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Intracellular Pathway for the Generation of Functional MHC Class II Peptide Complexes in Immature Human Dendritic Cells

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Binding of antigenic peptides to MHC class II (MHC-II) molecules occurs in the endocytic pathway. From previous studies in B lymphocytes, it is believed that most but not all of the newly synthesized MHC-II molecules are directly targeted from the trans-Golgi network to endosomal compartments. By using pulse-chase metabolic labeling followed by cell surface biotinylation, we show here that in contrast to an EBV-transformed B cell line and human monocytes, the majority of newly synthesized MHC-II molecules (at least 55 ± 13%) are first routed to the plasma membrane of dendritic cells derived from human monocytes. They reach the cell surface in association with the invariant chain (Ii), a polypeptide known to target MHC-II to the endosomal/lysosomal system. Following rapid internalization and degradation of Ii, these αβIi complexes are converted into αβ-peptide complexes as shown by their SDS stability. These SDS-stable dimers appear as soon as 15 to 30 min after internalization of the αβIi complexes. More than 80% of αβ dimers originating from internalized αβIi complexes are progressively delivered to the cell surface within the next 2 h. Depolymerization of microtubules, which delays the transport to late endosomal compartments, did not affect the kinetics of conversion of surface αβIi into SDS-stable and -unstable αβ dimers. Altogether, these data suggest that newly liberated class II αβ heterodimers may bind peptides in different compartments along the endocytic pathway in dendritic cells derived from human monocytes. The Journal of Immunology, 1998, 160: 2597–2607.

Immune response against exogenous Ag is initiated by presentation of antigenic peptides in the context of MHC class II (MHC-II) molecules. The specificity and efficiency of Ag presentation depend on the intracellular transport of MHC molecules (1). The α and β subunits of MHC-II molecules are assembled in the endoplasmic reticulum, where they associate with the invariant chains (Ii) (1). Ii MHC-II (αβIi) complexes then travel through the Golgi complex and reach the endocytic pathway, where degradation of Ii allows MHC-II to bind immunogenic peptides generated by proteolytic cleavage of internalized proteins. At the steady state, intracellular MHC-II molecules are predominantly located in endosomal compartments (2). In human APC, these intracellular compartments share characteristics with lysosomes (3). They appeared as multilamellar or multivesicular structures called MHC-II compartments (MIIC). Similar compartments have also been identified in human dendritic epidermal Langerhans cells (4, 5) and mouse dendritic cells (DCs) (6). MIIC are thought to represent a specialization of the prelysosomal or lysosomal compartments for efficient peptide loading onto newly synthesized MHC-II molecules (3, 7). They seem to be distinct from conventional early or late endosomal compartments and contain the majority of HLA-DM, a protein that catalyzes loading of peptide on MHC-II molecules (8, 9). This and current biochemical data, essentially obtained in B cells, have led to the generally accepted view of a direct transport of αβIi complexes from the trans-Golgi network (TGN) to the MIIC, before cell surface expression of peptide-loaded αβ dimers (7, 10). However, other studies report the presence of MHC-II αβ dimers in other compartments (11) or even throughout the whole endocytic route in B cells (12). The precise pathway taken by MHC-II molecules en route to peptide-loading compartments thus remains unclear.

All cell types that express MHC-II and Ii chains have the capacity to present Ags. Among them, DCs display unique characteristics for Ag presentation: 1) they synthesize high levels of MHC-II molecules (13, 14), 2) they express specialized receptors thought to potentiate the capture of diverse Ags and their specific delivery to the processing compartments (15, 16), 3) they efficiently present Ags in situ (17), and 4) they can prime virgin T lymphocytes in vitro and even in vivo (18). Moreover, low numbers of immature DCs and small amounts of Ag are sufficient to induce T cell stimulation in vitro (19). Upon maturation, these cells become even better APCs, when primed with Ags before their in vitro differentiation into mature DCs, while they lose their capacity to capture and process new Ags (19). In vivo, DCs are widely distributed in the body. They constitute a trace population of cells circulating between nonlymphoid and lymphoid tissues. In the nonlymphoid tissues, where they reside in an “immature” state, DCs are ideally placed to perform a “sentinel” function for the...
immune system (20). There, they capture Ags, process them into an immunogenic form, and present MHC-II-peptide complexes to sensitized T cells. Under some conditions, which remain to be defined, DCs can migrate from the nonlymphoid tissues to the T cell-dependent areas of the lymphoid organs and undergo maturation (21). Consequently, in the lymphoid tissues, “mature” DCs can initiate the sensitization of naïve/resting T cells.

A number of techniques have been developed allowing the isolation of relatively low quantities (1 to 2 x 10⁶) of cells from tissue human DCs or even highly purified lymphoid DCs (22, 23). Long-term mouse cell lines are also available (24, 25). However, due to the relative difficulty of isolating large quantities of immature human DCs, the precise dynamic of the intracellular transport of MHC-II molecules, which requires following the behavior of a small proportion of newly synthesized molecules from their site of synthesis to their final destination, has been less studied in these cells than in other MHC-II expressing cells. There have been conflicting reports on the nature of DC precursors in peripheral blood (for review see Ref. 26). One emerging explanation is that more than one cell type can develop into cells with the morphologic and functional characteristics of DCs (27, 28). Depending on the origin and on the in vitro method used for derivation, DCs with different phenotypes can also be obtained (29).

