The Mammalian AP-3 Adaptor-like Complex Mediates the Intracellular Transport of Lysosomal Membrane Glycoproteins*

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In mammalian cells, the mannose 6-phosphate receptors (MPRs) and the lysosomal glycoproteins, lysosomal-associated membrane protein (LAMP) I, lysosomal integral membrane protein (LIMP) II, are directly transported from the trans-Golgi network to endosomes and lysosomes. While MPR traffic relies on the AP-1 adaptor complex, we report that proper targeting of LAMP I and LIMP II to lysosomes requires the AP-3 adaptor-like complex. Overexpression of these proteins, which contain either a tyrosine- or a di-leucine-based-sorting motif, promotes AP-3 recruitment on membranes. Inhibition of AP-3 function using antisense oligonucleotides leads to a selective misrouting of both LAMP I and LIMP II to the cell surface without affecting MPR trafficking. These results provide evidence that AP-3 functions in the intracellular targeting of transmembrane glycoproteins to lysosomes.

The secretory and the endocytic pathways of eukaryotic cells are organized in several membrane-bound compartments connected by vesicular traffic. During vesicular transport, cytosolic coat components are recruited on a donor compartment in order to form a vesicle that buds and specifically docks and fuses with the acceptor compartment. The interaction of coat components with specific sorting determinants in the cytoplasmic domain of membrane proteins induces their clustering in the transport vesicle (reviewed in Refs. 1 and 2). A typical example is provided by the clathrin-coated vesicles that mediate either the endocytosis of transmembrane receptors or the sorting from the trans-Golgi network (TGN) of membrane proteins destined to endosomes/lysosomes (reviewed in Refs. 3–5). The TGN-derived and plasma membrane-derived vesicles contain distinct, heterotetrameric adaptor complexes, AP-1 and AP-2 respectively. In vitro, both AP-1 and AP-2 bind cytoplasmic domains of membrane receptors and interact with the tyrosine- or di-leucine-based sorting determinants (6) known to be important for the endocytosis or endosomal/lysosomal targeting of membrane proteins (reviewed in Sandoval and Bakke (7)). Ultrastructural studies have also revealed that clathrin-(8) and AP-1-coated buds (9) whose functions in vesicular transport are still unclear, are also present on membranes of early endosomes.

Early electron microscopic studies have illustrated that the TGN-derived clathrin-coated vesicles mediate the transport of the mannose 6-phosphate receptors (MPRs) to endosomal compartments (reviewed in Kornfeld and Mellman (3)). The proper lysosomal targeting of their bound acid hydrolases requires the presence of tyrosine-based and di-leucine-based motifs in their cytoplasmic domains (10, 11). Some other membrane proteins such as the lysosomal-associated membrane proteins (LAMP I, LAMP II, or lgp120), or the lysosomal integral membrane proteins (LIMP I, LIMP II) are also sorted in the TGN and follow an intracellular route to endosomes/lysosomes (reviewed in Refs. 3, 7, and 12–14). Their lysosomal targeting depends on either a critical tyrosine-based (LAMP I, LAMP II, LIMP I) or di-leucine-based (LIMP II) sorting determinants present in their 10–20-amino acid long cytoplasmic domains (15–20). By analogy with the MPRs, it has been proposed that their lysosomal targeting could involve an AP-1-dependent pathway. The presence of LAMP I in AP-1-coated structures may suggest that their exit from the TGN, like that of the MPRs, indeed depends on such a pathway (18). However, other membrane proteins may follow a different route. The lysosomal acid phosphatase (LAP) precursor, for example, has been proposed to be transported from the TGN to the cell surface and then endocytosed for subsequent transport to lysosomes (21). Its AP-2-dependent endocytosis is also mediated by a tyrosine-based sorting motif (22).

During the past years, the AP-3 adaptor-like complex has been characterized (for review see Odorizzi et al. (23)). Its δ, β3, μ3, and ω subunits are homologous to their counterparts in AP-1 and AP-2 (24, 25). Although a specific AP-3 variant has been identified in brain (26, 27) where it may function in synaptic vesicles biogenesis (28), AP-3 is ubiquitously expressed and is present on buds/vesicles associated with the TGN (29) as well as on intracellular structures occasionally labeled with endocytic tracers (24, 25, 30). The ubiquitously expressed AP-3 may function in protein sorting to lysosomes. In Drosophila, mutations in the Garnet gene whose product is closely related to the δ-subunit of AP-3 result in reduced pigmentation of pigment granules, lysosome-like organelles (25, 31) (for review see Refs. 23 and 32). More recently, it has been demonstrated that the yeast AP-3 complex plays a major role in the selective transport of the alkaline phosphatase (33, 34), which uses a di-leucine-based sorting signal contained in its cytoplasmic tail to follow the carboxy peptidase Y-independent pathway to the vacuole (35). Tyrosine-based sorting signals also interact in the yeast two-hybrid system with the μ3 subunit of AP-3 (24, 36, 37) as μ1 and μ2 of AP-1 and AP-2, respectively (38).

