Localization of the AP-3 adaptor complex defines a novel endosomal exit site for lysosomal membrane proteins

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Introduction

Heterotetrameric adaptor complexes play a role in the selection of cargo molecules and vesicle budding. In mammalian cells, there are at least four adaptor protein (AP) complexes (AP-1 through AP-4), each of which mediates a distinct membrane trafficking step (Robinson and Bonifacino, 2001). Adaptor complexes consist of four subunits: two large subunits (the so-called β subunit and the more divergent γ/δ/ε subunit in AP-1/2/3/4, respectively), a medium subunit (μ), and a small subunit (σ). The structure of the adaptor complexes resembles that of a “Mickey Mouse” head, with the COOH-terminal domains of the two large subunits protruding like ears (Heuser and Keen, 1988).

Although the AP-3 adaptor complex was identified relatively recently, a considerable amount of functional information has already been acquired, thanks to the availability of naturally occurring mutants. Loss of functional AP-3 complex leads to defects in the function of lysosomes and lysosome-related organelles. The most obvious trafficking defect in AP-3–deficient cells is that integral lysosomal membrane proteins (also known as lysosomal glycoproteins), including lysosome-associated membrane proteins (LAMPs) 1 and 2, LIMP-2, CD63, and CD1, show increased trafficking via the plasma membrane (Dell’Angelica et al., 1999; Rous et al., 2002; Sugita et al., 2002). Nevertheless, their steady-state distribution is still mainly lysosomal. Mannose 6-phosphate receptor–based transport of soluble lysosomal enzymes and cycling of the transferrin receptor (TfR) between the plasma membrane and endosomes are independent of AP-3 function (Le Borgne et al., 1998; Dell’Angelica et al., 1999).

Despite the accumulating evidence implicating AP-3 in the intracellular transport of lysosomal membrane proteins, the transport step that is mediated by AP-3 has remained unclear. In yeast, like in mammalian cells, AP-3 deletion leads to the mis-sorting of the vacuolar membrane proteins alkaline phosphate and Vam3p, but does not affect the trafficking of Vps10p, the receptor for the soluble hydrolase...
The role of clathrin in AP-3 function has also remained controversial. Two isoforms of the AP-3 β subunit are known, the ubiquitous β3A and the neuro (endocrine)-specific β3B, both of which contain a clathrin consensus-binding motif (Dell’Angelica et al., 1998; ter Haar et al., 2000). Biochemical analyses have shown that AP-3 can bind and recruit clathrin in vitro onto synthetic liposomes (Dell’Angelica et al., 1998; Drake et al., 2000). However, unlike AP-1 and AP-2, the AP-3 complex is not enriched in purified clathrin-coated vesicles (Simpson et al., 1996; unpublished data), and genetic and biochemical evidence suggest that the AP-3 complex does not require clathrin for function in vivo (Vowels and Payne, 1998; Wettey et al., 2002). Moreover, AP-3-deficient mammalian cells were shown to be rescued almost as well by a β3A construct lacking the consensus clathrin-binding site as by the wild-type construct, suggesting that clathrin binding to the β3A subunit of the AP-3 is not required for AP-3 function (Peden et al., 2002). Immuno-colocalization analyses of clathrin and AP-3 have also produced conflicting results (Simpson et al., 1996; Dell’Angelica et al., 1998; Peden et al., 2002). Thus, whether or not clathrin is required for AP-3 function still remains unclear.

To address these key questions on AP-3 function, we have generated an mAb against the δ subunit of the AP-3 complex, and have used this antibody to localize AP-3 by immuno-EM. This approach offers the unique possibility of localizing AP-3 in direct comparison to lysosomal membrane proteins, as well as with other cargo and coat proteins, at a resolution sufficient to define small membrane micro-domains involved in protein sorting. Our results provide evidence for the function of the AP-3 complex in the trafficking of lysosomal membrane proteins from endosomes, whereas no support for a role at the TGN was found. Unexpectedly, our data indicate that the endosomal AP-3 pathway arises from early endosome (EE)–associated tubules rather than the endosomal vacuole, implicating tubular endosomes also involved in protein recycling as an important intermediate station in lysosomal membrane protein traffic to the lysosomes.