Recently, it has been shown that low density mononuclear cells cultured with a combination of granulocyte-macrophage (GM)-CSF and IL-4 develop into cells that are highly efficient in the processing and presentation of diverse Ags to T cells. These cells also show phenotypic characteristics of immature DCs that can be further differentiated into mature DCs (19, 30). As already suggested (31), we found that these very same immature DCs can be differentiated into activated macrophages upon addition of macrophage-CSF (our unpublished observations), implying that these immature DCs are pluripotent cells.

We wanted to biochemically investigate the intracellular process leading to peptide formation in these cells. We thus compared monocyte-derived immature DCs with other APCs for the intracellular trafficking of MHC-II molecules.

We report that in strong contrast to an EBV-transformed B (BEBV) cell line and freshly isolated human monocytes, a large proportion (more than 55%) of functional MHC-II first traffic through the plasma membrane of human immature DCs in association with Ii. These αβIi complexes, transiently expressed at the plasma membrane of DCs, are rapidly internalized and are converted into αβ dimers in a microtubule-independent manner. Then, the αβ dimers can bind peptide, as assessed by their SDS stability (32), and are progressively delivered to the cell surface within the 2 h following synthesis of the polypeptide chains.

Materials and Methods

Cells, Abs, cell culture, and chemicals

DCs were derived from human blood monocytes using human recombinant (hr)GM-CSF and IL-4 (both generously provided by Schering-Plough, Union, NJ), as previously described (19). Monocytes were isolated by continuous flow centrifugation leukapheresis and counterflow centrifugation elutriation as previously described (33). They were maintained in RPMI 1640 medium supplemented with 2 mM glutamine, 1% sodium pyruvate, 1% nonessential amino acids, 10% heat-inactivated FCS (all from Life Technologies, Paisley, U.K.), 50 ng/ml hrGM-CSF, and 200 U/ml hrIL-4 (referred to as complete medium). Differentiated DCs were always used at day 7. Differentiation of monocytes was followed by flow cytometry analysis of different surface markers. For FACS analysis, cells were washed once in PBS and resuspended in PBS, 3% FCS, and 0.1% NaN₃ in 96-well plates. Cells were incubated with different primary Abs as specified, followed by species-specific FITC-labeled secondary Abs. After washing, cells were analyzed with a FACSscan (Becton Dickinson, Mountain View, CA).

At day 7, DCs express high levels of the MHC-II molecules HLA-DR and CD74 (Ii); MHC class I molecules (HLA-A, -B, -C); and the Ags CD1a, CD1b, CD1c, CD40, CD80 (B7-1), CD11b, CD11c, CD18 (β₂ integrin), CD54 (ICAM-1), and CD44. They express moderate amounts of CD32 (FcγRⅡ) and express only small amounts of the CD86 (B7-2) Ag. DCs do not express CD3, CD14, CD25, CD16 (FcγRⅢ), or 13C4 (CD140; E-cadherin). These results are in agreement with previously published observations (19) (data not shown). EBV-transformed B lymphocytes used in this study have been described elsewhere (34). The different Abs used were: L243 (35) (IgG2a, anti-Drαβ dimers; Becton Dickinson); W6/32 (IgG2a, anti-HLA class I; American Type Culture Collection, Rockville, MD); BU 45 (IgG1, anti-CD74 (Ii); Serotec, Kidlington, U.K.); MAB89 (IgG1, anti-CD40; Immunotech, Marseille, France); 1F2.2 and B11 (IgG2b, anti-CD86 (B7.2) and IgM, anti-CD80 (B7-1; Pharmingen, San Diego, CA); BL6, 4A7.6, and L161 (IgG1, IgG2a, IgG1, anti-CD1a, anti-CD1b, and anti-CD1c, respectively; all from Immunotech); Leu-15 (IgG2a, anti-CD11b; Becton Dickinson); Leu M5 (IgG2b, anti-CD11c; Becton Dickinson); Leu CD-54 (IgG2b, anti-CD54; Becton Dickinson); Leu M3 (IgG2b, anti-CD14; Becton Dickinson); Leu-4 (IgG1, anti-CD3; Becton Dickinson); 10.1, FLB 26, and 3G8 (IgG1, IgG2b, IgG1, anti-CD8, anti-CD3, and anti-CD16, respectively; Pharmingen); and G44-26 (IgG2b, anti-CD44; Pharmingen). Mouse IgG1, IgG2a, and IgG2b (all from Sigma Chemical Co., St. Louis, MO) were used for isotype controls and a FITC-conjugated affinity-isolated Fab_2 of a fraction of a sheep anti-mouse Ig Ab (Silenus, Hawthorn, Victoria, Australia) for the labeling procedures. The mouse hybridoma cell line cell DA6.147 (IgG2a, anti-HLA-DRα-chain) has been described (36). The rabbit antiseraum, RoHuli, was prepared by immunizing rabbits with the 191–212 peptide (PKESLELEPSGGLVTKQDLG) from human Ii linked to keyhole limpet hemocyanin (37). For electron microscopy studies, the following Abs were used: a polyclonal rabbit anti-Drα-chain Ab (kindly provided by Dr. J. Neefjes, The Netherlands Cancer Institute, Amsterdam, The Netherlands); and biotinylated goat anti-rabbit Ab (British BioCell Laboratories, Cardiff, U.K.), and gold-labeled Fab_2 fragments of the mAb anti-CD1b (Immunotech). Fab_2 fragments were prepared as described (38), and the labeling of the Fab_2 fragments with gold particles was performed as previously reported (38). Most chemicals used in this study were obtained from Sigma Chemical Co.