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1 The abbreviations used are: TGN, trans-Golgi network; MPR, mannose 6-phosphate receptor; LAP, lysosomal acid phosphatase; LAMP, lysosomal-associated membrane protein; LIMP, lysosomal integral membrane protein; VZV, varicella-zoster virus; FITC, fluorescein isothiocyanate; HA, hemagglutinin, GTPγS, guanosine 5’-O-(thiotriphosphate).
We have shown in previous studies that the MPRs are part of the key components required for the efficient translocation of cytosolic AP-1 onto membranes (9, 39, 40) and may regulate the formation of clathrin- and AP-1-coated vesicles (41). In vivo and in vitro, MPR expression in MPR-negative cells correlates to some extent AP-1 recruitment on membranes. In this study, we report that the overexpression of chimeric proteins made of the cytoplasmic domains of LAMP I, LIMP II, and LAP fused to the luminal and transmembrane domains of the varicella-zoster virus (VZV) envelope glycoprotein gpI, used as a reporter, is unable to promote AP-1 recruitment onto membranes. In contrast, the overexpression of gpI-LAMP I and gpI-LIMP II, but not that of the MPRs or gpI-LAP, promotes the recruitment of AP-3 on perinuclear membranes. This recruitment requires the presence of a tyrosine- or a di-leucine-based sorting motif in the cytoplasmic domains of LAMP I, LIMP II, and LAP fused to the cytoplasmic domains of AP-3 on perinuclear membranes. This recruitment requires the presence of a tyrosine- or di-leucine-based sorting motif in their cytoplasmic domains. Furthermore, inhibition of the synthesis of the μ subunit of AP-3 results in the selective misrouting of LAMP I and LIMP II to the plasma membrane. We conclude that, in mammalian cells, AP-3 is essential for the proper intracellular targeting of LAMP I and LIMP II to lysosomes.

**EXPERIMENTAL PROCEDURES**

**Materials**—All reagents were of analytical grade. Brefeldin A was from Sigma. DOTAP reagent was from Boehringer Mannheim GmbH (Mannheim, Germany). [35S]methionine/cysteine (EXPRESS) was from NEN Life Science Products. 30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide solution was from National Diagnostics (Atlanta, GA). Sulfo-NHS-S8-biotin and streptavidin beads were from Pierce.

**Cell Culture and Transfections**—HeLa and NRK cells (American Type Culture Collection, Rockville, MD) were grown in a minimum essential medium or Dulbecco’s modified Eagle’s medium complemented with 10% fetal calf serum, 2 mm glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin. For transfections, cells were split and grown onto coverslips the day before. Transient expression using calcium phosphate transfection was as described previously (42). For expression using the vaccinia virus, the protocol was as described previously (39). Briefly, the cells were first infected for 30 min with the vT7 recombinant virus and then transfected with the different cDNAs using DOTAP reagent. The cells were allowed to express the different chimeric proteins for 2 h in the presence of hydroxyurea to avoid cytopathic effects. Under those conditions, the bulk of the expressed lysosomal membrane proteins fills perinuclear compartments and has not reached the lysosomes.

**Plasmid Construction and Mutagenesis**—The cDNA encoding the signal peptide and the luminal domain of HA fused to the transmembrane and cytoplasmic domains of the murine CI-MPR fusion protein were constructed and inserted into the pGEM-2 vector as described previously (39).

The cDNA encoding the different gpI chimeric proteins were all constructed from the pGEMI vector containing the XbaI-HindIII fragment of the most truncated version of gpI (mutant gpI-3A) (42). To generate by PCR the gpI-LIMP II chimeras using the previous vector as a template, the following primers were used: forward primer (primer gpI-1) 5′-GGCTCTAGAGGGGACATTTAAATACCTGGTGA-3′; reverse primer, 5′-GCAAGCTTTTAACGCTGAgGAGGCGCCCTTTT- CATCGAGATTCCTCCACCTCTCAATGACCCTGTCGTCACATGGGCTGAC- AGATTTAAAATATCCGAG-3′. Introduction into the primers of XbaI and SacI sites allowed the cloning of the polymerase chain reaction product on the same sites into the pSPF6 and pGEMI vectors (42). The same strategy was used to obtain the LIMP II LG mutant except that the reverse primer was 5′-GCAAGCTTCTAGGGGACATTTAAATACCTGGTGA-3′; reverse primer was 5′-GCAAGCTTTTAACGCTGAgGAGGCGCCCTTTT- CATCGAGATTCCTCCACCTCTCAATGACCCTGTCGTCACATGGGCTGAC- AGATTTAAAATATCCGAG-3′. To inhibit the synthesis of the ubiquitin-3 containing fractions of a linear 5–20% sucrose gradient (data not shown).

