AP2 Clathrin Adaptor Complex, but Not AP1, Controls the Access of the Major Histocompatibility Complex (MHC) Class II to Endosomes

Marc Dugast†‡§, Hélène Toussaint‡§, Christelle Dousset, and Philippe Benaroch¶
From INSERM U520 Institut Curie, Section de Recherche, 75005 Paris, France

Received for publication, February 4, 2005, and in revised form, February 28, 2005
Published, JBC Papers in Press, March 4, 2005, DOI 10.1074/jbc.M501357200

Class II molecules of the major histocompatibility complex (MHC II) follow a particular intracellular trafficking, during which they are loaded with antigenic peptides and transported to the surface of the cell where they present these peptides (reviewed in Refs. 1–3). MHC II molecules are escorted through the biosynthetic pathway by the invariant chain (Ii). The association of Ii with MHC molecules begins just after synthesis in the endoplasmic reticulum, with binding of the MHC II α- and β-subunits to Ii homotrimer to form nonameric complexes referred to as immature MHC II. Ii is a type II membrane chaperone, promoting correct folding of the complexes and preventing premature peptide binding by occupying the peptide binding groove with its CLIP region. Following transfer to the Golgi apparatus and the trans-Golgi network (TGN), the αβIi complexes to endosomes. These signals also promote rapid internalization via clathrin-coated pits at the plasma membrane (8, 13). It has been suggested that there are two possible pathways for αβIi trafficking. Newly synthesized MHC II complexes may be sorted by an AP1-dependent pathway in the TGN, enabling them to reach the endosomes directly, bypassing the cell surface. Alternatively, such complexes may initially be directed to the cell surface and then rapidly internalized via an AP2-dependent pathway. In both cases, the signals carried by the cytoplasmic tail of Ii are required for sorting, which involves clathrin. Both pathways are consistent with early endosomes acting as intermediates in the transport of αβIi complexes to antigen-processing compartments (14, 15).

The possible existence of an AP1-dependent pathway is supported by the observation that αβIi complexes can recruit cytosolic AP1 on membranes (16). Importantly, this recruitment requires the three chains (α, β, and Ii) and is strictly dependent of the cytoplasmatic tail of Ii, which carries the two leucine-based motifs. These motifs bind both AP1 and AP2 in vitro, but have a higher affinity for AP1 (17–19). Usage of a potent inhibitor of vacuolar H+-ATPases in human B cells led to the conclusion that most αβIi complexes are targeted directly to the endocytic pathway without passing via the cell surface (20). A similar

bran. In this pathway, Ii is degraded in an ordered sequence reaction ending with the CLIP region, which is still lodged in the peptide binding groove (see Ref. 4). CLIP is then exchanged for peptides derived from antigens in the endocytic pathway, with the help of the HLA-DM chaperone. Once loaded with peptide, MHC II molecules are considered mature and are transported to the cell surface, where they present their bound peptides to CD4+ T cells. Thus, newly synthesized MHC II must be sorted at various intracellular stations to become mature and functional. The molecular machineries involved in these sorting processes are mostly unknown or a matter of debate. In particular, newly synthesized αβIi complexes can deviate from the constitutive exocytosis pathway to reach endocytic compartments at two cellular locations: the TGN or the plasma membrane, where various clathrin adaptors (AP) select cargo for inclusion in clathrin-coated vesicles.

AP are heterotetramers and are present on various membranes. AP1 consists of γ-, β1-, μ1-, and ε-subunits, and is found on the TGN and endosomes, whereas AP2 contains α-, β2-, μ2-, and ε2-subunits and is present on the plasma membrane (5). AP3 has been implicated in the transport of lysosomal proteins, but intracellular trafficking of MHC II and associated Ii appears to be normal in both human and mouse cells deficient for AP3 (6, 7).

Two independent leucine-based signals (Leu7–Ile8 and Pro15–Leu17) present in the short cytoplasmatic tail of Ii have been extensively characterized (8–12). They are required for the sorting of αβIi complexes to endosomes. These signals also promote rapid internalization via clathrin-coated pits at the plasma membrane (8, 13). It has been suggested that there are two possible pathways for αβIi trafficking. Newly synthesized MHC II complexes may be sorted by an AP1-dependent pathway in the TGN, enabling them to reach the endosomes directly, bypassing the cell surface. Alternatively, such complexes may initially be directed to the cell surface and then rapidly internalized via an AP2-dependent pathway. In both cases, the signals carried by the cytoplasmatic tail of Ii are required for sorting, which involves clathrin. Both pathways are consistent with early endosomes acting as intermediates in the transport of αβIi complexes to antigen-processing compartments (14, 15).