Radiolabeling, biotinylation, immunoprecipitation, and electrophoresis

Cells were pulse labeled with ³¹⁵S Promix (Amersham France, Les Ulis, France) for either 5 or 10 min at 37°C and chased for various periods of time, essentially as described previously (39). At indicated times, 2 x 10⁶ cells were chilled in cold PBS and cell surface biotinylated with a solution containing 25 mg of NHS-SS-biotin (Pierce Chemical Co., Rockford, IL) in 1 ml of cold PBS for 5 min at 4°C. The reaction was quenched with 50 mM of cold glycine in PBS. Cells were then chilled in cold X, 150 mM NaCl, 20 mM Tris-HCl, 5 mM EDTA, 0.2% BSA, and protease inhibitors. Postnuclear lysates were preclarified for 2 h with protein A-Sepharose at 4°C. Preclarified lysates were immunoprecipitated with the Abs RedHuli, L243, W6.32, and DA6.147 previously bound on protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden). Immunoprecipitates were washed as published elsewhere (39) and eluted in 10 ml of 10% SDS either at 95°C for 5 min or at room temperature for 30 min when indicated. Eluted material was then resuspended in 100 ml of lysis buffer without BSA. The efficiency of biotinylation being about 10 to 15% (data not shown), biotinylated proteins were recovered with streptavidin agarose on 90% of the eluted material, and the remaining 10% were left untreated. Except when specified, samples were boiled in Laemmli’s sample buffer (40) for electrophoresis on a 10 to 15% SDS-PAGE and run under reducing conditions (100 mM DTT) as described previously (41). Gels were processed for fluorography.

Quantification of sequential immunoprecipitations

All quantifications were based on the signal for a radiolabeled HLA-DR β-chain. To distinguish between the two forms of MHC-II molecules (αβIi complexes and αβ dimers), a first immunoprecipitation was performed. The recognized αβ dimers were solubilized from intact Ii chains (42, 43). Supernatants were then submitted to a second immunoprecipitation with the anti-Drα Abs DA6.147 mAb (36) to recover residual DR molecules corresponding to αβIi complexes. A hypothetical background of αβ dimers dissociated from Ii but recovered in the DA6.147 immunoprecipitation would be most detectable at a late time point in our pulse chase experiments (4 h), when the signal for these dimers is at a maximum. In B cells, the relative proportion between the DA6.147 and L243 immunoprecipitated β-chain at this time point was only 3%. In DCs,
some Ii chains associated with αβ dimers were still found at this late time point. They reach a proportion of 17% of the maximum signal for Ii, obtained at a chase time of 30 min. We thus subtracted 17% of the signal for the β-chain at the 30-min time point from the remaining signal detected with DA6.147 in the second immunoprecipitation after 4 h of chase. We calculated that only 12% of the β-chain signal detected in the DA6.147 immunoprecipitation could represent residual αβ dimers.

Autoradiographies exposed in the linear range of detection were scanned with a video camera (Bio-print system; Vilber Lourmat, Marne la Vallée, France). Optical densitometry was performed using the Bio-1D software (Vilber Lourmat). The background signal was calculated for each lane and subtracted from the ODs of the area corresponding to protein bands.

**Internalization and recycling experiments**

For internalization experiments, cells were pulse labeled, chased, and biotinylated as described above. They were then incubated for various times at 37°C to allow biotinylated material to endocytose. At the end of the chase period, ice-cold PBS was added and the cells were washed twice at 4°C. Lysis, immunoprecipitation, and streptavidin precipitation were performed as described above. When specified, 20 μM of nocardazole was added to the cells after biotinylation at 4°C and before further incubation at different temperatures to allow for internalization of the biotinylated material.

For recycling experiments, cells were pulse labeled, chased, biotinylated, and reincubated at 37°C for further transport. Reduction of remaining or recycled surface NHS-S-S-biotin was performed with 20 mM 2-mercaptoethanesulfonic acid (MESNA; Sigma Chemical Co.), 50 mM Tris, pH 8.6, 100 mM NaCl, and BSA 0.2% for 10 min at 4°C. The cells were solubilized, cell lysates were immunoprecipitated, and biotinylated immunoprecipitates were separated as described above.

**Immunogold-labeling procedure of DCs**

DCs (incubated in complete medium with or without 20 μM nocardazole) were cooled to 4°C for 10 min and incubated at 4°C for 60 min, in the presence of 10 nm of gold-labeled F(ab)² fragments of the mAb anti-CD1b (final dilution, 1:100). The former DCs were warmed to 20°C for 30 min, and either fixed at 20°C for electron microscopy or washed at 4°C, incubated at 37°C for 5 min in previously warmed complete medium (with or without 20 μM nocardazole), and fixed at 37°C for electron microscopy.

