Viitala,J., Matteson,J. and Carlsson,S.R. (1988) *J. Biol. Chem.*, **263**, 18920–18928.
Germain,R.N. and Hendrix,L.R. (1991) *Nature*, **353**, 134–139.
Geuze,H.J., Stoorvogel,W., Strous,G.J., Slot,J.W., Bleekemolen,J.E. and Mellman,I. (1988) *J. Cell Biol.*, **107**, 2491–2501.
Goldstein,J.L., Brown,M.S., Anderson,R.G.W., Russell,D.W. and Schneider,W.J. (1985) *Annu. Rev. Cell Biol.*, **1**, 1–39.
Griffiths,G. and Simons,K. (1986) *Science*, **234**, 438–443.
Harding,C.V. and Geuze,H.G. (1992) *J. Cell Biol.*, **119**, 531–542.
Harter,C. and Mellman,I. (1992) *J. Cell Biol.*, **117**, 311–325.
Kinashi,H., Someno,K. and Sakaguchi,K. (1984) *J. Antibiot.*, **37**, 1333–1343.
Klionsky,D.J. and Emr,S.D. (1989) *EMBO J.*, **8**, 2241–2250.
Klionsky,D.J., Herman,P.K. and Emr,S.D. (1990) *Microbiol. Rev.*, **54**, 266–292.
Klionsky,D.J., Nelson,H. and Nelson,N. (1992) *J. Biol. Chem.*, **267**, 3416–3422.
Knudsen,PJ. and Strominger,J.L. (1986) *Hum. Immunol.*, **15**, 150–163.
Koch,N., Moldenhauer,G., Hofmann,W.J. and Moller,P. (1991) *J. Immunol.*, **147**, 2643–2651.
Kornfeld,S. and Mellman,I. (1989) *Annu. Rev. Cell Biol.*, **5**, 483–525.
Kvist,S., Wiman,K., Claesson,L., Peterson,P.A. and Dobberstein,B. (1982) *Cell*, **29**, 61–69.
Lamb,C. and Cresswell,P. (1992) *J. Immunol.*, **148**, 3478–3482.
Lampson,L.A. and Levy,R. (1980) *J. Immunol.*, **125**, 293–299.
Lippincott-Schwartz,J. and Fambrough,D.M. (1986) *J. Cell Biol.*, **102**, 1593–1605.
Loss,G.E. and Sant,A.J. (1993) *J. Immunol.*, **150**, 3187–3197.
Lotteau,V., Teyton,L., Peleraux,A., Nilsson,T., Karlsson,L., Schmid,S.L., Quaranta,V. and Peterson,P.A. (1990) *Nature*, **348**, 600–605.

Machamer,C.E. and Cresswell,P. (1982) *J. Immunol.*, **129**, 2564–2569.
Mathews,P.M., Martinie,J.B. and Fambrough,D.M. (1992) *J. Cell Biol.*, **118**, 1027–1040.
Mellman,I. and Simons,K. (1992) *Cell*, **68**, 829–840.
Moriyama,Y. and Nelson,N. (1989) *J. Biol. Chem.*, **264**, 18445–18450.
Nabi,I., Le Bivic,A., Fambrough,D. and Rodriguez-Boulan,E. (1991) *J. Cell Biol.*, **115**, 1573–1584.
Neefjes,J.J. and Ploegh,H.L. (1992) *EMBO J.*, **11**, 411–416.
Neefjes,J.J., Doxiadis,I., Stam,N.J., Beckers,C.J. and Ploegh,H.L. (1986) *Immunogenetics*, **23**, 164–171.
Neefjes,J.J., Stollorz,V., Peters,P.J., Geuze,H.J. and Ploegh,H.L. (1990) *Cell*, **61**, 171–183.
Nelson,N. (1991) *Trends Pharmacol. Sci.*, **121**, 71–75.
Nelson,H. and Nelson,N. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 3503–3507.
Newcomb,J.R. and Cresswell,P. (1993) *J. Immunol.*, **150**, 499–507.
Nowell,J. and Quaranta,V. (1985) *J. Exp. Med.*, **162**, 1371–1376.