**Results**

**Generation of mAbs against the δ subunit of AP-3**

To investigate the localization of the AP-3 adaptor complex, it was necessary to generate antibodies that are suitable for immuno-EM experiments. To this end, we immunized mice with a GST fusion protein containing the hinge region (aa 608–800) of the δ subunit, as this region is the most immunogenic (Fig. 1 A, available at http://www.jcb.org/cgi/content/full/jcb.200311064/DC1). After fusion, the supernatants from the resulting hybridomas were screened by ELISA. Then, Western blotting and immunofluorescence were performed on both control and mocha fibroblasts (δ deficient) to confirm their specificity. SA4, the monoclonal used in this work, did not yield a Western or immunofluorescence signal in the mocha extracts or cells, confirming that the antibody is specific for the δ subunit and does not cross react with any other proteins (Fig. S1, B and C). Moreover, the monoclonal gives the characteristic staining pattern for AP-3 of punctate spots throughout the cytoplasm (Fig. S1 C).

**Immuno-EM localization of the AP-3 complex on EE-associated tubules**

To investigate the localization of the AP-3 adaptor complex in subcellular detail, ultrathin cryosections were prepared from HepG2 and normal rat kidney (NRK) cells, and were immunogold labeled with the different anti-δ subunit mAbs. Significant specific immunogold labeling was obtained with several anti-AP-3 monoclonals, but SA4 was selected for this work as it gave the highest labeling efficiency, especially in cells fixed only with formaldehyde. Although we obtained a similar staining pattern for AP-3 in both cell lines, we concentrated on HepG2 cells because they exhibited stronger staining.

First, we addressed whether AP-3 resides on the TGN and/or on endosome-associated membranes. The tubular membrane network within a distance of ~500 nm from the trans-most cisternae of a Golgi stack was considered as TGN (Geuze et al., 1985). These membranes are enriched in cation-independent mannose 6-phosphate receptor (CI-MPR; Fig. 1) and TGN46 (unpublished data), and show clathrin-coated and AP-1–coated buds at their cytoplasmic surface (Fig. 1 A) that mediate the exit of CI-MPR from the TGN (Klumperman et al., 1993; Doray et al., 1998). In mammalian cells, immunofluorescence localization experiments of the AP-3 complex have revealed intense staining in the perinuclear region, partially overlapping with markers of the TGN, but also significant staining out in the cell periphery, partially colocalizing with endocytic markers such as TIR (Simpson et al., 1996, 1997; Dell’Angelica et al., 1997, 1999). The degree of overlap with either TGN or endosomal markers varied considerably between these experiments. EM localization analyses also indicated a dual localization of the AP-3 complex on the TGN, as well as on endosomes (Simpson et al., 1996; Dell’Angelica et al., 1998).

At present, two (not mutually exclusive) models have been proposed to explain the role of the AP-3 complex in lysosomal membrane protein trafficking in mammalian cells (for review see Starcevic et al., 2002). In the first model, as in yeast, AP-3 is proposed to be responsible for recruiting lysosomal membrane proteins into a direct transport pathway from the TGN to endosomes/lysosomes. Lack of AP-3 function would then result in increased amounts of newly synthesized proteins traveling from the TGN via the constitutive pathway to the plasma membrane, from where it would be internalized by AP-2–dependent endocytosis and subsequently transported to the lysosomes. The second model proposes that AP-3 functions at an early endosomal compartment in which lysosomal membrane proteins must be segregated from other membrane proteins that recycle back to the plasma membrane. Lack of functional AP-3 would then lead to a buildup on the plasma membrane and in the recycling system.

**carboxypeptidase Y** (Cowles et al., 1997; Stepp et al., 1997). By contrast, yeast VPS mutants are still able to sort vacuolar membrane proteins to the vacuole. Hence, it was proposed that in yeast, AP-3 mediates a direct pathway for vacuolar membrane proteins from the TGN to the vacuole (Odorizzi et al., 1998). In mammalian cells, immunofluorescence localization experiments of the AP-3 complex have revealed intense staining in the perinuclear region, partially overlapping with markers of the TGN, but also significant staining out in the cell periphery, partially colocalizing with endocytic markers such as TIR (Simpson et al., 1996, 1997; Dell’Angelica et al., 1997, 1999). The degree of overlap with either TGN or endosomal markers varied considerably between these experiments. EM localization analyses also indicated a dual localization of the AP-3 complex on the TGN, as well as on endosomes (Simpson et al., 1996; Dell’Angelica et al., 1998).

