Decreased Endosomal Delivery of Major Histocompatibility Complex Class II-invariant Chain Complexes in Dynamin-deficient Cells*

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Kena Wang, Per A. Peterson, and Lars Karlsson‡

From The R. W. Johnson Pharmaceutical Research Institute, San Diego, California 92121

Major histocompatibility complex class II molecules are heterodimeric cell surface molecules which acquire antigenic peptides in the endosomal/lysosomal system. Invariant chain (Ii), a third chain which is associated with class II molecules intracellularly mediates the endosomal targeting, but it is debated whether class II molecules reach the endosomal system mainly from the trans-Golgi network or via the cell surface.

Dynamin is a cytosolic GTPase which is necessary for the formation of clathrin-coated vesicles from the plasma membrane, but which is not required for vesicle formation from the trans-Golgi network. Here we have used HeLa cells expressing a dominant negative form of dynamin to show that inhibition of clathrin-mediated uptake from the plasma membrane leads to accumulation of transfected Ii-class II complexes at the cell surface, while delivery of such complexes to endosomes/lysosomes is decreased. Our data therefore suggest that in this experimental system the majority of Ii-class II complexes traverse the cell surface before they reach the endosomal system.

Major histocompatibility complex (MHC)1 class II molecules are heterodimeric cell surface proteins which present antigenic peptides to CD4+ T cells. After synthesis in the endoplasmic reticulum, class II molecules associate with a third chain, the invariant chain (Ii) (1). Ii facilitates exit of class II molecules from the endoplasmic reticulum and mediates targeting of the Ii-class II complexes to the endosomal system where Ii is degraded and class II molecules acquire peptides (2). Complete removal of Ii and efficient peptide loading of class II molecules require the function of HLA-DM (3, 4), a class II-like molecule which is mainly, but not exclusively, located in lysosome-like MHC compartments (MHC class II compartments) (5–8). This structure has been proposed to be the location where class II molecules acquire peptides, but the actual evidence that peptide loading occurs preferentially in the MIIC is circumstantial and other endosomal compartments containing early and late endosomal markers have also been suggested to be compartments where class II-peptide association may occur (9–11).

Membrane proteins can be sorted for delivery to the endosomal/lysosomal system in two cellular locations; from the trans-Golgi network (TGN) and from the plasma membrane (12, 13). In both cases clathrin-coated vesicles are part of the sorting machineries, but the associated adaptor complexes are distinct in the two locations (14, 15). Thus AP1 is necessary for sorting and clathrin-coated pit assembly in the TGN, while AP2 has the same function at the plasma membrane. Although adaptor proteins from both complexes have been reported to bind lysosomal targeting signals, the sorting specificities of the two adaptor complexes are likely to be different (16, 17).

The N-terminal cytoplasmic tail of Ii contains two extensively characterized dileucine-based endosomal targeting motifs (18, 19). These motifs can clearly mediate internalization from the plasma membrane, but whether Ii sorting is occurring mainly in the TGN or at the cell surface under normal conditions is unclear. Several studies have suggested that the majority of Ii-class II complexes are transported directly from the TGN to endocytic compartments and in particular to the MIIC (5, 20, 21), while other reports have come to the conclusion that a large part of Ii-class II complexes reach the endosomal system via the cell surface (11, 22). The small number of Ii-class II complexes present at the cell surface at steady state has made it difficult to determine the relative importance of these pathways.

In this study we have used HeLa cells stably transfected with a dominant negative form of dynamin, K44A, under an inducible promoter (23) to study the intracellular transport pathways of Ii and Ii-class II complexes. Dynamin is a cytosolic GTPase which is required for the formation of constricted clathrin-coated pits at the plasma membrane (24). Cells which express the K44A form of dynamin accumulate elongated clathrin-coated pits, but are unable to form clathrin-coated vesicles and are thus incapable of clathrin-mediated endocytosis. Since dynamin is not required either for transport in the secretory pathway or for the clathrin-mediated vesicular transport from the TGN to the endosomal system (23), the use of dynamin-mutant cells provides an opportunity to address the question where endosomal sorting of Ii occurs. Although HeLa cells are not antigen presenting cells and do not normally express MHC class II molecules or Ii, transfected class II molecules are sorted to endosomal/lysosomal compartments in cells expressing class II and Ii (7, 25).

In the presence of the mutant form of dynamin we found that Ii, as well as Ii-class II complexes, accumulated at the cell surface, while only a minor amount of Ii appeared to reach the endosomal system. Our data suggest that in HeLa cells the sorting of Ii-class II complexes for delivery to the endosomal system occurs mainly at the cell surface, rather than in the TGN.