For Western blotting and immunofluorescence analyses, γ-adaptin was detected as described previously (9, 39, 41). The 100/3 monoclonal anti-γ-adaptin was a kind gift from Dr. Ungewickell. The human LAMP I was detected using the HA3 mouse monoclonal antibody (Developmental Studies Hybridoma Bank, Iowa, IA). Rat LIMP II was detected using a monoclonal antibody (kindly provided by Dr. I. Sandoval, Universidad Autónoma de Madrid, Spain). HA was detected using a polyclonal serum or a monoclonal antibody kindly provided by Drs. Roth and Skehel). The mouse monoclonal antibody SG1 (Viro Research Inc., Rockford, IL) or a polyclonal anti-gpI were used to detect the VZV gpI. CI-MPR was detected using a rabbit polyclonal anti-CI-MPR antibody (45). All the secondary antibodies against the Fe fragment of mouse, rat, and rabbit IgGs were used in a Western blotting corresponding to the α5 and B subunits as judged by their coincidence with gpI by gel filtration chromatography and by their presence in gpI-containing fractions of a linear 5–20% sucrose gradient (data not shown).

**Antibodies**—The β-subunit of AP-3 was detected using an affinity purified rabbit polyclonal antibody raised against a fusion protein consisting of glutathione S-transferase residues 22–756 of the β-subunit (25). μ A,B were detected using an affinity purified rabbit polyclonal antibody raised against a GST fusion protein (29). α5 A,B were detected using a polyclonal serum prepared by immunizing rabbits with the 166–193 peptide (KINLPEPRINIG) coupled to keyhole limpet hemocyanin. This polyclonal antisera recognizes a doublet of Western blotting corresponding to the α5 and β subunits as judged by their coincidence with gpI by gel filtration chromatography and by their presence in gpI-containing fractions of a linear 5–20% sucrose gradient (data not shown).

**Antisense Oligonucleotides**—To inhibit the synthesis of the ubiquitin-3 containing fractions of a linear 5–20% sucrose gradient (data not shown).

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The following antibodies were then added to the culture media, the HA3 monoclonal anti-LAMP I antibody (HeLa cells), or the anti-LIMP II monoclonal antibody (NRK cells) or an anti-CI-MPR (HeLa cells) antibody. The cells were incubated at 37 °C with these antibodies for the indicated periods of time, then washed and processed for immunofluorescence. The internalized antibodies were detected with the appropriate Texas Red-conjugated secondary antibodies. To quantitate the extent of misrouting to the cell surface, untreated or oligonucleotide-treated HeLa or NRK cells were labeled with [35S]methionine/cysteine and incubated with the HA3 anti-LAMP I (HeLa cells) or the anti-LIMP II (NRK cells) monoclonal antibodies or anti-CI-MPR (HeLa cells) antibodies. After 1 h (anti-CI-MPR) or 4 h (LAMP I and LIMP II), cells were treated with 200 μg/ml of 3-methyladenine, 5 μg/ml leupeptin, and 1 μg/ml leupeptin (Protein A-Sepharose) was then added for 2 h at 4 °C. The immune complex were subjected to SDS-polyacrylamide gel electrophoresis followed by fluorography. The values were normalized to the total newly synthesized LAMP I, LIMP II, or CI-MPR.

**Flow Cytometry**—Oligonucleotide-treated cells were incubated at
37 °C with 5 mM EDTA for 5 min. The detached cells were fixed with 4% paraformaldehyde, stained with primary antibodies against CI-MPR, LAMP I, LIMP II (see above), or control IgGs followed by secondary antibodies coupled to R-phycoerythrin and analyzed using a Coulter Epics ELITE (Coultronics, Miami, FL).

Miscellaneous—The in vitro recruitment of AP-3 was performed as described previously for AP-1 (46) except that an anti δ-subunit antibody was used. Transport of cathepsin D was measured as described (40). SDS-polyacrylamide gel electrophoresis and Western blotting were performed as described previously (41).

RESULTS

The expression of physiological levels of the MPRs in MPR-negative cells triggers the recruitment of AP-1 on TGN membranes both in vivo and in vitro (9, 39, 41). This property is also shared with the VZV envelope glycoprotein gpI, a TGN membrane protein which recycles between the TGN and the plasma membrane via the endosomes (42). To investigate the sorting of the newly synthesized lysosomal membrane glycoproteins...
LAMP I and LIMP II along an AP-1-dependent pathway, we have tested the possibility that the expression of these proteins could also trigger AP-1 recruitment. For this, we fused the cytoplasmic domains of LIMP II, LAMP I, or LAP to the luminal and transmembrane domains of the VZV gpI, used as a reporter molecule devoid of any trafficking information (42). When transiently expressed in HeLa cells, these chimeric proteins were properly targeted to lysosomes as indicated by their colocalization with the endogenous LAMP I (Fig. 1). As expected from previous studies (reviewed in Sandoval and Bakke (7)), a point mutation in the di-leucine-based motif of LIMP II (mutant LG) or in the tyrosine-based motif of LAMP I (mutant YA) results in the accumulation of the chimeric proteins at the cell surface (Fig. 1). Thus, the chimeric proteins and the corresponding mutants faithfully reflect the behavior of the full-length lysosomal membrane glycoproteins.