**Preparation of DCs for transmission electron microscopy**

DCs maintained at either 20°C or 37°C in 1 ml of the above-mentioned medium were fixed by adding an equal volume of fixative solution, previously warmed to 37°C or 20°C, respectively, composed of 2.5% glutaraldehyde (Electroly Microscopy Sciences, Euromedex, Strasbourg, France) in 0.1 M sodium cacodylate buffer containing 2% sucrose (305 mOsm, pH 7.3) (both from Merck, Darmstadt, Germany). After 5 min, the mixture was centrifuged, the supernatant discarded, and the pellet resuspended and further fixed for 45 min with the same fixative solution maintained at 20°C or 37°C, respectively. DCs were then washed in the 0.1 M sodium cacodylate buffer and postfixed for 1 h at 4°C, with 1% osmium tetroxide (Merck) in 0.1 M sodium cacodylate buffer. After additional washing in the 0.1 M sodium cacodylate buffer, DCs were dehydrated in successively increasing (50, 70, 80, 95, and 100%) ethanol concentrations. Finally, the cells were incubated overnight in Epon (Electron Microscopy Sciences)-absolute alcohol (1:1, v/v) and embedded in Epon. Ultrathin sections were cut, collected as above. They were pretreated for 1 h at room temperature with PBS containing 1% goat serum and incubated overnight with the rabbit anti-DRα chain Ab (dilution 1/400). Thereafter, the sections were washed with PBS/1% goat serum and further incubated for 1.5 h at room temperature with the gold-labeled goat anti-rabbit Ab (dilution 1/20). Finally, the grids were washed two times with PBS/0.5% goat serum, two times with PBS, postfixed for 30 min at room temperature with 2.5% glutaraldehyde in PBS, washed with distilled water, and stained with 1:8% uranyl acetate/0.2% methylcellulose. Sections were then examined under a Philips CM 120 BioTwin electron microscope (120 kV). Controls were performed by substituting nonimmune rabbit IgG for the primary Ab.

**Immunofluorescence and confocal microscopy**

DCs were first allowed to adhere on glass coverslips precoated with a 0.1% poly-L-lysine solution in water for 30 min at 37°C. Adherent cells were incubated with 10 μg/ml of IgGs purified from the RaHaL antisem for 30 min at 4°C. They were then incubated for 30 min at 37°C for the indicated times in the presence or absence of 20 μM nocardazole. Cells were fixed in 3% paraformaldehyde for 10 min. After permeabilization with 0.05% saponin in PBS supplemented with 0.2% BSA, they were incubated with the L243 mAb and finally stained with both FITC and Texas Red-donkey antiserum directed against mouse and rabbit IgGs (Jackson ImmunoResearch, West Grove, PA). The coverslips were then mounted in Mowiol (Merck, Darmstadt, Germany).

Confocal laser scanning microscopy and immunofluorescence analysis were performed using a TCSP4D confocal microscope based on a DM microscope interfaced with an Argon/Krypton laser. Simultaneous double fluorescence acquisitions were performed using the 488- and 568-nm laser lines to excite FITC and Texas Red dyes using a 100× oil immersion Plan Apo objective (numerical aperture = 1.4). The fluorescence was selected with the appropriate double fluorescence dichroic mirror and band pass filters and measured with blue-green sensitive and red side sensitive one photomultipliers.

**Results**

A high proportion of αβIi complexes reaches the plasma membrane of DCs

We first compared the cell surface appearance of newly synthesized MHC-II molecules in BEBV cells, in freshly isolated blood monocytes and in DCs obtained by culturing the latter cells in the presence of hrGM-CSF and hrHL-4. These three types of cells express both MHC-II molecules (not shown) and Ii chain at their cell surface. As shown in Figure 1A, larger amounts of Ii were always detected at the cell surface of immature DCs. We found that most if not all cell surface Ii was associated with αβ dimers of MHC II molecules (not shown). MHC-II molecules exist as two major forms where αβ dimers can be complexed with diverse isoforms of Ii in a nonameric precursor structure (45) and as free αβ dimers from which Ii has been released by proteolytic cleavage. To distinguish between these two forms of MHC-II molecules, we took advantage of the restricted specificity of the L243 mAb for the αβ dimers of HLA-DR devoid from intact Ii chains (42, 43). Cells were surface biotinylated at chosen times of a pulse-chase experiment (Fig. 1B). Lysates were first immunoprecipitated with the L243 mAb. Supernatants were then submitted to immunoprecipitation with the DA6.147 mAb to recover residual DR molecules corresponding to αβIi complexes. The partition between αβ and αβIi forms of MHC-II molecules using sequential immunoprecipitations was ascertained by our quantification analysis, as described in Materials and Methods. Biotinylated cell surface molecules were compared with the total immunoprecipitated material.

The expression of free αβ dimers at the cell surface reached a plateau between 2 and 4 h after synthesis (Fig. 1B, Biot. L243) in both monocytes and DCs. This rather long kinetic of cell surface appearance corresponds to the time necessary for free αβ dimers to move from their intracellular site of formation to the plasma membrane of these cells. We observed no major differences between the three cell types, except for a slight but reproducibly longer lag time in the appearance of αβ dimers at the cell surface of BEBV cells. This was probably due to a longer delay in the oligomerization of newly synthesized αβIi complexes in BEBV cells (Fig. 1B, Total DA6.147).