EXPERIMENTAL PROCEDURES

Reagents—The stable tTA-HeLa cell line with tetracycline-regulated expression of dynamin mutant K44A was provided by Sandra Schmid (23). The expression plasmids for HLA-DR, DBB, human lip31, lip41, and lip31(AA-A) have been described (19, 26). lip41(AA-AA) was generated by replacing a BsrGI-MunI fragment in the lip31(AA-AA) plasmid with the corresponding fragment from a lip41 plasmid. Human CD8α and mouse CD40 were cloned in the expression vector pCMU.
RESULTS

Cell Surface Accumulation of Invariant Chain in K44A-expressing Cells—To determine whether invariant chain localization was dependent on dynamin function we transiently transfected wild-type or targeting-deficient forms of invariant chain (lips1 or lips1AA-AA) into HeLa cells stably transfected with the K44A dynamin mutant under a tetracycline-regulated promoter. Overexpression of proteins with endosomal or lysosomal targeting motifs has been reported to result in mis-sorting and accumulation of transfected and endogenous lysosomal proteins at the plasma membrane (30, 31). To avoid this problem a moderately strong a-globin promoter was used to control the expression of Ii. The cells were grown in the presence of tetracycline (to suppress K44A expression) or in the absence of tetracycline (to induce K44A expression). 72 h after transfection the cell surface expression of invariant chain was analyzed by flow cytometry after staining with mAb B44 and cultured in the presence or absence of tetracycline as indicated. The cell surface Ii was stained with B44 and analyzed by flow cytometry.

We next examined the uptake of Ii from the cell surface in the presence or absence of mutant dynamin. Cells transfected with Ii and CD40 (as a cell surface control) were plated on cover slips and these were incubated on ice with mAb B44. After washing, the cells were allowed to internalize the bound antibodies for 1 h in complete medium at 37 °C. Cells were then fixed and stained with fluorescent isothiocyanate-labeled ant-mouse IgG. The stained cells were analyzed by immunofluorescence microscopy. Fig. 2a shows the distinct vesicular staining of non-induced cells after permeabilization. When the cells were not permeabilized, Ii staining could not be detected (Fig. 2b), indicating that B44 which had bound to the cell surface had been internalized. CD40 staining was present at the cell surface whether the cells had been permeabilized or not (Fig. 2c), indicating that B44 which had bound to the cell surface had been internalized.
The Ii staining pattern after permeabilization (Fig. 2e) was not significantly different from the pattern seen in the non-permeabilized cells (Fig. 2g) and in both cases the staining was largely coincident with the CD40 control staining (Fig. 2, f and h). The shape of K44A-expressing cells is different from non-induced cells, probably due to the accumulation of coated pits which are unable to pinch off to form vesicles (23). Molecules which are normally internalized are concentrated in these pits, thus giving a dotty cell surface staining pattern. The data from these experiments show that Ii internalization from the cell surface depends on the formation of clathrin-coated vesicles.

Functional Dynamin Is Required for Efficient Endosomal Localization of Invariant Chain—The subcellular distribution of Ii under steady state conditions was analyzed by indirect immunofluorescence of cells transfected with Iip31 and CD40 cDNAs. Staining of permeabilized non-induced cells with mAb Bu¨ 45 showed Ii to be localized intracellularly in reticular and vesicular structures (Fig. 3a, c). CD40 staining was detectable at the cell surface in both cases (Fig. 3a, b and e). When K44A expressing cells were stained with mAb Bu45, it could only be detected at the cell surface (coincident with the CD40-staining), whether the cells were permeabilized or not (Fig. 3a, e and g). No vesicular staining was seen in the permeabilized cells, suggesting that a large part of Ii was located at the plasma membrane. Immunofluorescence staining of cells transfected with Ip41 showed that this form of Ii, like Ip31, accumulated at the cell surface in K44A expressing cells (compare Fig. 3, B, panel a, with A, panel b).
Thus, the degree of II dissociation from class II molecules can be used as an indirect measure of the delivery of II-class II complexes to the endosomal system. Iip41 was used in these transfections instead of Ip31, since it is easier to distinguish from the class II α and β chains in SDS-PAGE gels due to its larger size. The two forms of II appear to be equally efficient in promoting class II folding and targeting, although they may influence II and antigen degradation in different ways (35, 36).