Expression of gpI Chimeras and AP-1 Recruitment—Unlike the MPRs and gpI which continuously recycle back to the TGN, the newly synthesized LAMP I, LIMP II, and LAP undergo a single round of protein sorting at the exit of the TGN. Therefore, these membrane proteins are expected to contribute only to a minor extent to AP-1 recruitment. Thus, gpI-LIMP II, gpI-LAMP I, and gpI-LAP chimeric proteins were overexpressed in HeLa cells using the T7 RNA polymerase recombinant vaccinia virus as was done previously (39, 46). As a positive control, a chimeric protein made of the luminal domain of the influenza virus hemagglutinin (HA) fused to the transmembrane and cytoplasmic domains of the Man-6-P/IGF II receptor (HA-MPR) and the full-length gpI were also overexpressed (39, 42). Pulse-chase experiments indicated that gpI and the different gpI chimeric proteins were synthesized to the same extent and processed to mature forms in the secretory pathway with similar kinetics (data not shown). The transfected cells were fixed and labeled with an anti-gpI or an anti-HA polyclonal antibody to distinguish between transfected and non-transfected cells. The bulk of these different chimeric proteins was found concentrated in the perinuclear region (Fig. 2, a, c, e, and g), whereas a faint staining of transfected cells was observed in the cytoplasm (Fig. 2, b, d, f, and h). The gpI chimeras and the HA-MPR were detected with a FITC-conjugated goat anti-rabbit antibody. γ-Adaptin was detected using a Texas Red-conjugated goat antimouse antibody. Note that, under the experimental conditions, the bulk of the expressed chimeric lysosomal proteins is detected in the perinuclear region and has not yet reached lysosomes. Untransfected cells are indicated with an asterisk.

FIG. 2. AP-1 staining of HeLa cells overexpressing different gpI chimeras. HeLa cells were infected with a recombinant T7 RNA polymerase vaccinia virus, and transfected with gpI-LIMP II (a and b), gpI-LAMP I (c and d), gpI-LAP (e and f), or HA-MPR (g and h). After 2 h of expression, the cells were fixed and labeled with the anti-gpI polyclonal antibody (a, c, and e) or an anti-HA polyclonal antibody (g) and the anti-γ-adaptin 100/3 monoclonal antibody (b, d, f, and h). The gpI chimeras and the HA-MPR were detected with a FITC-conjugated goat anti-rabbit antibody. γ-Adaptin was detected using a Texas Red-conjugated goat antimouse antibody. Note that, under the experimental conditions, the bulk of the expressed chimeric lysosomal proteins is detected in the perinuclear region and has not yet reached lysosomes. Untransfected cells are indicated with an asterisk.
AP-3 and Lysosomal Glycoproteins Transport

29455

described previously (39, 42). Typically, cells overexpressing HA-MPR or gpI exhibited a 2-fold increase in the perinuclear AP-1 staining when compared with untransfected cells (Fig. 3A). This AP-1 staining also depends on the presence of the ARF-1 GTPase since it is sensitive to brefeldin A (data not shown). In contrast, gpI chimeric proteins did not affect γ-adaptin staining (Figs. 2 and 3A). Collectively, these results suggest that the bulk of gpI-LIMP II, gpI-LAMP I, and gpI-LAP may be transported via an AP-1 independent pathway.

Expression of gpI Chimeras and AP-3 Recruitment—The results described above prompted us to investigate the possibility that AP-3 could be involved in the trafficking of these lysosomal membrane glycoproteins. For this purpose, gpI-LIMP II, gpI-LAMP I, gpI-LAP, gpI, or HA-MPR were overexpressed as before, and the cells were then fixed but labeled with a polyclonal antibody directed against the δ-subunit of AP-3 (25). In nontransfected HeLa cells, this antibody labeled mostly punctate structures scattered throughout the cytoplasm and faintly perinuclear membranes as previously observed in NRK cells with the same polyclonal antibody (25). The overexpression of gpI-LIMP II and gpI-LAMP I induces a 3-fold increase in the staining of the endogenous AP-3 on membranes of the perinuclear region without significantly affecting the punctuate peripheral staining (Figs. 3B and 4). This perinuclear AP-3 staining partially colocalized with AP-1 at the fluorescence level and became soluble when HeLa cells were treated with brefeldin A prior to fixation (data not shown), as expected for an ARF-dependent recruitment (24, 25). Similar results were also obtained with polyclonal sera against peptides corresponding to the αδA,B and δ subunits of AP-3 (data not shown). We interpret these data as reflecting a translocation of cytosolic AP-3 on membranes rather than a simple redistribution of membranes with bound AP-3. The overexpression of similar amounts of gpI-LAP or gpI or HA-MPR remained without effect on AP-3 staining (Figs. 3B and 4). These interactions could also be reproduced using an in vitro assay in which transfected HeLa cells were permeabilized with streptolysin O and incubated with a bovine brain cytosol. In vitro, the distribution of the newly recruited AP-3 was identical as in vivo and not affected by the addition of GTPγS (data not shown). The quantitation of these in vitro experiments shows that AP-3 recruitment was stimulated by GTPγS, inhibited by brefeldin A (Fig. 3C) and stimulated by the expression of gpI-LIMP II or gpI-LAMP I (Fig. 3D), as observed in vivo. Therefore, it would appear that there is a direct coupling between gpI-LIMP II or gpI-LAMP I expression and AP-3 recruitment both in vivo and in vitro, as we previously observed for the MPRs and AP-1, and that AP-1 and AP-3 recognize membrane proteins destined to endosomes/lysosomes differently.