This paper is available on line at http://www.jbc.org

* This work was supported by grants from the SIDACTION and Agence Nationale de Recherche sur le SIDA (ANRS). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The online version of this article (available at http://www.jbc.org) contains Supplementary Materials.
‡ Both authors contributed equally to this work.
§ Recipient of a fellowship from SIDACTION.
¶ Recipient of a fellowship from INSERM-region.
To whom correspondence should be addressed: Institut Curie INSERM U520, 28 rue d’Ulm, 75248 Paris Cedex 05, France. Tel.: 33-1-4234-6432; Fax: 33-1-4234-6438; E-mail: benaroch@curie.fr.
1 The abbreviations used are: MHC II, class II molecules of the major histocompatibility complex; BFA, brefeldin A; EGF, epidermal growth factor; Ii, invariant chain; MFI, mean fluorescence intensity; MPR, mannose-6-phosphate receptor; PE, phycoerythrin; shRNA, short hairpin RNA; Tf, transferrin; TGN, trans-Golgi network; MES, 4-morpholineethanesulfonic acid; GFP, green fluorescent protein; mAb, monoclonal antibody.
conclusion was drawn from a study showing that the maturation rate of MHC II was unaffected by the expression of a dominant negative mutant of the dynamin (21). However, another study using the same dynamin mutant generated opposite conclusions (22). This discrepancy may be caused by differences in experimental set-up and in the levels of expression of the dynamin mutant and MHC II chains.

The use of dominant negative mutants such as the hub mutant of the clathrin heavy chain represented an attractive means to block clathrin-dependent pathways. Accumulation of the hub mutant in HeLa cells before the expression of αβII complexes did not prevent some of these complexes from reaching the endocytic pathway, suggesting the involvement of a clathrin-independent pathway (23). However, previous leakage of the block imposed by the hub mutant and a lack of quantification for this transport step reduced the significance of these data. Indirect evidence for an AP2-dependent pathway is provided by several studies reporting the rapid internalization of surface αβII complexes in various cell types (13, 24–26).

Recent studies based on RNA interference have challenged our view of AP-dependent and AP-independent pathways (5, 27). The clathrin-mediated endocytosis of several receptors, including the EGF receptor, may occur in the absence of AP2 (28, 29), although the EGF receptor has been shown to bind AP2 (30). Because the cytoplasmic tail of a given transmembrane protein has affinity for an AP subunit, this does not mean that AP is necessary for sorting of the transmembrane protein concerned. This may be the case for the cytoplasmic tail of Ii, which has been shown by a variety of techniques to bind AP1 (16, 17, 31), although no study has demonstrated a direct role for this complex in the sorting of αβII. Furthermore, our view of AP recruitment by cytoplasmic tails is influenced by the many experiments that have been performed with purified subunits. Recent data obtained with three-hybrid technology have indicated that the binding of a single AP subunit to membrane proteins does not necessarily indicate AP recruitment in vivo (32).

Thus, despite the many studies performed in various cell types, the precise pathway by which αβII complexes reach the endocytic system remains unclear. We used an RNA interference strategy to obtain insight into the mechanisms involved.

Materials and Methods—Plasmids and Transfections—Plasmids were synthesized (Proligo, Sigma Genosys), annealed, and ligated into pSUPER (Clontech). HeLa-CIITA cells were transfected with pSUPER constructs by electroporation, using a Bio-Rad gene pulser II as previously described (34). We transfected 8 × 10^6 cells at 230 V and 975 microfarad, with up to 27.5 μg of the various expression vectors mixed with 2.5 μg of PEGFP (Clontech), giving a total of 30 μg of DNA per electroporation. Cell cultures were split after 2 days and used 4 days after transfection.

Antibodies and Reagents—The following mAbs were used: anti-α-tubulin (Merck), anti-β2 receptor (Santa Cruz Biotechnology), anti-MPR300 (clone 2G11 from Abcam), anti-γ-adaptin (clone 100/3 from Sigma-Aldrich), and anti-μ2-subunit (BD Biosciences). Affinity-purified anti-MPR46 rabbit antibodies were kindly provided by P. Schu (University of Göttingen, Göttingen, Germany). The following anti-Ii-specific antibodies were used: the Ab PIN1 (36), the mAb BU45 (37), the mAb By2 coupled to PE (Santa Cruz Biotechnology) and a rabbit immune serum kindly provided by J. Salamero (Curie Institute, Paris). The following anti-HLA-DR mAbs were used: TC36 (38), L243 (39), and IBI (40). In addition, we used the MHC I-specific mAb W6/32 (41). Secondary antibodies labeled with AlexaFluor 647 or AlexaFluor 488 were purchased from Molecular Probes. Cy3-labeled secondary antibodies were purchased from Jackson Immunoresearch Laboratories. Human transferrin (Sigma-Aldrich) was coupled to AlexaFluor 647, using a protein labeling kit from Molecular Probes.