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The average diameter of the CI-MPR and AP-1–positive buds on the TGN was 77 nm \((n = 42)\). No significant AP-3 labeling was found in these TGN profiles (Fig. 1, B–D).

By contrast, AP-3 was present on highly convoluted tubules that were often found in close vicinity to endosomal vacuoles (Fig. 1 C and Fig. 2). On these tubules, AP-3 was associated with buds that were significantly smaller than those on the TGN, i.e., only 33 nm \((n = 40;\) Fig. 2, A–E). The AP-3–positive tubules were reminiscent of the EE recycling tubules as described by others (Geuze et al., 1983; Marsh et al., 1986; Griffiths et al., 1989). Therefore, to assay the nature of these tubules, we performed colocalization experiments with internalized transferrin (Tf) and asialoglycoprotein receptor (ASGPR), both markers of the endosomal recycling pathway. After a 20-min internalization, biotinylated Tf (Tf-biotin) was found at the limiting membrane of EE vacuoles, as well as in the associated tubules (Fig. 2, A and B). By double labeling, we found AP-3–positive buds on the Tf-biotin–containing recycling tubules. Similar results were obtained when cells were double labeled for AP-3 and endogenous ASGPR (Fig. 2, C and D). Primary endocytic vesicles labeled with the endocytic tracer BSA-gold were consistently devoid of AP-3 label (unpublished data). AP-3–positive tubules also contained small amounts of CI-MPR (Fig. 2 E). The CI-MPR label was mostly found dispersed in the noncoated areas of the AP-3–positive tubules, but occasionally AP-3–negative buds were observed where CI-MPR was concentrated (Fig. 2, F and G; arrowheads). Together, these findings show that AP-3 resides on a tubular endosomal compartment that contains cargo proteins destined for recycling to the plasma membrane or the TGN.

**AP-3 localizes to clathrin-coated membranes**

Previous reports have not clearly resolved whether AP-3 uses clathrin as its structural scaffold in vivo. Therefore, we performed double labelings of AP-3 and clathrin (Fig. 3, A and B). AP-3 was found on membranes that were (Fig. 3 A) and were not (Fig. 3 B) labeled for clathrin. Moreover, AP-3–positive buds were also seen on tubules that displayed clath-
rin-coated buds not labeled for AP-3 (Fig. 3 B). This prompted us to investigate whether different APs may associate with distinct buds of a continuous endosomal tubule. Indeed, double labeling of AP-3 and AP-1 in HepG2 cells revealed that these two coat complexes could be found on distinct buds arising from a single, continuous tubular membrane (Fig. 3 C). By triple labeling, the presence of internalized Tf-biotin was shown in the tubular membranes displaying AP-1- and AP-3-positive buds, confirming their endosomal origin (Fig. 3 D). Similar observations were
made in NRK cells (Fig. 3 E). The average size of AP-1 buds on endosomal tubules was 43 nm (n = 37), which is considerably smaller than AP-1 buds in the TGN (77 nm) and in the same range as the AP-3 buds (33 nm). Clathrin coats are readily visible in ultrathin cryosections (Fig. 1, A–D; Fig. 3 B; Fig. 4 A). In the AP-3 and AP-1 double-labeled cells, 46.3 ± 7.4% (average ± SEM) of AP-3 was found on clathrin-coated membranes (as defined by the presence of a dense cytosolic coat), whereas in these same cells 91 ± 8.5% of AP-1 label resided in coated membranes. Colocalization of AP-3 and AP-1 onto a single membrane bud or vesicular profile occurred to a very minor extent (<10%). We conclude from these data that AP-3 does localize with clathrin, but to a lesser extent than does AP-1, and that AP-3 and AP-1 mediate different exits from a single endosomal tubule.