Non-induced or induced transfected cells were labeled for 30 min then washed and either lysed immediately or chased in non-radioactive medium as indicated (1–8 h) before lysis. MAb DA6.147 was used to immunoprecipitate DRαβ and DRαβII complexes. The precipitates were separated by SDS-PAGE and analyzed by autoradiography (A). Bands of Iip41, Iip41⁺ (the terminally glycosylated form of Iip41), DRα, and DRβ are indicated to the right, molecular masses in kilodalton (kDa) is shown at the left. The radioactivities associated with the Iip41 (dotted) and Iip41⁺ (solid) were quantified by PhosphorImager analysis (B).

Decreased Delivery of II-Class II Complexes to the Endosomal System—Although the majority of II was not delivered to endosomes in the presence of the mutant dynamin, it was not clear to what extent II-class II complexes did reach the endosomal system, since the protease sensitivity of II results in rapid degradation after arrival in these compartments. To estimate the endosomal delivery of II-class II complexes in the absence or presence of the mutant dynamin, we took advantage of the fact that in the presence of the protease inhibitor leupeptin invariant chain degradation is partly inhibited (32). The resulting II-derived fragments, LIP (leupeptin-induced proteins) migrating at 21–23 kDa and SLIP (small leupeptin-induced proteins) migrating at 12 kDa, remain associated with class II molecules in the endosomal system (37, 38). The relative abundance of the two fragments appears to vary depending on experimental conditions.

To quantify the amount of II-class II complexes that did reach the endosomal system we made pulse-chase experiment as above, but included leupeptin in the chase medium. Fig. 5 shows that in the non-induced cells (i.e. +tet), a distinct LIP fragment was present in the immunoprecipitates from leupeptin-treated cells after 8 h of chase. No distinct SLIP fragment could be detected. The immunoprecipitates from the induced cells (−tet) showed, as expected, that most of the II was still intact. However, a small amount of LIP could be detected after 8 h of chase also in this case. Quantification by PhosphorImager analysis showed that the LIP fragment constituted 80% of the total transported invariant chain remaining after 8 h in the non-induced cells (after compensating for the lower number of methionines in the LIP fragment; 9 versus 13) while it constituted 15% in the induced cells. Comparison with the amount of II precipitated after 1 h of chase showed that only a minor amount of II was unaccounted for in the leupeptin-treated cells. This experiment shows that some II-class II complexes reached the endosomal system in the presence of mutant dynamin, although the majority of complexes did not.

Accumulation of DR-II Complexes at the Cell Surface—Internalization of II was blocked by K44A expression and it was therefore likely that the slow dissociation of II from DR in the K44A expressing cells was a reflection of the blocked internalization from the plasma membrane, leading to accumulation of DR-II complexes at the cell surface. To verify that this was the case we analyzed the cell surface expression of cells transfected with DBA, DRB, and either wild-type Iip41 or Iip41(ΔA-ΔA), which lacks endosomal targeting information. 72 h after transfection, the cell surface proteins of induced or non-induced cells were labeled with sulfo-NHS-biotin. After lysis, DR molecules
were immunoprecipitated with DA6.147, as above. The samples were separated on SDS-PAGE gels, then blotted onto nitrocellulose filters. Immunoprecipitated proteins labeled with biotin residues (i.e. molecules derived from the cell surface) were detected using alkaline-phosphatase-conjugated streptavidin in combination with a colorimetric substrate. Fig. 6 shows that little Ii was coprecipitated with the cell surface streptavidin in combination with a colorimetric substrate. Cell surface lip41 (indicated as p41+), DRα and DRβ are indicated to the right, molecular masses (kDa) are indicated to the left.

DISCUSSION

It is a well accepted fact that MHC class II molecules acquire peptides in endosomal or lysosomal compartments, although the exact location where peptide loading occurs is debated. Several studies have tried to establish whether II-class II complexes reach these loading compartments directly from the TGN or whether they are delivered to the endosomal system via the cell surface. Studies based on immunoelectron microscopy and subcellular fractionation as well as the use of inhibitors of proteolysis or endosomal acidification have concluded that the majority of II-class II complexes are delivered directly to the endosomal/lysosomal system and the slow rate of II dissociation suggested that the majority of II-class II complexes did not reach this system within 8 h of chase. Pulse-chase experiments with leupeptin-treated cells confirmed that under these conditions <20% of II-class II complexes did enter endosomal compartments where Ii degradation could occur. Analysis of cell surface molecules in the same transfecants showed that most DR molecules at the plasma membrane were indeed associated with Ii. The fraction of II-class II complexes that did reach the endosomal system may represent material directly transported from the TGN to endosomes. Alternatively, endocytosis of II-class II complexes from the cell surface was not totally blocked, either because the mutant dynamin may not completely block the formation of clathrin-coated vesicles or because some II-class II complexes may be internalized in non-clathrin-coated vesicles. Clathrin-independent pinocytosis of fluid phase markers is increased in cells where dynamin function is inhibited (40).