Flow Cytometry—For surface staining, cells were detached and stained with various mouse mAbs in FACS buffer (phosphate-buffered saline supplemented with 3% fetal calf serum and 0.05% azide) on ice. Flow cytometry was performed on a FACScalibur machine, with analysis by CellQuest software (BD Biosciences).

Biochemical Analyses—Cell surface biotinylation followed by immunoprecipitation was carried out as previously described (34). All immunoprecipitations of cell lysates were preceded by preclearing once with normal mouse and rabbit sera mixed with protein A- and G-coupled to Sepharose beads (Amersham Biosciences) and twice with only the beads. Then specific immunoprecipitations on the cell lysates were performed either sequentially, or in parallel. A preclear with protein A- or G-coupled to Sepharose beads was performed between two sequential immunoprecipitations. For Western blotting, cells were lysed in Nonidet P-40 lysis buffer (0.5% Nonidet P-40, 50 mM Tris-HCl, pH 7.4, 5 mM MgCl2) supplemented with 1 mM phenylmethylsulfonyl fluoride and 10 mM N-ethylmaleimide (Pierce). Lysates were diluted in reducing Laemmli buffer and heated at 95 °C for 5 min before analysis by SDS-PAGE in 10 or 12% polyacrylamide gels, or in precast 10% acrylamide gels (Invitrogen). The protein bands were transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was incubated in blocking solution, followed by primary and, finally, horseradish peroxidase-labeled goat anti-mouse or anti-rabbit IgG secondary antibody (Jackson Immunoresearch Laboratories). Antibody binding was detected with an enhanced chemiluminescence (ECL) kit (Roche Applied Science).

Endocytosis Assays—HeLa-CIITA cells transfected with pSUPER plasmids were harvested after 4 days and incubated with Tf-AlexaFluor 488 or with the By2-PE mAb for 30 min at 4 °C, washed, and shifted to 37 °C for various periods of time in culture medium supplemented with 10 mM Hepes. The medium was removed by washing and half the samples were washed in 25 mM glycine-HCl, 125 mM NaCl, pH 2.8 and rapidly neutralized with 25 mM Tris, pH 10. Samples were then washed three times with PBS, stained with various mouse mAbs in FACS buffer (phosphate-buffered saline supplemented with 3% fetal calf serum and 0.05% azide) on ice, and stained with various mouse mAbs in FACS buffer (phosphate-buffered saline supplemented with 3% fetal calf serum and 0.05% azide) on ice. Forward/side light scatter. Events corresponding to at least 5000 live cells positive for GFP were accumulated per sample. Flow cytometry was performed on a FACScalibur machine, with analysis by CellQuest software (BD Biosciences).

Fluorescence—Two days after transfection, we seeded glass coverslips in 24-well plates with cells (1 × 10^5). Lysosomes were stained by incubating the cells for 90 min with Lysotracker Red DND99 (Molecular Probes) at 37 °C. Early endocytic compartments were labeled by incubation with Tf-AlexaFluor 647 for 5 or 10 min. Fixation, staining, and confocal analysis were performed as previously described (42). Cells were

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa-CIITA cells are HeLa cells expressing CIITA in a stable manner. This transactivator drives the expression of genes encoding several proteins related to MHC II: HLA-DRo and β2, HLA-DMo, and β. These cells were maintained in culture as previously described (34). DG75 is an EBV-negative human B cell line (35); it was maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum and antibiotics.

Plasmids and Transfections—Synthetic oligonucleotides (64-mers) containing the human γ-adaptin (ACGGAATTAAGAAAGTGGT) or μ2 (GGGATCTTGCTTGGGTCA) target sequences for cloning in pSUPER were

APE-dependent Entry of MHC Class II in Endosomes
were fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline. After quenching in 0.1M glycine in phosphate-buffered saline, permeabilization, and saturation were performed in phosphate-buffered saline supplemented with 0.2% bovine serum albumin and 0.05% (w/v) saponin. Cells were incubated with primary antibodies for 1 h and with secondary antibodies for 30 min. The coverslips were mounted in Mowiol on glass slides and analyzed with a TCS4D scanning laser confocal microscope (Leica Microscopy and Scientific Instruments, Heerbrugg, Switzerland).