AP-3 Recruitment Requires Either Tyrosine- or Di-leucine-based Sorting Signals—Lysosomal localization of LAMP I and LIMP II is determined by the presence of sorting signals in their cytoplasmic tails, a tyrosine- and a di-leucine-based motif respectively (7). To investigate the importance of these two types of sorting signals in AP-3 recruitment, we overexpressed gpI-chimeric proteins mutated on the tyrosine residue of LAMP I (LAMP I YA) and on the di-leucine-based signal of LIMP II (LIMP II LG). Pulse-chase experiments indicated that these mutants were expressed at the same level and transported through the secretory pathway with similar kinetics as the wild type chimeras (data not shown). The cells were then labeled with the anti-gpI monoclonal antibody and the polyclonal antibody against the δ-subunit of AP-3. The mutated chimeric proteins failed to trigger AP-3 recruitment on perinuclear membranes both in vivo (Figs. 3B and 5) or in vitro (Fig. 3D).

This result suggests that AP-3 may recognize the tyrosine- and di-leucine-based sorting signals of LAMP I or LIMP II.

Inhibition of AP-3 Function Leads to a Selective Misrouting of LAMP I and LIMP II—We then reasoned that inhibition of AP-3 synthesis may cause a misrouting of LAMP I and LIMP II to the cell surface. Because HeLa, NRK cells, and mouse fibroblasts used in this study appear to express relatively low amounts of AP-3 when compared with other cell lines, we adopted an antisense oligonucleotide approach. Oligonucleotides were designed to inactivate the synthesis of the μ3 A

Fig. 3. Quantitation of AP-1 and AP-3 immunostaining in HeLa cells overexpressing different gpI chimeras. The intensity of the fluorescence signals corresponding to the γ subunit of AP-1 (panel A) or the δ-subunit of AP-3 (panel B) as shown in Figs. 2, 4, and 5 was quantitated from 100 untransfected cells (MOCK) or −100 cells overexpressing HA-MPR or the full-length gpI, gpI-LIMP II wild type (LAMP I wt), mutant (LAMP I YA), gpI-LIMP II wild type (LIMP II wt), mutant (LIMP II LG), or gpI-LAP (LAP). In panels C and D, the transfected cells were permeabilized with streptolysin O, incubated with cytosol, and stained with an antibody against the δ-subunit of AP-3. The results were processed as described under “Experimental Procedures.” The values represent the means ± S.E. of three to six different experiments. The confidence limits of the results obtained were assessed by the Student’s t test (N.S., not significant; ***, confidence limits >99% (p < 0.01), when compared with nontransfected cells).
subunit of AP-3, the subunit interacting with tyrosine-based sorting signals in the yeast two-hybrid system (24). A pair of 15-nucleotide-long antisense DNA-oligonucleotides were designed from a cDNA covering the ATG initiation codon (ATG- and inner-antisense). A control oligonucleotide was designed by reversing the sequence of the ATG oligonucleotide (reversed ATG-antisense). Phosphorothioate-modified oligonucleotides were used because they have been shown to selectively inhibit expression of a variety of genes including Ras p21 (47, 48) and Rab 8 (49). Several cell lines, such as HeLa cells, NRK cells, and mouse fibroblasts, were treated with the reversed-antisense oligonucleotide as a negative control or with a combination of the two antisense oligonucleotides for 48 h. The steady state expression level of the subunit was then monitored by Western blotting and compared with that of the γ-adaptin used as a marker for the AP-1 complex, and σ3A,B as a marker for AP-3 complex. Fig. 6 shows that the treatment of the three different cell lines tested with the antisense oligonucleotides reduces the synthesis of the subunit by ~60% when compared with untreated cells or cells treated with the reversed-antisense oligonucleotide without affecting the synthesis of γ-adaptin and σ3A,B.

To test if the inhibition of AP-3 synthesis could lead to surface expression of endogenous LAMP I and LIMP II, we probed the ability of treated and untreated cells to internalize anti-LAMP I or LIMP II antibodies added to the incubation medium. After washing, the cells were fixed and stained with secondary antibodies. Fig. 7 shows that untreated cells or those treated with reversed-antisense oligonucleotides do not efficiently internalize the exogenously added anti-LAMP I or anti-LIMP II antibodies, consistent with previous observations showing that the bulk of those lysosomal membrane proteins follow an intracellular pathway to lysosomes (16, 19, 20, 50, 51). In contrast, cells treated with antisense oligonucleotides exhibited a strong LAMP I or LIMP II immunostaining inside punctuate structures very reminiscent to lysosomes. This fluorescence pattern was identical to that of the endogenous LAMP I and LIMP II whose overall steady state distributions remained unchanged in treated cells as determined by indirect immunofluorescence (data not shown). This result indicates

