Expression of Major Histocompatibility Complex Class II Molecules in HeLa Cells Promotes the Recruitment of AP-1 Golgi-specific Assembly Proteins on Golgi Membranes*

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The newly synthesized major histocompatibility complex (MHC) class II molecules, an αβ dimer associated with the Ii invariant chain, must be targeted to endosomal, lysosomal enzyme-rich compartments in order to bind and present immunogenic peptides. The precise route followed by this complex at the exit of the trans-Golgi network, the last sorting station of the biosynthetic pathway, is poorly understood. We show here that overexpression of αβIi complexes in HeLa cells promotes the first step of clathrin-coat assembly in vitro, that is the ARF-dependent translocation of AP-1 Golgi-specific assembly proteins on membranes. In contrast, αβ dimers alone or associated with Ii lacking most of its cytoplasmic domain fail to recruit AP-1. This study strongly suggests that the invariant chain (Ii) is responsible for the AP-1-dependent sorting of the αβ dimers in the trans-Golgi network of HeLa cells and that the MHC class II molecules are, like the mannose 6-phosphate receptors, transported directly from this compartment to endosomes via clathrin-coated vesicles.

The major histocompatibility complex (MHC) class II molecules bind peptides produced by the proteolysis of foreign antigens in endocytic, hydrolase-rich compartments and mediate their presentation to competent T-lymphocytes (1–3). The α and β polypeptides that form the MHC class II complex associate with the invariant chain (Ii) in the endoplasmic reticulum. The whole complex is then transported through the biosynthetic pathway for delivery to endosomal/lysosomal compartments where Ii dissociates from αβ dimers. The invariant chain contains sorting signals in its cytoplasmic domain that confer endosomal/lysosomal targeting to the MHC class II molecules (4–6). While the fate of the MHC class II molecules in the early secretory and in the endocytic pathways has been described extensively, little is known about their vesicular transport at the exit of the trans-Golgi network. Whether the MHC class II molecules are targeted directly to endosomal compartments via clathrin-coated vesicles like lysosomal hydrolases bound to the mannose 6-phosphate receptors (MPRs) (7) or via other types of carrier vesicles, as recently proposed for I-cell disease B-lymphoblasts (8), has been difficult to evaluate.

The first step in the formation of TGN-derived, clathrin-coated vesicles is the translocation of the cytosolic AP-1 Golgi-specific assembly proteins onto membranes of this compartment (9), a process regulated by the small GTPase ARF-1 (10, 11). We have shown previously that the mannose 6-phosphate receptors, the major cargo membrane proteins sorted along this clathrin-dependent pathway, are part of the membrane components that permit the efficient recruitment of AP-1 (12, 13), thereby strongly suggesting that MPR sorting is highly coupled to the first step of clathrin coat assembly. Moreover, efficient AP-1 binding to membranes requires the presence of specific determinants in the MPR cytoplasmic domain (14). The property that the expression of cargo transmembrane proteins triggers AP-1 recruitment has been used here to examine whether the MHC II molecules could be sorted from the trans-Golgi network via an AP-1-dependent pathway. We now report that the expression of the MHC class II molecules in HeLa cells promotes the recruitment of cytosolic AP-1 on membranes and that the cytoplasmic tail of Ii is mostly responsible for this effect.

EXPERIMENTAL PROCEDURES

Overexpression and Detection of MHC Class II in HeLa Cells—HeLa cells were from ATCC and grown in complete medium containing 5% fetal calf serum. HeLa cells were first infected for 30 min with the V7 recombinant vaccinia virus and then transfected with various cDNAs using DOTAP reagent (Boehringer Mannheim) as described (12). Each cDNA encoding Ii, HLA-DRα or -DRβ, or Ii16 (5) was subcloned into the PGEM-1 plasmid (Promega, Madison, WI). The cDNAs encoding either the wild type hemagglutinin (HA) of the influenza virus (A/3Japan/305/57 strain) or the luminal domain of HA fused with the trans-membrane and the cytoplasmic domain of the mannose 6-phosphate/IGF II receptor (HA/MPR) were as described (12). In double and triple transfections, 80% of the cells expressed the different protein complexes tested.