**RESULTS**

**Knockdown of AP1 and AP2 Expression, Using Short Hairpin RNA**—We depleted cells of specific proteins by inserting sequences encoding shRNA molecules specific for the µ2-chain of AP2 and for the γ-chain of AP1 into pSUPER (33). These correspond to previously characterized and validated small interfering RNA (28, 43), respectively. We analyzed the kinetics of protein extinction in HeLa-CIITA cells transfected with the various pSUPER constructs by Western blotting (Fig. 1A). These extinctions were specific, as tubulin levels were similar in all samples (Fig. 1A). Furthermore, AP2-µ2 depletion did not affect AP1-γ levels and vice versa (not shown).

Transfection of siRNA can activate interferon-stimulated genes and thereby might modify MHC expression (44). Total levels of Tf receptor, MHC I and II chains were similar in our three cell populations (see Supplemental Fig. S1A) indicating that transfection of our pSUPER constructs does not affect the production of MHC proteins. We analyzed subsequent experiments 3–4 days after transfection. Adding a plasmid encoding EGFP (one-twelfth the total amount of DNA used) to the various pSUPER constructs for electroporation made it possible to estimate electroporation efficiency (generally around 80%) and to identify the transfected cells in flow cytometry and immunofluorescence analyses.

We studied the functional effects of AP1-γ depletion by localizing both mannose-6-phosphate receptors (MPR) 46 and 300, by immunofluorescence (Fig. 1B). The distribution of MPR300 was not affected by either of the knockdowns, whereas the depletion of AP1-γ, but not of AP2-µ2, shifted MPR46 from a perinuclear distribution to a totally scattered distribution. These observations are consistent with previous reports indicating that, in fibroblasts derived from µ1A-deficient mice, MPR46 are not recycled from endosomes back to the TGN and therefore accumulate in endosomes, whereas the distribution of MPR300 is not affected to the same extent (45, 46). We investigated the functional consequences of AP2-µ2 depletion by

![FIG. 1. Effects of depleting AP2-µ2 and AP1-γ in HeLa-CIITA cells. A, HeLa-CIITA cells were transfected with the indicated pSUPER constructs. They were harvested at the time points indicated and lysed. We loaded equivalent amounts of protein for each cell lysate onto a gel, subjected them to SDS-PAGE, and Western-blotted the gels with antibodies against the indicated proteins. B and C, confocal sections of HeLa-CIITA cells 4 days after transfection with pSUPER constructs, stained for the indicated proteins. For transferrin staining, cells were exposed to Tf-AlexaFluor 647 for 5 min at 37 °C before fixation and analysis. Similar data were obtained in at least three independent experiments.](http://www.jbc.org/doi/suppl/10.1074/jbc.M118.000270)

**FIG. 1. Effects of depleting AP2-µ2 and AP1-γ in HeLa-CIITA cells. A, HeLa-CIITA cells were transfected with the indicated pSUPER constructs. They were harvested at the time points indicated and lysed. We loaded equivalent amounts of protein for each cell lysate onto a gel, subjected them to SDS-PAGE, and Western-blotted the gels with antibodies against the indicated proteins. B and C, confocal sections of HeLa-CIITA cells 4 days after transfection with pSUPER constructs, stained for the indicated proteins. For transferrin staining, cells were exposed to Tf-AlexaFluor 647 for 5 min at 37 °C before fixation and analysis. Similar data were obtained in at least three independent experiments.**
assessing the internalization of Tf-AlexaFluor 647 by confocal microscopy. As expected (28), the depletion of AP2, but not of AP1, totally inhibited the uptake of Tf-AlexaFluor 647 over a 5-min period (Fig. 1C). The same held true for longer periods of time (not shown, see kinetics below). We conclude that our pSUPER constructs efficiently knocked down the production and expression of AP1 and AP2.

**Fig. 2. Effects of depleting AP2-μ2 and AP1-γ on Ii cell surface expression.** A, HeLa-CIITA cells cotransfected with the indicated pSUPER and pEGFP (one-twelfth the total DNA electroporated) were analyzed 4 days after transfection by Western blotting (upper panels) for the presence of the indicated proteins and by flow cytometry for surface expression of the indicated proteins, using PE-conjugated secondary antibodies. Typical flow cytometry profiles are presented for each cell population for Ii staining (middle panels). We used R1 gating on GFP+ cells to determine MFI for each marker, normalized with respect to the MFI obtained with control pSUPER-transfected cells. The histograms show data obtained from six independent experiments with S.E. Asterisk, the MFI obtained was 57 ± 7. B, similar experiments performed with the human B-cell line DG75. The histograms show data obtained from three independent experiments with S.E. Asterisk, the MFI obtained was 44 ± 16.