**LAMP-1 and LAMP-2 are concentrated in AP-3-positive buds on endosomal tubules**

So far, our data indicate a role for AP-3 in protein traffic from the tubular recycling endosomes evolving from EEs. This was surprising because previous reports had found no effect of AP-3 deficiency on recycling proteins. Moreover, endosomal tubules are not known to be involved in the delivery of lysosomal membrane proteins to the lysosomes. To address this issue, we performed double labelings of AP-3 and LAMP-1 in HepG2 cells (Fig. 4, A–C, E, and F). The vast majority of LAMP-1 label was, as expected, found in the lysosomes (Fig. 4, E and F). LAMP-1 located in the TGN showed no colocalization with AP-3 (Fig. 4 A). Importantly, low but consistent amounts of LAMP-1 label were present in the AP-3–bearing endosomal tubules (Fig. 4, B and C). LAMP-1 colocalized with internalized Tf-biotin in EE vacuoles and in the surrounding tubules, reinforcing the notion that LAMP-1 is present in EE-derived recycling tubules (arrows). EE, early endosome; G, Golgi complex; L, lysosome; M, mitochondrion; N, nucleous; P, plasma membrane. Bars, 200 nm.

**Figure 4.** LAMP-1 is concentrated in AP-3-positive budding profiles. (A–C) Double labelings of AP-3 (15-nm gold) and LAMP-1 (10-nm gold), showing colocalization in endosome (E)-associated tubular-vesicular profiles (arrows). Low labeling densities of LAMP-1 are present in the AP-3–positive tubules. Note that in A, the LAMP-1/AP-3 positive tubule (arrow) has a smaller diameter than the typical TGN-associated clathrin-coated buds (arrowheads). (D) Colocalization of LAMP-1 (10-nm gold) and Tf-biotin internalized for 20 min (15-nm gold) unequivocally shows that LAMP-1 is present in EE-derived recycling tubules (arrow). (E and F) Double labelings of AP-3 (15-nm gold) and LAMP-1 (10-nm gold), showing that LAMP-1 is found within the AP-3–positive regions of the endosome-derived tubules (arrows). EE, early endosome; G, Golgi complex; L, lysosome; M, mitochondrion; N, nucleous; P, plasma membrane. Bars, 200 nm.
umn C), which amongst others will include the AP-1–coated buds and AP-3–independent concentration sites such as is shown for CI-MPR (Fig. 2, F and G). To measure the enrichment for cargo proteins in AP-3–positive buds, we therefore divided the labeling density in the AP-3–positive buds by that in the nonconcentrating, noncoated regions of the tubule (Table I, column A/B). This clearly showed that LAMP-1 and LAMP-2 are concentrated in AP-3 buds over the attached AP-3–negative membrane tubules, by factors of 8.5 and 3.6, respectively. By contrast, the ASGPR and CI-MPR are not enriched in the AP-3–positive buds. These results suggest that AP-3 plays a role in the segregation and concentration of LAMP-1 and LAMP-2 (but not CI-MPR and ASGPR) within the tubular endosomal compartment.

Loss of AP-3 leads to increased levels of recycling of LAMP-1 and CD63 back to the cell surface

From our immuno-EM experiments, we would predict that loss of AP-3 should lead to increased recycling of LAMP-1 and CD63 via the cell surface due to the decreased efficiency of lysosomal delivery from the tubular endosomes. To test this hypothesis, we decided to look at the trafficking of both LAMP-1 and CD63 in AP-3–deficient and rescued fibroblasts, using flow cytometry. Due to the lack of an antibody against mouse CD63, a mocha cell line was generated that expressed human CD63 at a similar level to that of endogenous LAMP-1 (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200311064/DC1). This cell line was then subsequently infected with either empty virus (Fig. 6 A, mock) or virus expressing the β subunit of the AP-3 complex (Fig. 6 A, rescued). By immuno-EM, AP-3 in rescued mocha cells showed a similar labeling pattern as in HepG2 cells (Fig. S3); AP-3 was readily detected on endosome-associated tubules where it colocalized with LAMP-1. Quantitation of LAMP-1 labeling density over AP-3–bearing tubules revealed a marked concentration of LAMP-1 in the AP-3–positive buds (Table I). Using the mocha cells as negative control, the relative distribution of AP-3 in rescued mocha cells was established (Table II). This quantitative approach revealed low