Fig. 4. AP-3 staining of HeLa cells overexpressing different gpl chimeras. HeLa cells were infected with a recombinant T7 RNA polymerase vaccinia virus and transfected with gpl-LIMP II (a and b), gpl-LAMP I (c and d), gpl-LAP (e and f), or HA-MPR (g and h). After 2 h of expression, the cells were then fixed and labeled with a polyclonal antibody against the σ-subunit of AP-3 (b, d, f, and h) and the SG1 anti-gpl monoclonal antibody (a, c, and e) or the anti-HA monoclonal antibody (g). The gpl chimeras and the HA-MPR were detected using a FITC-conjugated goat anti-mouse antibody. The anti-σ-subunit antibody was detected using a Texas Red-conjugated goat anti-rabbit antibody. Untransfected cells are indicated with an asterisk.
that, under those conditions, LAMP I and LIMP II were partially misrouted to the cell surface prior to their lysosomal delivery. This result is consistent with flow cytometry analyses performed on fixed cells showing that endogenous LAMP I and LIMP II can be detected on the surface of antisense oligonucleotide-treated cells (Fig. 8A). In order to quantify this mistargeting, untreated and oligonucleotide-treated cells were labeled with [35S]methionine and chased for 4 h in the presence of exogenously added anti-LAMP I or anti-LIMP II antibodies. The immunoprecipitation of the internalized antibodies indicated that ~40% of the newly synthesized LAMP I or LIMP II had now access to the cell surface when cells were treated with antisense oligonucleotides (Fig. 8B and C). Because divalent anti-LAMP I or anti-LIMP II monoclonal antibodies were used, we estimate that at least ~20% of the newly synthesized LAMP I or LIMP II traffic via the cell surface in antisense oligonucleotide-treated cells.

In contrast, such a treatment did not perturb trafficking of the MPRs, which are in dynamic equilibrium between the TGN, endosomes, and the cell surface. Treatment of cells with antisense oligonucleotides did not significantly enhance the uptake of anti-Man-6-P/IGF II receptor antibodies which, in every case, localized to similar punctuate perinuclear structures (Fig. 7) or modified the steady state distribution of this MPR as determined by immunofluorescence (not shown), and flow cytometry analysis of fixed cells (Fig. 8A). Fig. 8B and C, shows that the internalized antibody could immunoprecipitate 25% of the newly synthesized Man-6-P/IGF II receptor under every condition tested. We also determined the sorting function of the MPRs (Fig. 8B and D). For this purpose, mouse fibroblasts were pulse-labeled and chased to monitor the intracellular transport of the newly synthesized cathepsin D. Efficient sorting of MPRs results to the intracellular retention of cathepsin D while a misrouting via the cell surface leads to cathepsin-D secretion if mannose 6-phosphate is added in the medium to displace the MPR-bound ligands. Thus, cathepsin D was immunoprecipitated from the culture medium and the cell lysates. In mock- or reversed-antisense oligonucleotide-treated cells, ~30% of the newly synthesized cathepsin D was secreted in the medium, indicating that the bulk of cathepsin D was

**Fig. 5.** AP-3 recruitment and expression gpI-LIMP II and gpI-LAMP I mutants. Cells were infected and transfected as indicated in the legend of Figs. 2 and 3 with gpI-LIMP II (LIMP II wt; a and b), gpI-LIMP II LG (LIMP II LG; c and d), gpI-LAMP I (LAMP I wt; e and f), or gpI-LAMP I YA (LAMP I YA; g and h). The cells were then processed for indirect immunofluorescence and labeled with the SG1 anti-gpI monoclonal antibody followed by a FITC-conjugated goat antimouse antibody (a, c, e, and g). AP-3 was detected using the polyclonal antibody against the δ-subunit followed by a Texas Red-conjugated goat anti-rabbit antibody (b, d, f, and h). Untransfected cells are indicated with an asterisk.
directly transported to lysosomes. The treatment of cells with antisense oligonucleotides had no effect on the intracellular transport of this lysosomal enzyme. Collectively, these results indicate that the partial inactivation of the μ3A subunit leads to a substantial misrouting of the endogenous LAMP I and LIMP II to the cell surface without affecting MPR trafficking. A similar antisense approach to reduce AP-1 synthesis did not result in a detectable modification of MPR trafficking (not shown), probably due to the high expression level of AP-1 in the cells used in this study.

**DISCUSSION**

We have expressed chimeric proteins made of the cytoplasmic domains of LAMP I and LIMP II fused to the luminal and transmembrane domains of VZV gpl. We show here that their lysosomal targeting involves an AP-3-dependent pathway. These interactions require the presence of either a tyrosine- or a di-leucine-based lysosomal targeting signal in the cytoplasmic tail of these proteins. In contrasts, MPR or VZV gpl trafficking does not involve this AP-3-dependent pathway. While these results further support the notion that membrane protein sorting is coupled to coat assembly, they suggest that the AP-1-and AP-3-dependent pathways regulate the intracellular traffic of two distinct subsets of transmembrane proteins destined to be delivered to endosomes and lysosomes in mammalian cells.