Effects of AP1 and AP2 Depletion on Ii Cell Surface Expression—We analyzed the potential involvement of AP1 and AP2 complexes in MHC II trafficking by transfecting HeLa-CIITA cells with pSUPER-CTL, -AP1-γ, or -AP2-μ2, together with pEGFP (one-twelfth the total amount of DNA), and using flow cytometry to analyze cell surface levels of Ii, Tf receptor, MHC I, and mature MHC II (Fig. 2A). The R1 gating used on GFP+
cells for calculation is shown in the middle panels of Fig. 2A, which also show that HeLa-CIITA control cells express very low levels of surface Ii. AP2-μ2 knockdown induced an increase in Tf receptor expression by a factor of five and a dramatic increase in TfII surface expression (by a factor of 57). In contrast, AP1-γ knockdown increased the surface expression of the Tf receptor by a factor of 1.5 and of II by a factor of four. Surface levels of MHC I were only slightly reduced by the depletion of AP1-γ or AP2-μ2 (Fig. 2A). Similarly, surface levels of mature MHC II were largely insensitive to the depletion of AP1-γ or AP2-μ2 (Fig. 2A).

We carried out similar experiments with DG75, a human EBV-negative B-cell line, to extend these results to another cell type. The expression of AP1-γ and AP2-μ2 was efficiently and specifically knocked down in DG75 cells (not shown). As observed in HeLa-CIITA cells, the depletion of AP2-μ2 led to a large increase (by a factor of 44) in surface II expression in DG75 cells, whereas AP1-γ depletion had a much weaker effect (increase by a factor of two) (Fig. 2B). Surface levels of MHC I and of mature MHC II were largely unaffected by these protein depletions (Fig. 2B). We conclude that, in two different cell types, AP2 depletion led to a major increase in Ii cell surface expression with no effect on cell surface levels of mature MHC II.

A possible explanation of these data is that, in the absence of AP2, a fraction of the II pool reaches the cell surface without associated MHC II α- and β-chains. We tested this hypothesis by carrying out cell surface biotinylation experiments in cells transfected with pSUPER constructs. The cell surface was first biotinylated. We then lysed the cells, immunoprecipitated the relevant proteins, and carried out Western blotting with streptavidin-peroxidase detection. Immunoprecipitation with the 1B5 mAb, which is specific for DRα, revealed that cells depleted of AP2-μ2 had larger amounts of DRα associated with the DRβ chain than did control cells and AP1-γ-depleted cells (Fig. 3A). However, comigration of the II and DRα chains prevented clear identification, for which alternate immunoprecipitation was required. Surface II and DRβ chains were clearly detected in the anti-II precipitate from AP2-μ2-depleted cells, indicating that surface II was associated with DRβ and, therefore, probably with DRα (Fig. 3B). On longer exposures, II and DRβ chains were also detected in the control and AP1-γ-depleted lanes, consistent with the flow cytometry results. Interestingly, we did not detect any II degradation fragments in anti-II precipitates, suggesting that αβIi complexes that accumulate at the cell surface in the absence of AP2 did not travel through the endosomes. The supernatants of the anti-II precipitates were analyzed sequentially with anti-DRαβ (L243) and anti-Tf receptor (H68.4) mAbs (Fig. 3B). Consistent with flow cytometry data, DRα and β levels were similar in all three cell types, whereas Tf receptor levels were slightly increased by AP2 and more strongly increased by AP2-μ2 depletion. Fig. 3C shows that no II was detectable in anti-II precipitates, following the removal of all mature (i.e., peptide-loaded) and immature HLA-DR complexes by sequential immunoprecipitation with the L243 and T636 mAbs, respectively. T636 is specific for the DRαβ complex, with or without II, and cannot recognize any of the three chains alone (47). All II molecules that reach the cell surface are therefore probably associated with DRα and DRβ chains, and correspond to αβIi complexes. Thus, AP2-μ2 depletion results in a large increase in the number of αβIi complexes on the cell surface, with no major effect on the number of mature αβ complexes. In contrast, AP1-γ depletion has only limited effects on αβIi and αβ complexes at the cell surface.