Odorizzi,G.C., Trowbridge,I.S., Xue,L., Hopkins,C.R., Davis,C.D. and Collawn,J.F. (1994) *J. Cell Biol.*, **126**, 317–330.
Parham,P., Barnstable,C.J. and Bodmer,W.F. (1979) *J. Immunol.*, **123**, 342–349.
Peters,C., Braun,M., Weber,B., Wendland,M., Schmidt,B., Pohlmann,R., Waheed,A. and von Figura,K. (1990) *EMBO J.*, **9**, 3497–3506.
Peters,P.J., Neefjes,J.J., Oorschot,V., Ploegh,H.L. and Geuze,H.J. (1991) *Nature*, **349**, 669–676.
Pieters, J., Bakke, O. and Dobberstein, B. (1993) *J. Cell Sci.*, 106, 831–846.
Qiu,Y., Xu,X., Wandinger-Ness,A., Dalke,D.P. and Pierce,S.K. (1994) *J. Cell Biol.*, **125**, 595–605.
Quaranta,V., Majdic,O., Stingl,G., Liszka,K., Honigsmann,H. and Knapp,W. (1984) *J. Immunol.*, **132**, 1900–1905.
Riberdy,J.M., Newcomb,J.R., Surman,M.J., Barbosa,J.A. and Cresswell,P. (1992) *Nature*, **360**, 474–476.
Roche,P.A. and Cresswell,P. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 3150–3154.
Roche,P.A., Marks,M.S. and Cresswell,P. (1991) *Nature*, **354**, 392–394.
Roche,P.A., Teletski,C.L., Stang,E., Bakke,O. and Long,E.O. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 8581–8585.
Sandoval,I.V. and Bakke,O. (1994) *Trends Cell Biol.*, **4**, 292–297.
Shaw,S., Ziegler,A. and DeMars,R. (1985) *Hum. Immunol.*, **12**, 191–211.
Springer,T.A., Kaufman,J.F., Siddoway,L.A., Mann,D.L. and Strominger,J.L. (1977) *J. Biol. Chem.*, **252**, 6201–6207.
Stern,L.J. and Wiley,D.C. (1992) *Cell*, **68**, 465–477.
Teyton,L., O'Sullivan,D., Dickson,P.W., Lotteau,V., Sette,A., Fink,P. and Peterson,P.A. (1990) *Nature*, **348**, 39–44.
Trowbridge,I.S., Collawn,J.F. and Hopkins,C.R. (1993) *Annu. Rev. Cell Biol.*, **9**, 129–161.
Tulp,A., Verwoerd,D., Dobberstein,B., Ploegh,H.L. and Pieters,J. (1994) *Nature*, **369**, 120–126.
Unanue,E.R. (1984) *Annu. Rev. Immunol.*, **2**, 395–428.
Unanue,E.R. (1992) *Curr. Opin. Immunol.*, **4**, 63.
Van de Rijn,M., Geurts van Kessel,A.H.H., Kroezen,V., Van Agthoven,A.J., Verstijnen,K. Terhorst,C. and Hilgers,J. (1983) *Cytogenet. Cell Genet.*, **36**, 525–531.
Viville,S., Neefjes,J., Lotteau,V., Dierich,A., Lemeur,M., Ploegh,H., Benoist,C. and Mathis,D. (1993) *Cell*, **72**, 635–648.
West,M., Lucocq,J. and Watts,C. (1994) *Nature*, **369**, 147–150.
Wraight,C.J., Van Endert,P., Moller,P., Lipp,J., Ling,N.R., MacLennan,I.C., Koch,N. and Moldenhauer,G. (1990) *J. Biol. Chem.*, **265**, 5787–5792.
Woo,J.T., Shinohara,C., Sakai,K., Hasumi,K. and Endo,H. (1992) *Eur. J. Biochem.*, **207**, 383–389.
Yilla,M., Tan,A., Ito,K., Miwa,K. and Ploegh,H.L. (1993) *J. Biol. Chem.*, **268**, 19092–19100.
Zachgo,S., Dobberstein,B. and Griffiths,G. (1992) *J. Cell Sci.*, **103**, 811–822.
Zeuzem,S., Feick,P., Zimmermann,P., Haase,W., Kahn,R.A. and Schultz,I. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 6619–6623.

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