The invariant chain Ii was detected using an antiserum, ReHu1, prepared by immunizing rabbits with the 191–212 peptide (FKESLELDPPSSGLGVRQDGL) from Ii coupled to keyhole limpet hemocyanin. The α subunit of MHC-II molecules was detected with the rabbit anti-DRs antisera that recognizes its cytoplasmic tail (15). The bound antibodies were detected with fluorescently labeled donkey anti-rabbit secondary antibodies. Triple or double transfections were systematically assessed with the mouse L243 monoclonal antibody (16) recognizing the αβ dimer on separated coverslips and a secondary, fluorescently labeled, donkey anti-mouse antibody. HA and HA/MPR were stained with an anti-HA polyclonal antibody.

Metabolic Labeling and Immunoprecipitation—Labeling of proteins and immunoprecipitations were performed essentially as described (17). Four hours after transfection, the cells were pulse-labeled with...
In Vitro Binding of AP-1 and Quantitation—The in vitro binding of AP-1 was as described (12,13). Briefly, the transfected HeLa cells were allowed to express the different constructs for 4 h, washed, and then incubated at 4 °C with streptolysin O (20). After washing, pore formation was induced by incubating the cells at 37 °C. At washing with KOAc buffer at 25 mM Hepes-KOH, pH 7.1, 115 mM potassium acetate, 2.5 mM MgCl2, the permeabilized cells were incubated for 10 min at 37 °C with bovine brain cytosol (15 mg/ml) in KOAc buffer in the presence or the absence of 200 μM GTP-γS as indicated. The permeabilized cells were washed with KOAc buffer, fixed with 3% paraformaldehyde, and subsequently immunoprecipitated with the DA6.147 monoclonal antibody against its α-subunit (mAb 100/3) and Texas Red-coupled anti-mouse secondary antibodies.

The fluorescence associated to the bound AP-1 was quantitated at the single cell level by confocal laser scanning microscopy and immunofluorescence analysis using a TCS4D confocal microscope based on a DM microscope interfaced with an argon/krypton laser. Simultaneous double fluorescence acquisitions were performed using the 488 nm and the 568 nm laser lines to excite fluorescein isothiocyanate and Texas Red dyes using a 63 × oil immersion Neofluor objective (numeric aperture = 1.4). The fluorescence was selected with appropriate double fluorescence dichroic mirror and band pass filters and measured with blue-green-sensitive and red side-sensitive one photomultipliers. In each separated experiment, the laser beam and the photomultipliers were first adjusted on the AP-1 signal of streptolysin O-permeabilized HeLa cells expressing αβII incubated with cytosol together with GTP-γS, in order to avoid saturation in recording the signal for γ-adaptin binding on a 0–250 gray values color scale. For each samples, medial optical slices of 100–150 different cells were recorded as 512 × 512 pixel images with presetted parameters. A region of interest corresponding to the perinuclear staining for γ-adaptin was then created and the intensity of fluorescence was calculated within this area using a 0–250 gray value color scale. Then, this fixed area serves as an outline for fluorescent measurements of other areas in the image, i.e. from one cell to the other and from one field to another one. All data were saved in different series and statistical analysis (mean intensities and standard deviation) was performed. This method to determine the amount of bound AP-1 at a single cell level gave results similar to our previous enzyme-linked immunosorbent assay-based assay (12, 13).

RESULTS AND DISCUSSION

Unlike the mannose 6-phosphate receptors (MPRs) which provide a continuous supply of AP-1 binding sites due to their constitutive recycling between the TGN and endosomes, the newly synthesized MHC II molecules are expected to be minor components in transit through the TGN. However, this can be circumvented by using overexpression systems such as the vaccinia virus expression system. Thus, HeLa cells were infected with the T7 polymerase recombinant virus and then triple-transfected with cDNAs encoding the human DRα, DRβ, and p33 T1 chain.

We first tested the oligomerization of αβ and Li, the conversion of this complex into mature αβ dimers, and the cell surface appearance of these dimers. Transfected HeLa cells were pulse-labeled, chased, and surface-biotinylated at various times of the chase (Fig. 1). Then, the cell lysates were immunoprecipitated with the L243 monoclonal antibody specific for αβ dimers. The αβ dimers associated with Li or IiΔ16 complexes were detected at the cell surface. However, this can be done by confocal laser scanning microscopy and immunofluorescence microscopy, as indicated under “Experimental Procedures” and is expressed as a mean of two independent experiments. Open triangles, αβII; filled circles, αβIIΔ16; filled triangles, αβ.