Fate of Surface αβIi Complexes—We monitored the fate of αβIi complexes on the cell surface by means of an uptake assay using an anti-II mAb (By2) directly coupled to PE. Control cells and cells depleted of AP2-μ2 or AP1-γ were stained with By2-PE or with Tf-AlexaFluor 647 for 30 min at 0 °C. The cells were washed and shifted to 37 °C for various lengths of time. Half of each sample was washed with an acidic buffer to remove surface-bound mAb or Tf. Transfected cells were analyzed by flow cytometry, with gating on the basis of GFP expression (Fig. 4). Tf was rapidly internalized in control cells and in AP1-γ-depleted cells, reaching the cell surface by 5 min. Thereafter, recycling and Tf detachment from its receptor at low pH resulted in a gradually decrease in levels of internalized Tf in both cell populations (Fig. 4A). The II-specific mAb By2-PE was also decreased in levels of internalized Tf in both cell populations (Fig. 4A).
raptly internalized in control cells and in AP1-γ-depleted cells, with 50% internalization achieved within 3 mins (Fig. 4B). Over time, almost 100% of the By2-PE mAb accumulated within the cells of both populations. In sharp contrast, the uptake of Tf and By2-PE was completely blocked in AP2-depleted cells (Fig. 4). These data suggest that, in AP2-depleted cells, αβII complexes accumulate at the cell surface because of impaired internalization.

The transport of αβII complexes within the endocytic pathway is reflected in the sequential degradation of II. We therefore followed the fate of αβII complexes in the endocytic pathway by analyzing steady-state levels of II and its degradation products in lysates from HeLa-CIITA and DG75 cells (Fig. 5). AP2-depleted cells and control cells behaved similarly, whereas AP1 depletion resulted in higher levels of full-length, unde-graded II. In addition, lysates from HeLa-CIITA cells contained Ii degradation fragments, which were more abundant in the absence of AP1. Thus, AP2 depletion induces a large increase in the number of surface αβII complexes but does not modify total II levels at steady state, suggesting that there are compensatory mechanisms ensuring the normal degradation of this chaperone. AP1 depletion leads to a slight increase in the number of αβII complexes at the cell surface and to an accumulation of II and its degradation products.

We followed the fate of surface αβII complexes after internalization by incubating transfected cells with anti-II mAb and Tf-Alexa-647 for 10 min at 37 °C. Confocal microscopy analysis showed that, in control and AP1-depleted cells, anti-II mAb was in part colocalized with Tf, suggesting that surface αβII complexes had reached early endosomes (Fig. 6A). When internalized anti-II mAb was chased for 90 min at 37 °C, a significant proportion of mAb staining was co-distributed with LysoTracker in control cells, suggesting that αβII complexes had reached lysosomal compartments by that time (Fig. 6B). In AP1-γ-depleted cells, anti-II mAb was often present in large endocytic structures, which were also partly colocalized with LysoTracker. In contrast, AP2-depleted cells were frequently round and did not internalize anti-II mAb or Tf from the plasma membrane at any time. These data indicate that surface αβII complexes reach lysosomal compartments in the absence of AP1 whereas they remain at the cell surface for at least 90 min in the absence of AP2. However, steady-state surface levels of mature MHC II were largely unaffected by AP2 depletion.

Generation of Mature MHC II Complexes—The knockdown of expression for the AP1-γ- and AP2-μ2-subunits was maximal only after a few days. Over these days, mature MHC II complexes were probably produced at a close to normal level because of the remaining functional adaptors available. As surface MHC II molecules have a very long half-life, usually around 40 h (48), assessing steady-state levels of MHC II may underestimate the effects of depletion. We carried out a morphological pulse-chase experiment, based on BFA exposure, to avoid this problem. BFA blocks the supply of nascent MHC II molecules to the endocytic pathway by disrupting the secretory pathway and inhibits the formation of most peptide-MHC-II complexes in late endocytic compartments (49–52). Preliminary experiments showed that overnight exposure to BFA was required for the efficient depletion of mature MHC II complexes from the endocytic pathway of HeLa-CIITA cells. Transfected cells exposed to BFA overnight were washed and chased for various periods of time, at the end of which, cells were processed for the detection of mature peptide-loaded complexes by immunofluorescence, with the L243 mAb. Confocal microscopy analysis revealed that, following BFA exposure, most of the L243-reactive material had disap-
peared from its intracellular location. This was the case in all three cell types analyzed (Fig. 7 at time 0). After a 6-h chase, L243− vesicles were clearly observed in control cells and AP1-α,β-depleted cells, reflecting the arrival of a new supply of nascent MHC II in the endocytic pathway and the loading of these molecules with antigenic peptides. In sharp contrast, AP2-depleted cells did not recover after 6 h of chase (Fig. 7). However, after a 24-h chase, all cells had recovered large numbers of intracellular L243+ vesicles (Fig. 7). These data suggest that generation of most of the newly formed MHC II-peptide complexes after release of the BFA blockade required functional AP2. The absence of AP2 might, over extended time, lead either to some internalization (if the block imposed by RNA interference is not perfect) or to the use of alternative transport routes, thereby merely delaying the production of mature MHC II-peptide complexes.