AP-1 and AP-3 Mediate the Intracellular Transport of Distinct Membrane Proteins—LAMPs and LIMPfs follow a direct intracellular pathway from the TGN to endosomes/lysosomes (16, 18–20, 50–53). Whether these newly synthesized lysosomal glycoproteins enter the AP-1-dependent pathway at the exit of the TGN like the MPRs has remained unclear. In previous studies, we have reported that MPR expression can promote to some extent the ARF-1-dependent translocation of AP-1 on its target membranes (9, 39, 41). Therefore, we made use of this assay to determine the ability of LAMP I, LIMP II, and LAP en route to lysosomes to follow the AP-1-dependent pathway.

Our study indicates that gpl-LAMP I and gpl-LIMP II are transported to lysosomes by an AP-3-dependent sorting mechanism. Our in vivo study would agree with recent in vitro binding experiments showing that the cytoplasmic domain of LIMP II can interact with cytosolic AP-3 and that these interactions involve a di-leucine-based sorting signal (54). However, part of our results would differ from other in vitro studies showing that the cytoplasmic domain of LAMP I interacts strongly with purified AP-1 (18) but only very weakly with cytosolic AP-3 (54). The reasons for these discrepancies remain unclear at present. Our assay might not be sensitive enough to detect low affinity interactions between AP-1 and some transmembrane proteins. In the yeast two-hybrid system, however, the tyrosine-based sorting motif of LAMP I interacts better with μ3 than with μ2 or μ1 (36), and the tyrosine-based sorting motifs of the envelope glycoprotein (Env) complex of HIV-1 interact with μ1, μ2, and μ3 (37). Therefore, it is also possible that the interaction of cytosolic AP-3 with the tyrosine-based signal of LAMP I is not of a sufficient strength to be detected in vitro. Consistent with the proposal that a significant fraction of full-length LAP is excluded from AP-1 coated vesicles in the TGN and traffics via the cell surface (21), our study shows that gpl-LAP does not interact with AP-1 or AP-3. Because MPR and gpl trafficking does not appear to involve AP-3, our results would therefore argue that, in mammalian cells, AP-1 and AP-3 are involved in the intracellular trafficking of different sets of membrane proteins destined to be transported from the TGN to endosomes and lysosomes.

Although AP-3 has not been localized in gpl-LAMP I or gpl-LIMP II overexpressing cells, it is clear that AP-3 is involved in the selective intracellular targeting of transmembrane proteins to lysosomes in mammalian cells. This interpretation is consistent with the phenotype observed in the Drosophila garnet mutant flies in which the structure of lysosome-like pigment granules is altered (25, 31) and with more recent genetic studies performed in Saccharomyces cerevisiae (33, 34). In yeast, two pathways lead to the intracellular delivery of newly synthesized proteins to the vacuole/lysosome (55, 56). Several proteins including carboxypeptidase Y are delivered to the vacuole via a pathway which requires several VPS gene products such as Vps45p (a Sec1p homologue), the Vps15p protein kinase or the Vps 34p PtdIns 3-kinase, and the t-SNARE Pep12p. In contrast, the vacuolar delivery of the alkaline phosphatase does not require these gene products indicating that an alternative pathway must exist for its intracellular targeting. Genetics in yeast has revealed that AP-3 is required for the vacuolar delivery of alkaline phosphatase (33, 34) which requires a critical di-leucine-based sorting motif (35). Although vacuolar delivery is not affected in AP-1 and AP-2 deletion mutants, deletion of each of the AP-3 subunits resulted in the mislocalization of alkaline phosphatase and the t-SNARE Vam3p without affecting carboxypeptidase Y transport. Therefore, alkaline phosphatase in yeast behaves as LAMP I and LIMP II in mammalian cells and it is tempting to speculate that carboxypeptidase Y and its Vps10p receptor in yeast behave as the lysosomal enzymes and their MRs in mammalian cells.

**Sorting Signals and Interaction with Adaptor Complexes**—We show here that, like AP-2 and AP-1 AP-3 may recognize both tyrosine- and di-leucine-based signals.
these related adaptor complexes exhibit different interactions with membrane proteins which contain similar sorting determinants in their cytoplasmic domains. A single tyrosine-based motif mediates the transport of the newly synthesized LAP to lysosomes (22), and both a tyrosine- and a di-leucine-based motif are involved in the recycling pathway of the MPRs (10, 11). However, none of these proteins is able to trigger AP-3 recruitment to detectable levels. The AP-1 adaptor complex recognizes the cytoplasmic domain of MPRs and other membrane proteins such as the VZV gpl but does not efficiently recognize those of the newly synthesized gpl-LAMP I, gpl-LIMP II or gpl-LAP which contain either a tyrosine- or a di-leucine-based sorting determinants. Thus, there is an apparent selective recognition of cargo membrane proteins. What could be the molecular basis for this selectivity? A first explanation could involve the relative affinities of the different assembly proteins for similar sorting signals. For example, both the two-hybrid system in yeast (38) and biochemical studies (57) suggest that AP-2 exhibits a higher affinity for tyrosine-based motifs than AP-1. Thus, a membrane protein with a tyrosine-based motif such as LAP could escape sorting in the TGN along the AP-1 dependent pathway and be efficiently recycled back to the cell surface. The proteolytic cleavage at the putative furin cleavage site (42) and/or the spacing of the YxxI motif of LAMP I relative to the lipid bilayer, could explain its lack of interaction with AP-3. However, electron microscopic studies have also shown that AP-3 is recruited to the sorting motifs could easily explain such differences in affinities.