FIG. 1. Expression, oligomerization, and cell surface appearance of MHC-II molecules. HeLa cells overexpressing the complexes αβII or αβIIΔ16 or αβ were pulse-labeled, chased, and cell surface-biotinylated after the indicated chase times. A, the αβ dimers were immunoprecipitated with the L243 monoclonal antibody. The αβII complexes, left in the supernatants of this immunoprecipitation, were subsequently immunoprecipitated with the DA6.147 monoclonal antibody. For each sample, 10% of the immunoprecipitated material was directly analyzed by SDS-PAGE (Total) and 90% was incubated with streptavidin beads to recover the biotinylated material (Biot.) before analysis on SDS-PAGE and autoradiography. The exposure times for autoradiography of the material immunoprecipitated with the L243 monoclonal antibody were 18 h and 3 days, respectively. The positions of α, β, mature (mIi), or immature (Ii) invariant chain are indicated. B, cell surface appearance of MHC II. The quantitation was performed as indicated under “Experimental Procedures” and is expressed as a mean of two independent experiments. Open triangles, αβII; filled circles, αβIIΔ16; filled triangles, αβ.

We then tested the oligomerization of αβ and Li, the conversion of this complex into mature αβ dimers, and the cell surface appearance of these dimers. Transfected HeLa cells were pulse-labeled, chased, and surface-biotinylated at various times of the chase (Fig. 1). Then, the cell lysates were immunoprecipitated with the L243 monoclonal antibody specific for αβ dimers deprived of associated Ii chain and subsequently immunoprecipitated with the anti-DRα DA6.147 monoclonal antibody in order to recover the αβII precursors. The biotinylated αβ dimers were fractionated on streptavidin beads. Fig. 1A shows that, in HeLa cells expressing αβII, the DA6147 monoclonal antibody could immunoprecipitate the α and β chains associated with Ii shortly after their synthesis. The disappearance of αβII complexes correlated with the detection of free αβ dimers which appeared at the cell surface with delayed kinetics. Under these conditions, 75–85% of αβII matured into αβ dimers which reached the cell surface within 2–4 h (Fig. 1B). The same experiment was performed with cells expressing the α and β chains and an Ii mutant (IiΔ16) lacking the 16 N-terminal amino acids containing the essential signals for endosomal/lysosomal targeting (5). Within 2–4 h, 65% of the αβIIΔ16 complexes were detected at the cell surface (Fig. 1B). Interestingly, a small proportion of αβIIΔ16 complexes (~10%) were converted into αβ dimers which gained access to the plasma membrane. In the absence of Ii, αβ dimers could still assemble, as detected with the L243 monoclonal antibody, and ~50% of the newly synthesized dimers appeared at the cell surface (Fig. 1B). Thus, the absence of Ii or the truncation of its cytoplasmic domain do not preclude the appearance of MHC II at the cell surface.

These different transfected HeLa cells were permeabilized and the L243 monoclonal antibody was used to quantify the cell surface proteins. The cells were lysed, and MHC II were first immunoprecipitated with the L243 monoclonal antibody specific for αβ dimers. This method to determine the amount of bound AP-1 at a single cell level gave results similar to our previous enzyme-linked immunosorbent assay-based assay (12, 13).
HeLa cells expressing either the αβ dimers or Ii alone or the complex αβIi or the complex αβIIΔ16 were permeabilized, incubated with bovine brain cytosol in the presence of 200 μM GTPγS, and then processed for fluorescence analysis. As negative and positive controls, the hemagglutinin (HA) of the influenza virus or the luminal domain of HA fused to the transmembrane and cytoplasmic tail of the Man-6-P/IGF II receptor (HA/MPR) were expressed in HeLa cells in parallel experiments. The amount of membrane-bound AP-1 was then determined by confocal microscopy and image analysis as described under “Experimental Procedures.”