**DISCUSSION**

In this study, we addressed the long-debated question of which AP is involved in the sorting of MHC II complexes to the endocytic pathway and by which route they reach this pathway. Two opposing hypotheses have been put forward concerning the way in which nascent MHC II α- and β-chains associated with Ii reach the Golgi apparatus; they may be directly targeted to endosomes by means of an AP1-dependent pathway bypassing the cell surface, or they may first be directed to the plasma membrane and then rapidly internalized via an AP2-dependent pathway. Many studies on the potential involvement of AP complexes in the sorting and exact intracellular
routing of MHC II molecules have been published in the last 10 years, with conflicting conclusions. Only indirect evidence has been accumulated so far, which favors the use of the AP1-dependent pathway.

We used a RNA interference approach to knockdown AP1 and AP2 expression, and to evaluate the consequences of this knockdown for MHC II trafficking. To our surprise, AP1 knockdown had only minor effects on MHC II trafficking as compared with AP2 knockdown. As expected, transfection with a plasmid encoding a shRNA specific for AP2-μ2 efficiently knocked down μ2 expression and strongly inhibited Tf endocytosis, as shown by confocal microscopy and flow cytometry. Levels of Tf receptors at the surface were increased (by a factor of 2–5) but the major modification in the two cell models studied concerned the cell surface expression of αβII complexes, which was markedly increased (by a factor of about 50), as shown by flow cytometry and biochemical analysis. AP2 knockdown also efficiently inhibited the uptake of an anti-Ii antibody, suggesting that the internalization of αβII complexes at the plasma membrane via an AP2-dependent pathway is an important step in MHC II trafficking. This conclusion is supported by a quantitative study on Ii internalization, which suggested that all αβII complexes briefly appear on the cell surface before becoming mature MHC II (26). The two leucine-based signals carried by the cytoplasmic tail of Ii are potent endocytosis signals that may function independently (8). Consistent with our interpretation, the mutation of these signals affects their capacity to bind AP2 in vitro and the capacity of the molecule to undergo endocytosis in vivo (19). Finally, AP2 depletion delayed the appearance of mature MHC II in intracellular compartments, suggesting that AP2 plays a central role in the trafficking and maturation of MHC II molecules.

The efficiency of AP1-γ depletion induced by specific shRNA was demonstrated by the loss of Golgi localization for MPR46, with no effect on the distribution of MPR300. The cytoplasmic tail of MPR300 is known to have a higher affinity than that of MPR46 for GGAs (53). This may account for the weaker effect of AP1 depletion on the distribution of MPR300 than on the distribution of MPR46. Our data are also consistent with those of a previous study on fibroblasts derived from mice deficient for AP1, in which the overall distribution of MPR300 was unaffected (46). Surface levels of Ii were slightly increased by AP1 knockdown (by a factor of about four in HeLa-CIITA cells and about two in DG75 cells). However, as αβII complexes are poorly expressed at the surface of normal cells, these increases correspond to relatively low numbers of complexes. AP1 depletion also resulted in similar increases in the total amount of full-length Ii in HeLa-CIITA and DG75 cells, as shown by Western blotting of cell lysates. Moreover, cell lysates from HeLa-CIITA contained Ii degradation fragments that increased in abundance following AP1 depletion, which was not the case for DG75 cells. The difference between HeLa-CIITA and DG75 cells may be caused by the lack of certain proteases in HeLa-CIITA cells, as has been suggested for cathepsin S (54).

The rate of Ii degradation and the production of Ii fragments was normal in the absence of AP1, as shown by metabolic pulse chase experiments (see Supplemental Fig. S1). This suggests that AP1 depletion exerts its effect on Ii degradation very slowly, and therefore has little effect on newly synthesized αβII complexes. AP1 depletion may decrease the efficiency of protease transport by modifying MPR46 trafficking. However, AP1 knockdown did not inhibit general transport within the endocytic pathway because the kinetics and extent of EGF degradation were not affected by AP1 depletion (see Supplemental Fig. S2), consistent with the findings of a recent study (55). Moreover, cysteine protease activities were not affected by the depletion of AP1 or AP2, as shown by experiments with an active site labeling probe (DCG04), which links a biotin to these enzymes for detection on Western blots (Ref. 56 and data not shown). Thus, our data obtained with AP1-depleted cells do not support, although they cannot exclude, the possibility that some of the newly synthesized αβII complexes are routed directly from the Golgi apparatus to the endosomes by an AP1-dependent pathway.