In contrast, the analysis of remaining biotinylated DR molecules immunoprecipitated with the DA6.147 mAb showed that much...
higher amounts of αβ dimers complexed with li chains were detected at the surface of DCs, compared with monocytes and BEBV cells (Fig. 1B, Biot. DA6.147). In human cells, li is present in four different isoforms: two alternatively spliced forms, p33 and p41 and, due to two different initiation sites, a p35 and a p43 form (46). All isoforms can assemble with MHC-II molecules (46). As previously shown in human B cells (47), trace amounts of αβli complexes devoid of the p35 li isoform were detected at the plasma membrane of BEBV cells and monocytes (Fig. 1, Biot. DA6.147). The transient expression of αβli complexes at the plasma membrane of DCs reached its maximum within 30 min to 1 h after their synthesis. A polypeptide chain of 47 kDa (p47) (Fig. 1) was also strongly detected at the plasma membrane of DCs. It represents the mature sialylated p41 form (48), as assessed by neuraminidase desialylation after immunoprecipitation with a rabbit antisera directed against human li (not shown). This isoform was barely detectable in BEBV cells and monocytes. During further chase times, all li chains, including cell surface p47 and the 35-kDa proteins (corresponding to comigrating sialylated p33 (mp33 for mature p33) and MHC-II DRα-chains), progressively declined (Fig. 1). Immature β-chain signals also declined over the time course of the experiment. In fact, as is better seen after a longer exposure (Fig. 1C), this molecule is first converted into a more mature form, which migrates between immature β-chain and p33 li. Then, it is transiently expressed at the plasma membrane in an αβli complex. Finally, αβ dimers are formed as detected here in the first immunoprecipitation with the L243 mAb (Fig. 1, left panel).

A quantitative analysis, performed on DCs from six blood donors, showed that more than half (55 ± 13%) of the newly synthesized αβli complexes gained access within 30 min to the DCs surface, where they are transiently expressed (Fig. 2A). The disappearance of αβli complexes from the plasma membrane of DCs correlates with the formation of free αβ dimers and the subsequent arrival of these dimers at the plasma membrane (Fig. 2A). Using the same quantitative analysis, we found that only 7.8% of the newly synthesized αβli complexes also gained access to the plasma membrane of BEBV cells (Fig. 2B).
The rapid cell surface appearance of newly synthesized αβII complexes in DCs suggests that they follow a constitutive pathway for plasma membrane delivery. To verify this observation, DCs were pulse labeled for only 5 min and chased for short periods of time. As shown in Figure 3, the newly synthesized αβII complexes reached the plasma membrane as rapidly as MHC class I molecules, detected here with a monomorphic mAb (W6/32). This indicates that αβII could be directly transported from the Golgi to the plasma membrane, as documented for MHC class I molecules (7).

Altogether, these results are consistent with a specific pathway of transport for MHC-II molecules in DCs, whereby a significant amount of newly synthesized αβII complexes are first delivered to the cell surface.

αβII complexes transiently expressed at the plasma membrane of DCs are converted into SDS-stable αβ dimers

We analyzed in further detail the conversion of αβII complexes transiently expressed at the plasma membrane into αβ-peptide complexes. This was possible by taking advantage of the capacity of L243 mAb to recognize undisassociated αβ-peptide complexes in an SDS stability assay (32). DCs were labeled for 10 min, chased for 30 min, and surface biotinylated at 4°C. As already shown, this chase time corresponds to the arrival of significant amounts of metabolically labeled αβII complexes at the plasma membrane (Figs. 1, 2A, and 3). However, at this time no radiolabeled αβ dimers were detected on the cell surface (Figs. 1 and 2A). After given times of further incubation at 37°C, cells were lysed and the lysates immunoprecipitated with L243 mAb. The immunoprecipitates were then dissociated in the presence of SDS for 30 min at room temperature and adsorbed onto streptavidin-agarose beads. To test the SDS stability of the αβ dimers, the final material was eluted from the beads in the presence of SDS and the reducing agent DTT at room temperature. After the first immunoprecipitation with the L243 mAb, the lysates were reimmunoprecipitated with the DA6.147 mAb. Surface-biotinylated αβII complexes rapidly disappeared following their internalization at 37°C. At the same time, newly synthesized p41, present in the total fraction of αβII complexes, still matured into the p47 sialylated form (compare Fig. 4A total vs biotinylated, DA6.147). This showed that an excess of free-labeled Ii can still assemble with nonradioactive α- and β-chains in the endoplasmic reticulum during subsequent chase times, while biotinylation of cell surface proteins synchronizes further transport of radiolabeled biotinylated αβII complexes. Consequently, the biotinylated αβ dimers detected in this experiment (Fig. 4B) were generated from αβII complexes previously expressed at the plasma membrane (Fig. 4A Biot., 0'). Newly formed biotinylated-αβ dimers, including both SDS-stable and -unstable forms, appeared within 15 (data not shown) to 30 min (Fig. 4B, Biot.) after internalization of biotinylated αβII complexes. The proportion of SDS-stable dimers in DCs widely differed from one blood donor to the next (not shown), probably reflecting the various levels of SDS stability for the different DR molecules associated with peptides. However, the rate of conversion of αβII complexes into αβ dimers did not vary between donors. These molecules, which appeared as both SDS-sensitive and -resistant dimers, increased in concentration over the next 30 min. Interestingly, the kinetics of formation for biotinylated and total αβ dimers were undistinguishable.