A second possibility could reside in the relative accessibility of these sorting signals when the proteins are embedded in the membranes. This accessibility could first involve the spacing of a given sorting signal relative to the membrane: changes in the spacing of the YxxI motif of LAMP I relative to the lipid bilayer, almost completely block its lysosomal targeting (58). Alternatively, post-translational modifications could modulate the accessibility of sorting signals. For example, the proper trafficking of the cation-dependent mannose 6-phosphate receptor requires the reversible palmitoylation of one cysteine residue in its cytoplasmic domain (59). The cytoplasmic domains of both MPRs contain casein kinase II phosphorylation sites which are transiently phosphorylated on serine residues (60, 61). Such a phosphorylation site in the cation-dependent MPR tail, adjacent to a di-leucine motif is required for the high affinity interaction of AP-1 with membranes (40). A casein kinase II phosphorylation site also regulates the trafficking of the varicella-zoster virus gpl (42) and furin (62–64). The endocytosis of CD3γ which is mediated by a di-leucine-based sorting signal also requires phosphorylation of an adjacent serine residue (65). Thus, it is possible that post-translational modifications and/or the spacing of the sorting determinants relative to the lipid bilayer could be critical for the efficient recognition of tyrosine- and di-leucine-based signals by different but related adaptor complexes. If these modifications were compartment specific, they could then modulate the interaction of membrane proteins with different types of adaptor complexes.

**Function of AP-3 in Membrane Traffic**—In the neuron, the AP-3 complex has been proposed to function in protein transport between the cell body and the nerve terminals (27) and in the biogenesis of synaptic vesicles (26, 28). While these results could reflect a specialized function of the neuron-specific AP-3 complex, genetic evidence in Drosophila (25, 31) and S. cerevisiae (33, 34) clearly indicates that the ubiquitous AP-3 functions in protein targeting to lysosomes. In mammalian cells, the targeting of newly synthesized membrane proteins to lysosomes requires several steps of membrane traffic involving their sorting in the secretory and endocytic pathways. The expression of gpl-LAMP I or gpl-LIMP II induces AP-3 recruitment on perinuclear membranes. However, several compartments are known to be located in the perinuclear region, in particular the TGN, recycling endosomes and late endosomes. In mammalian cells, AP-3 localizes to peripheral structures occasionally containing endocytic tracers (24, 25, 30). An AP-3 homologue in yeast has been identified as a suppressor of casein-kinase I deficiency (66). Since casein-kinase I is somehow required for the efficient degradation of the endocytosed α-factor, this suggests that AP-3 could first function in endocytosis in yeast (internalization and/or recycling to the plasma membrane). A lower synthesis of functional AP-3, as seen in our study, could then result in the more important recycling of LAMP I and LIMP II toward the cell surface. The proteolytic cleavage of the LAP cytoplasmic domain which occurs during its transport in the endocytic pathway after its endocytosis (67) could explain its lack of interaction with AP-3. However, electron microscopic studies have also shown that AP-3 is recruited to the...
in vivo and in vitro on specialized subdomains of a late Golgi compartment (29). Although this has not been determined at the electron microscopy level, it is tempting to speculate that the perinuclear compartment onto which AP-3 is recruited upon LAMP I or LIMP II overexpression represents the TGN which, under physiological conditions contains only few LAMP I, LIMP II or other related molecules in transit. If this interpretation were correct, AP-3 would then function in protein sorting in the TGN in a similar fashion as AP-1 functions in MPR sorting. The hyperglycosylation of alkaline phosphatase in the yeast AP-3 deletion mutant would also support this interpretation (29). Two distinct consequences could well contribute to endosomes and lysosomes. In this respect, it is interesting to note that LAMP I, LIMP II, gpl and the MRPs have different fates and exhibit different steady state distributions. LAMP I and LIMP II mostly reside in lysosomes while gpl and MRPs are in equilibrium between the TGN, endosomes and the plasma membrane but are excluded from lysosomes. It is evident that, at the present stage additional studies including electron microscopy are required to elucidate the precise function of AP-3 in mammalian cells and to decipher which step of membrane traffic it controls. Also of interest will be to determine how distinct but related adaptor complexes can mediate the differential sorting of membrane proteins containing similar sorting determinants in their cytoplasmic domains.

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