The key question therefore concerns the proportion of MHC II complexes following the AP1- or the AP2-dependent pathways. It is difficult to estimate the proportion of MHC II complexes concerned because (i) MHC II complexes have a long half-life (48) and (ii) RNA interference results in slow depletion of the protein concerned, possibly allowing a compensatory mechanism to take over. We tried to address this question of proportion by analysis in cells at maximum protein depletion. Knockdown was initiated 3 days before overnight exposure of the cells to BFA to eliminate most of the MHC II-peptide complexes from the endocytic pathway. Once BFA had been removed, we monitored the appearance over time of new mature MHC II complexes in intracellular compartments. The depletion of AP2 inhibited this reappearance, whereas that of AP1 did not, strongly suggesting that the major pathway by which nascent MHC II complexes are transported to the endosomes is AP2-dependent. However, beyond a certain level of accumulation at the plasma membrane, αβII complexes probably follow an alternative maturation route. Indeed, 24 h after the removal of BFA, intracellular levels of MHC II molecules were similar in AP2-depleted cells and control cells. In addition, 4 days after transfection with pSUPER AP2-μ2, the intracellular and surface distribution of mature MHC II molecules appeared normal in transfected cells. The rates of Ii degradation (Supplemental Fig. S1) and of mature MHC II production (not shown) did not seem to be affected by AP2 depletion, as shown by metabolic pulse chase experiments. These data appear to conflict with those obtained in BFA experiments. However, if alternative adaptors can compensate for the absence of AP2, routing αβII complexes to endosomes, but at a lower rate, then AP2-depleted cells would appear normal at steady state. The main difference from control cells would be a tendency for αβII to accumulate at the cell surface, which was indeed observed. Thus, the delay imposed by the lack of AP2 may have been too short to be detected in most of our experiments, because of the participation of alternative adaptors. Only BFA blocking and release can generate enough αβII complexes to saturate the alternative pathways and to reveal the sorting defect in the absence of AP2. Importantly, 24 h after BFA release, most of the αβII complexes released from the endoplasmic reticulum had reached the endosomes and been processed into mature MHC II, probably because of alternative pathways of transport to the endosomes.

One could propose to explain our data that newly synthesized αβII complexes pass rapidly through the early endosomes on their way to the plasma membrane using the recycling pathway, as has been shown for some Tfr receptors (57). In the absence of AP2, these “recycling” MHC II complexes would be blocked at the cell surface and accumulate. In the absence of AP1, transport of αβII would pass via the plasma membrane, via the AP2-dependent pathway leading to a slight increase of the surface levels of αβII as observed. To test this hypothesis, we carried out simultaneous depletions of AP1 and AP2 in HeLa-CIITA cells. Half-doses of pSUPER-AP1-γ and pSUPER-AP2-μ2 were co-transfected in HeLa cells. Such cells were compared with single AP-depleted cells that had also received half-doses but only of one of the two plasmids. AP2 depletions.
were efficient whereas AP1 knockdowns were only partial (see Supplementary Fig. S3). Expression of cell surface markers: ii, Tf receptor, MHC I and II, in single AP-depleted cells (Fig S3) was similar to what we observed in cells treated with a full dose of pSUPER plasmid (Fig. 2). Importantly, doubly (AP1+AP2)-depleted cells exhibited cell surface levels of the various markers similar to the ones observed in AP2-depleted cells (Fig. S3), supporting the idea that AP1 has no major role in the trafficking of MHC II. Taken together with the results of the BFA experiments, this also suggests that in AP2-depleted cells, MHC II complexes do not use the AP1 pathway to reach the endosomes but rather use an alternative unknown pathway.

The next benchmark will be to extend these data to MHC II-expressing cells other than HeLa-CIITA and B cell lines. However, our conclusions are supported by published studies using other cell systems. In immature human dendritic cells, most newly synthesized αβII complexes transit via the cell surface, where they are actively internalized (24). Even in human EBV-transformed B cells, which express low levels of surface, where they are actively internalized (24). Even in that

*content of the page*
AP2 Clathrin Adaptor Complex, but Not AP1, Controls the Access of the Major Histocompatibility Complex (MHC) Class II to Endosomes
Marc Dugast, Hélène Toussaint, Christelle Dousset and Philippe Benaroch

J. Biol. Chem. 2005, 280:19656-19664. doi: 10.1074/jbc.M501357200 originally published online March 4, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M501357200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2005/03/10/M501357200.DC1

This article cites 57 references, 37 of which can be accessed free at http://www.jbc.org/content/280/20/19656.full.html#ref-list-1