Intracellular αβ dimers generated from internalized αβII complexes return to the plasma membrane of DCs

We next addressed whether αβ dimers generated from internalized αβII complexes underwent further transport to the DCs plasma membrane. Cells were pulse labeled, chased for 30 min, and biotinylated with cleavable NHS-SS-biotin at 4°C as described above.
The cells were then warmed to 37°C (Fig. 5), allowing biotinylated proteins to internalize. At given times, cells were cooled to 4°C and one-half of each sample was treated with MESNA, a membrane-impermeant reducing agent. Immunoprecipitations were conducted as in Figure 4. A. Only the biotinylated material found in DA6.147 immunoprecipitates is shown. The time 0’ was used to calculate the reduction efficiency of the MESNA treatment, which was estimated at about 80%. B. Both total (Total) and biotinylated (Biot.) material immunoprecipitated with L243 were run on SDS-PAGE. Note that very little material is present at time 0’ in both Total and Biot. samples. MESNA treatment does not lead to a loss of L243-immunoprecipitated material, as seen in the upper part of the figure (compare −MESNA and +MESNA in Total).

FIGURE 5. αβ dimers generated from internalized αβIi complexes return to the cell surface. DCs were pulse labeled (10 min), chased for 30 min, and surface biotinylated with cleavable NHS-SS-biotin. Before lysis, half of the samples were treated with MESNA, a membrane-impermeant reducing agent. Immunoprecipitations were conducted as in Figure 4. A. Only the biotinylated material found in DA6.147 immunoprecipitates is shown. The time 0’ was used to calculate the reduction efficiency of the MESNA treatment, which was estimated at about 80%. B. Both total (Total) and biotinylated (Biot.) material immunoprecipitated with L243 were run on SDS-PAGE. Note that very little material is present at time 0’ in both Total and Biot. samples. MESNA treatment does not lead to a loss of L243-immunoprecipitated material, as seen in the upper part of the figure (compare −MESNA and +MESNA in Total).

In conclusion, internalized αβIi complexes are rapidly converted into both SDS-stable and -unstable αβ dimers, which then progressively reach the plasma membrane of DCs.

Microtubules are not required for the conversion of cell surface αβIi complexes into αβ dimers

It is already known that presentation of different peptides may involve distinct pools of MHC II molecules and may depend on the way Ags are delivered into APC (49, 50). The rather fast generation of αβ dimers from internalized αβIi complexes in DCs is compatible with the loading of αβ dimers in different endosomal compartments with different proteolytic and physiologic properties (12). This led us to better characterize where, in the endocytic pathway of DCs, cell surface αβIi can be converted into αβ dimers. For this purpose, we took advantage of the fact that migration of transmembrane proteins along the endocytic pathway can be inhibited, or modulated, by incubating cells under two different experimental conditions. First, cells can be incubated at relatively low temperatures. At this temperature, prelysosomal late endosomes no longer fuse with newly formed endosomes, at least as observed in Chinese hamster ovary cells (51). Second, delivery of ligands (52, 53) or transmembrane proteins (54, 55) from early endosomes to degradative compartments of the endocytic pathway, probably lysosomes, can be slowed by inducing depolymerization of microtubules in the presence of nocodazole.

The effect of low temperature incubation or nocodazole on the migration of transmembrane proteins in DCs was first checked by following under the electron microscope the fate of CD1b molecules. Indeed, we have recently demonstrated that CD1b molecules expressed at the DC surface are internalized by receptor-mediated endocytosis and reach successively early endosomes, multivesicular/late endosomes, and finally the MIIC (our manuscript in preparation) (56). Thus, DCs were incubated, for 60 min at 4°C then for 30 min at 20°C, with gold-labeled anti-CD1b F(ab’)2. Progression of CD1b was blocked in the early endosomes as illustrated by the fact that gold-labeled anti-CD1b F(ab’)2 accumulated in these
compartments when DCs were further incubated for 30 min at 37°C. Thereafter, DCs were warmed up to 20°C for 30 min with or without nocodazole and chased for indicated times at 37°C, still in the presence or absence of nocodazole. We found no differences between nocodazole-treated and untreated cells on the rate of αβ dimer generation from internalized biotinylated αββI complexes (Fig. 10A, Biot.). In both cases, biotinylated SDS-stable dimers started to appear within 5 min at 37°C (Fig. 10B). In contrast, both the degradation of Ii associated with αβ (not shown) and the generation of free αβ dimers were slowed down during the course of the chase in the total fraction of cells treated with nocodazole (Fig. 10A, Total). This suggests that the depolymerization of microtubules only affects the transport of MHC-II molecules before they leave the exocytic pathway. The lack of effect of microtubule depolymerization on the generation of αβ-peptide complexes from plasma membrane αββI precursors strongly suggests that DCs have the capacity to bind peptides onto their MHC-II molecules before the αββI complexes gain access to the late endocytic compartments and in particular to the MIIC. This does not demonstrate that all αβ-peptide complexes are generated in early endosomes. However, since a large proportion of αβ-peptide complexes are formed from plasma membrane αββI complexes in DCs (Figs. 2 and 6), this alternative pathway of MHC-II trafficking may influence Ag processing and presentation in these cells.