Although the intracellular transport of transfected II-class II complexes, as well as Ii dissociation and final subcellular distribution of class II molecules, is similar in HeLa cells to what has been reported for both B lymphoblastoid cell lines and different types of transfected cell lines (1, 34, 41). HeLa cells are not normally antigen presenting cells and we cannot conclude that a majority of II-class II complexes reach endosomal and lysosomal compartments via the cell surface also in professional antigen presenting cells. It is apparent, however, that difficulty to detect a substantial amount of II-class II complexes at the plasma membrane does not allow the conclusion that a majority of these complexes reach the endosomal system via a direct pathway from the TGN.

The route of transport to the endosomal system may matter in the case of class II molecules since both lysosomal compartments and lighter compartments containing early endosome markers have been indicated in peptide loading (8, 9, 42, 43). Internalization of II-class II complexes together with antigenic proteins from the cell surface may allow the most extensive contact between class II molecules and internalized antigens, molecules created during a short pulse labeling will have become spread out by the time the molecules reach the later stages of the secretory pathway.

In this study we have addressed the question of how II-class II complexes travel to endosomes by taking advantage of the fact that formation of clathrin-coated vesicles during endocytosis from the cell surface require the function of the cytosolic GTPase dynamin, whereas formation of clathrin-coated vesicles from the TGN does not. HeLa cells stably transfected with a dominant negative form of dynamin (K44A) expressed under the control of a tightly regulated inducible promoter have previously been well characterized (23). Expression of the K44A dynamin blocks receptor-mediated endocytosis from the cell surface, apparently without disturbing the vesicular transport from the TGN to endosomes. Delivery of the lysosomal protease cathepsin D is normal in these cells and the distributions of cation-independent mannose 6-phosphate receptor and γ-adaptin (which is part of the TGN-specific AP1 complex) have normal distributions (Ref. 23 and not shown).

Using transiently transfected K44A-expressing HeLa cells we found that Ii internalization from the cell surface was dependent on the function of normal dynamin and that most II molecules as well as II-class II complexes were delivered to the cell surface instead of the endosomal system. Pulse-chase experiments with K44A-expressing cells transfected with DR and Ii showed that the rate of invariant chain dissociation from DR molecules was very slow, with ~80% of the transported II chains still associated with DR molecules after 8 h of chase. Ii is very sensitive to proteolytic degradation in the endosomal system and the slow rate of Ii dissociation suggested that the majority of II-class II complexes did not reach this system within 8 h of chase. Pulse-chase experiments with leupeptin-treated cells confirmed that under these conditions <20% of II-class II complexes did enter endosomal compartments where Ii degradation could occur. Analysis of cell surface molecules in the same transfecants showed that most DR molecules at the plasma membrane were indeed associated with Ii. The fraction of II-class II complexes that did reach the endosomal system may represent material directly transported from the TGN to endosomes.

FIG. 6. Accumulation of II-class II complexes at the cell surface. K44A-transfected HeLa cells were transiently transfected with DRα, DRβ, and lip41 (lanes 1 and 2) or with DRα, DRβ, and mutant lip41(AA-AA) (lane 3) in the presence (lane 1 and 3) or absence (lane 2) of tetracycline. 72 h after transfection cells were surface-labeled with biotin before lysis. DR molecules, including II-DR complexes, were precipitated with mAb DA6.147 before SDS-PAGE. The gel was blotted to nitrocellulose filters and cell surface proteins were detected using alkaline phosphatase-conjugated streptavidin in combination with a colorimetric substrate. Cell surface lip41 (indicated as p41+), DRα and DRβ are indicated to the right, molecular masses (kDa) are indicated to the left.
Invariant Chain Sorting Is Dynamin-dependent

thus increasing the chances that class II molecules will be able
to bind and present some epitope from a given protein.

In conclusion, we have used a new experimental approach to
analyze the intracellular transport of Ii-class II complexes. We
find that in transfected HeLa cells a majority of Ii-class II
complexes, as well as free Ii reach the endosomal system via
the plasma membrane. This approach shows that the ability to
bind and present some epitope from a given protein.

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