Table I. Lysosomal membrane proteins are concentrated in AP-3–labeled regions of tubular sorting endosomes

| Protein   | (A) AP-3<sup>+</sup> | (B) AP-3<sup>-</sup> (noncoated) | (C) AP-3<sup>-</sup> (coated) | A/B |
|-----------|---------------------|---------------------------------|-------------------------------|-----|
| HepG2 LAMP-1 | 0.34 ± 0.03         | 0.04 ± 0.03                     | 0.03 ± 0.02                   | 8.5 |
| HepG2 LAMP-2 | 0.36 ± 0.04         | 0.10 ± 0.05                     | 0.05 ± 0.02                   | 3.6 |
| HepG2 CI-MPR | 0.15 ± 0.03         | 0.15 ± 0.04                     | 0.15 ± 0.02                   | 1.0 |
| HepG2 ASGPR | 0.13 ± 0.03         | 0.14 ± 0.07                     | 0.16 ± 0.02                   | 0.9 |
| Rescued mocha LAMP-1 | 0.34 ± 0.01 | 0.01 ± 0.01                     | 0.03 ± 0.03                   | 34  |

Numbers represent the labeling densities ± SEM of the indicated cargo proteins in distinct regions of AP-3–positive compartments. Within AP-3–positive membrane tubules, three membrane categories were discriminated: (A) AP-3–positive membrane buds and vesicles; (B) AP-3–negative, noncoated often tubular membranes; and (C) AP-3–negative but visibly coated membrane buds. B and C were only defined when seen in continuity with A.
levels (4%) of staining in the TGN (defined as within 500 nm of the trans-side of a Golgi complex), whereas 43% of the label was found on the characteristic tubular membranes near endosomal vacuoles (Table II, Fig. S3). 40% of the AP-3 label was associated with tubulo-vesicular profiles that within the plain of the section were not in close vicinity of an endosomal vacuole or the Golgi complex.

Notably, we found that the levels of LAMP-1 and CD63 were greatly increased on the cell surface in mock-infected cells (AP-3 deficient) compared with rescued mocha cells (approximately eight- and fivefold, respectively; Fig. 6 B). Interestingly, the level of CD63 at the cell surface is far greater than that of LAMP-1 both in mock and rescued cells (Fig. 6 B and Fig. S2). In agreement with the steady-state levels, the amount of LAMP-1 and CD63 delivered to the cell surface is also greatly increased in mock compared with rescued cells (Fig. 6 C; for triplicate experiments see Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200311064/DC1). These data confirm previous reports showing increased trafficking of LAMP-1 and CD63 over the plasma membrane of AP-3–deficient cells (Dell’Angelica et al., 1999), but do not discriminate between proteins derived from the TGN or endosomes. To address this point, we next looked at the levels of recycling of internalized anti-TfR, anti-LAMP-1, and anti-CD63 antibodies fed to the cells in a 20-min pulse (Fig. 7). No significant difference was detected between TfR recycling in mock and rescued cells. This result was confirmed by assaying Tf recycling (unpublished data). However, in agreement with the proposed role for AP-3 on tubular endosomes, we detected a significant increase in antibody recycling for LAMP-1 and CD63 in mock compared with rescued cells (1.8–3.3- and 1.7–2.1-fold increase, respectively; experiments were performed in triplicate and similar results were obtained and shown in Fig. S5).

**Discussion**

Despite significant progress in our understanding of AP-3 structure, function, and regulation, several important questions have remained unanswered: (1) the subcellular localization of AP-3; (2) the transport step of lysosomal membrane proteins that is mediated by AP-3; and (3) the physiological significance of clathrin in AP-3–dependent trafficking. In this work, we have generated an mAb against the ε subunit of AP-3 to localize the complex by immuno-EM, and have studied the trafficking of lysosomal membrane proteins in AP-3–deficient cells. Collectively, our data reveal that the AP-3 complex marks a thus far unrecognized exit site for lysosomal membrane proteins from endosome-associated tubules.
First, we set out to address to what compartment (or compartments) the AP-3 complex localizes in mammalian cells. In HepG2 cells, AP-3 was typically found on budding profiles emerging from endosomal tubules containing internalized Tf-biotin and ASGPR, two classical markers of the endosomal recycling pathway (Dautry-Varsat et al., 1983; Geuze et al., 1983; Klausner et al., 1983; Hopkins et al., 1994), and CI-MPR, which recycles between endosomes and the TGN (Kornfeld and Mellman, 1989). In addition, AP-3–bearing tubules contained the lysosomal proteins LAMP-1 and LAMP-2 and displayed distinct, small-sized AP-1–positive buds. AP-1 on endosomal tubules has been implicated in retrograde transport of MPR46 and Shiga toxin B subunit to the TGN (Mallard et al., 1998; Meyer et al., 2000), as well as recycling of internalized Tf (Mallard et al., 1998; van Dam and Stoorvogel, 2002). The presence of distinct types of recycling proteins and the association of multiple adaptor complexes suggest that from the AP-3–containing tubular endosomes, proteins are sorted to distinct destinations in the cell. Therefore, the AP-3–positive compartment is best referred to as tubular sorting endosome. A point of future interest will be to define the physical and functional boundaries between the tubular sorting endosome, vacuolar sorting endosomes, and tubular recycling endosomes.