**Discussion**

The trafficking of MHC-II molecules was examined in immature DCs derived in vitro from human blood monocytes and compared with their trafficking in freshly isolated monocytes and in BEBV cells. This study clearly establishes that the intracellular pathways of MHC-II molecules widely diverge among different APC. We showed that more than half (55%) of the functional αβ-peptide complexes in immature DCs are derived from αββI complexes transiently expressed at the plasma membrane. It should be pointed out that this value may be underestimated. Indeed, αββI complexes are known to reside for a very short time at the plasma membrane (57). Our biotinylation assay only allows the detection of a fraction of the newly synthesized αββI transiently present at the cell surface at a given time. Consequently, the entire fraction of the newly synthesized MHC-II molecules labeled within a 10-min pulse that passes through the cell surface before reaching Ag-processing compartments cannot be precisely calculated. Moreover, as clearly shown in Figure 3, the whole process of oligomerization of newly synthesized αββI complexes and egress from the endoplasmic reticulum is a rate-limiting step in the transport of MHC II molecules. Oligomerization of all αββI complexes and complete maturation of the DRβ-chain synthesized within a 5-min pulse will then take at least 30 min (Fig. 3). We conclude that a fraction larger than 55 ± 13% of the new MHC-II molecules must traffic through the cell surface of DCs.

The kinetics of delivery of αββI complexes on the plasma membrane of DCs were very similar to those of MHC class I molecules. This suggests a direct transport from the Golgi complex to the plasma membrane. We cannot exclude an arrival at the plasma membrane after targeting from the TGN to early endosomes, as recently shown for newly synthesized transferrin receptor (58). The αββI complexes reaching early endosomes could either enter a recycling pathway to the cell surface or be transported to late endocytic compartments. However, Ii has been shown to be very sensitive to proteases (59, 60). The fact that cell surface Ii is intact, as well as the rapid kinetics of transport from the TGN to the different compartments when cells were incubated with 20 μM nocodazole (Fig. 9, e and f). Cells were pulse labeled and chased for 30 min at 37°C before cell surface biotinylation at 4°C. Thereafter, DCs were warmed up to 20°C for 30 min with or without nocodazole and chased for indicated times at 37°C, still in the presence or absence of nocodazole.
FIGURE 8. Localization along the endocytic pathway of an internalizing transmembrane protein in the presence or absence of 20 μM nocodazole. A–C, DCs were incubated, in the presence of gold-labeled F(ab')2 fragments of an anti-CD1b mAb, for 60 min at 4°C, 30 min at 20°C, and finally 5 min at 37°C in the absence of nocodazole. A, Visualization of gold-labeling of an early endosome (center) as well as a multivesicular/late endosome (left) and a MIIC (right). B and C show a gold-labeled multivesicular/late endosome and a typical gold-labeled multilamellar MIIC, respectively. D–F, DCs incubated as in A–C but in the presence of nocodazole show gold-labeling of early endosomes (D) and of some multivesicular/late endosomes (E), while gold-labeled MIIC are only rarely observed (F). Note in F the abundance of homogenous-appearing unlabeled MIIC. These MIIC, whose content in MHC-II molecules has not been determined in this experiment, are clearly visible in G and H. G–H, Lowicryl K4 m-embedded DCs were incubated successively with 1) a rabbit anti-DRα-chain Ab and 2) a gold-labeled goat anti-rabbit Ab. Homogenous-appearing MIIC are numerous in the monocyte-derived DCs (G), which contain, in some areas, typical multilamellar MIIC (H).
plasma membrane of mature sialylated Ii forms, would imply that newly synthesized αβIi complexes reside for a very short time in endosomes before cell surface appearance in DCs.

Interestingly, we demonstrate that within 30 min after the internalization, most of the cell surface αβIi complexes (74%) are already converted into αβ dimers, part of them being stable in SDS. However, we observed that, although the conversion of most cell surface αβIi complexes into αβ dimers was relatively rapid, the deposit of these dimers at the plasma membrane of DCs was progressive, reaching its completion only 2 h after αβIi internalization. These results are consistent with a rapid sorting and active targeting of cell surface αβIi complexes to processing compartments such as MIIC. In these compartments, the rapid dissociation of Ii from internalized αβIi complexes will give rise to αβ dimers. Then, the progressive return of newly formed αβ dimers to the plasma membrane would suggest that egress from MIIC is a rate-limiting step in the delivery of MHC-II molecules at the cell surface. Alternatively, endocytosed αβIi complexes could also be converted into αβ dimers in different endocytic compartments (11), including early endosomes (12). The fact that nocodazole depolymerization of microtubules blocks the access of αβIi complexes to αβ-dimer-rich compartments without affecting the conversion of αβIi into αβ-peptides complexes favors this latter hypothesis. Although recent studies performed on another type of MHC-II-expressing cell do not argue in favor of the transport of αβ dimers from different endocytic compartments to the plasma membrane (61), it was shown that peptide loading by itself can take place in endocytic compartments upstream from the MIIC (12), independently of polymerized microtubules (62). A process in which a large proportion of newly formed αβ dimers originating

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*Immunogold labeling on DCs was performed as described in Materials and Methods. Cells were treated as shown in Figure 8. Briefly, they were incubated in the presence of gold-labeled anti-CD1b for 1 h at 4°C, then 30 min at 20°C in the absence or presence of nocodazole, and finally 5 min at 37°C with the same treatment (+/- nocodazole). The values represent percentages of the total number of gold particles counted in 60- and 40-cell profiles, respectively, for the two conditions.