FIG. 3. The invariant chain Ii cytoplasmic domain determines AP-1 recruitment. Mock-transfected or transfected HeLa cells expressing either αβ dimers or Ii alone or the complex αβIi or the complex αβIIΔ16 were permeabilized, incubated with bovine brain cytosol in the presence of 200 μM GTPγS, and then processed for fluorescence analysis. The amount of bound AP-1 was then measured by confocal microscopy and image analysis as described under “Experimental Procedures.”

FIG. 4. ARF-dependent AP-1 recruitment in MHC II expressing cells. Mock-transfected or transfected HeLa cells expressing the complex αβIi were permeabilized, incubated with bovine brain cytosol in the absence or the presence of 200 μM GTPγS or 100 μg/ml brefeldin A as indicated. In this latter case, the cells were pretreated for 10 min with 100 μg/ml brefeldin A and permeabilized in the presence of 100 μg/ml brefeldin A. The cells were then processed for fluorescence analysis. The amount of bound AP-1 was then measured by confocal microscopy and image analysis as described under “Experimental Procedures.”

The amount of AP-1 bound to membranes of the perinuclear region was quantitated using confocal microscopy and image analysis (Fig. 3). When compared to mock-transfected cells, the expression of αβIi complexes induced a 2.6-fold increase in AP-1 binding. As expected from previous studies (12), a similar recruitment was seen upon expression of a chimeric protein made of the luminal domain of the influenza virus hemagglutinin (HA) fused to the transmembrane and cytoplasmic domain of the mannose 6-phosphate/IGF II receptor (HA/MPR). In contrast, no specific increase was detected in cells expressing αβ dimers or HA known to follow the constitutive secretory pathway toward the plasma membrane. Interestingly, the deletion of the 16 N-terminal amino acids of Ii was sufficient to fully preclude AP-1 recruitment induced by the αβIi complexes. We conclude from these results that the MHC-II molecules are, like the mannose 6-phosphate receptors, able to promote AP-1 recruitment on membranes of HeLa cells and that this activity depends on the presence of an intact Ii cytoplasmic domain. This would be consistent with the observation that the overexpression of Ii alone is able to promote AP-1 recruitment, although to a small but significant extent (1.3-fold increase). This probably reflects the limited transport of Ii through the biosynthetic pathway when expressed alone (4, 5). As show in Fig. 4, the AP-1 recruitment induced by the expres-
sion of the αβIi complexes was ARF-dependent since it was stimulated by the addition of the slowly hydrolyzable GTPγS which stabilizes ARF proteins onto membranes and inhibited by the presence of brefeldin A which impairs the membrane insertion of this GTPase (21).

The cell surface appearance of the MHC II molecules requires several steps of membrane transport. We have used here an ARF-dependent AP-1 binding assay on permeabilized cells that permits the dissection of a single step of membrane transport at the exit of the TGN. Our previous studies showed a good correlation between MPR expression, efficient AP-1 binding in vitro, and transport of lysosomal enzymes in vivo (14). In the present study, we show that the expression of MHC class II molecules, like that of the mannose 6-phosphate receptors, promotes the recruitment of AP-1. Previous investigations on the intracellular transport of the Ii chain have shown that the same sorting signals in its cytoplasmic tail mediate both lysosomal targeting (4–6) and endocytosis (22). We provide here direct evidence that the Ii cytoplasmic domain is essential for AP-1 recruitment and that it likely determines the sorting of the whole αβI complex in the TGN. Interestingly, αβ dimers associated with Ii lacking its lysosomal targeting signals which can be endocytosed (23) are unable to trigger AP-1 recruitment. Thus, our study strongly suggests that the MHC II molecules and the mannose 6-phosphate receptors can potentially be packaged in the same TGN-derived clathrin-coated vesicles. Recently, biochemical and morphological studies performed on I-cell disease B lymphoblasts which use a mannose 6-phosphate-independent intracellular pathway for lysosomal enzyme targeting (24), have shown that the MHC II and the newly synthesized cathepsin D can be packaged into non-clathrin-coated vesicles at the exit of the TGN (8). Therefore, it would appear that the MHC class II molecules can follow different intracellular pathways at the exit of the trans-Golgi network as also suggested by the heterogeneity of the endocytic structures that contain the MHC class II molecules (25).

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