FIGURE 9. Effect of microtubule depolymerization on transport of internalized αβIi complexes. DCs were incubated with RaHuII at 4°C for 30 min (a and b), then washed and incubated at 37°C for 30 min without (c and d) or with (e and f) nocodazole (see Materials and Methods). Cells were processed for immunofluorescence staining as described in Materials and Methods. They were then processed for confocal laser scanning microscopy, a, c, and e represent Texas Red labeling (showing internalized RaHuII); b, d, and f represent FITC labeling (showing MHC-II molecule staining). Representative images of three independent experiments corresponding to superpositions of four medial optical slices are shown (bars: 10 μm).

FIGURE 10. Depolymerization of microtubules do not affect the kinetics of conversion of surface αβIi into SDS-stable and unstable αβ dimers. DCs were pulse labeled (10 min), chased for 30 min, and surface biotinylated. Cell surface molecules were then allowed to internalize by further incubation with or without nocodazole (+ or −NOCO) at 20°C for 30 min. MHC-II molecules behavior was then observed as described in Figure 4, DCs being incubated at 37°C for the indicated time with half of the cells treated with nocodazole. A. The samples were boiled before run on the SDS-PAGE. Total and biotinylated fractions are shown (Total and Biot.). B. The samples were not boiled to test the SDS stability of αβ dimers. Only the biotinylated material is shown.
from cell surface-internalized αβIi complexes can bind peptides in endocytic compartments with different proteolytic and physiologic properties would extend the range of peptides presented to CD4+ T cells by these APCs. Consequently, this would reinforce the suspected “sentinel” role of DCs throughout the body by helping these cells to identify and activate trace amounts of Ag-reactive T cell clones. This latter hypothesis does not exclude a predominant accumulation of most endocytic MHC-II molecules in the MHC of DCs at the steady state, nor does it preclude the possibility that αβ dimers continuously recycling between endocytic compartments and the plasma membrane of these cells can bind newly encountered peptides (30).

It was already known that newly synthesized αβIi complexes can reach the plasma membrane before rapid internalization and targeting to the endosomal compartments. Support for this hypothesis was obtained in studies showing that endosomal targeting signals present in the cytoplasmic domains of all Ii isoforms also function as internalization signals (63, 64). In B lymphocytes, this results in the rapid endocytosis of class II αβIi from the cell surface (57). The fraction of the αβIi complexes reaching the cell surface does not contain p35, revealing that isoforms of the Ii chain may modulate the different transport pathways taken by newly synthesized αβIi complexes en route to processing compartments (47). However, the relative importance of these different pathways in B cells remains to be accurately quantified.

Remarkably, we found a differential level of expression for the three main isoforms of Ii in the three different MHC-II-expressing cell types. We show that, like murine Langerhans cells (65, 66), human immature DCs derived in vitro from blood monocytes naturally express higher levels of the alternatively spliced p41 isoform of Ii. An attractive hypothesis would be that the exon 6b, which codes for additional luminal amino acid residues exclusively present in p41, accounts for the enhancement of the transport of MHC-II molecules between Golgi and the plasma membrane in DCs. In this respect, p41 has been found to act as a better potentiator of Ag presentation than p31 (67). It has been suggested that p41 could do so by modifying the transport and/or processing of MHC-II molecules (68). However, such a hypothesis is not consistent with the generally accepted models of intracellular targeting of membrane proteins in which sorting or retention signals are found in cytoplasmic tails. Moreover, if the presence of p41 in the nonameric complexes leaving the TG-N (45) was responsible for delivery at the cell surface, nonamers reaching the plasma membrane should contain at least one p41. From our estimation this should lead to a twofold enrichment of p41 in the surface fraction of αβIi complexes, which did not occur (not shown). Finally, we cannot formally exclude that the direct trafficking of newly synthesized αβIi complexes to the cell surface of immature DCs is due to their high level of synthesis saturating the specific machinery that targets them to MHC. However, overexpression of these complexes in HeLa cells (37), corresponding to about 10 times the rate of synthesis in DCs, does not lead to an equivalent amount of αβIi transiently expressed at the plasma membrane. A better knowledge about the cytosolic machinery that interacts with the Ii cytoplasmic tails and that mediates the intracellular targeting of MHC-II molecules will clarify the respective roles of the different Ii isoforms and/or the differences in the intracellular trafficking of MHC II molecules in different cell types.

The assay described here, which allowed us to follow and quantify the conversion of cell surface αβIi into functional αβ-peptide complexes and their arrival to the plasma membrane, designates the human monocyte-derived DCs as the appropriate material to study the mechanisms underlying the transport of newly synthesized MHC-II molecules via the plasma membrane and its role in Ag presentation.

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