Our quantitative analyses revealed that of the different types of cargo proteins present in the tubular sorting endosomes, only LAMP-1 and LAMP-2 were concentrated in the AP-3–positive buds and associated vesicular profiles (Table I). By contrast, ASGPR and CI-MPR were equally distributed over the AP-3–bearing and nonlabeled regions of the endosomal tubules. These findings imply that AP-3 on tubular sorting endosomes mediates an exit specific for lysosomal membrane proteins. Lysosomal membrane proteins may reach EEs via a direct pathway from the TGN or by following the constitutive secretory pathway to the cell surface and subsequent endocytosis (for review see Peters and von Figura, 1994; Hunziker and Geuze, 1996; Rouille et al., 2000). In rat hepatocytes, 45% of newly synthesized LAMP-2 and at least 25% of LAMP-1 passes through EEs before it reaches later compartments (Akasaki et al., 1995, 1996). A first possible pathway to the lysosome is then by incorporation into the intra-endosomal vesicles that form by inward budding of the limiting membrane, and this leads to the formation of multivesicular bodies (reviewed by Geuze, 1998). Sorting into intra-endosomal vesicles is an active, signal-mediated event, whereas recycling from EEs back to the cell surface can occur by default (Dunn et al., 1989; Jing et al., 1990; Mayor et al., 1993; Verges et al., 1999; Sachse et al., 2002). Lysosomal membrane proteins that are not sorted into the intra-endosomal vesicles will enter the tubular sorting endosomes. Our data suggest that from here a second, AP-3–mediated sorting step to the lysosomes originates (see Fig. 8 for a model). As suggested previously (Dell’Angelica et al., 1999; Starcevic et al., 2002), the model in Fig. 8 also accounts for the increased transport of lysosomal proteins via the plasma membrane in AP-3–deficient cells. Specifically, if AP-3 deficiency impairs transport from tubular sorting endosomes to lysosomes, it will lead to an increased incorporation of lysosomal membrane proteins into the default recycling pathway to the plasma membrane. Indeed, this was what we observed when we compared the level of recycling of endocytosed LAMP-1 and CD63 to the plasma membrane in AP-3–deficient and rescued fibroblasts, providing additional strong evidence that AP-3 acts on an endosomal compartment. Consistent with previous papers (Le Borgne et al., 1998; Dell’Angelica et al., 1999), recycling of the TfR is unaffected. See also Fig. S4 (available at http://www.jcb.org/cgi/content/full/jcb.200311064/DC1).
A role for AP-3 in lysosomal membrane protein traffic from tubular sorting endosomes is in agreement with previous immuno-EM data in PC12 and A431 cells (Dell’Angelica et al., 1998), and with the recent observation that in AP-3–deficient cytotoxic T-lymphocytes, tubular endosomes elongate (Clark et al., 2003). Our observations are in contrast to a previous immuno-EM experiment in NRK cells by Simpson et al. (1996), showing that AP-3 labeling is mainly associated with the TGN. However, some of the localization experiments of Simpson et al. (1996) were done in the presence of GTPγS, which can lead to the mislocalization of coat complexes (Seaman et al., 1993). By in vitro recruitment experiments in mocha cells in the presence of GTPγS, it was found that the AP-3 complex indeed distributes to a more perinuclear location than normal (unpublished data). We defined the TGN as the membrane network within ~500 nm of the trans-side of the Golgi stack (Geuze et al., 1985). Our quantitations in rescued mocha cells revealed that 4% of total AP-3 label was found on membranes that by this definition were classified as TGN. 41% of the AP-3 label was found on tubulo-vesicular membranes associated with endosomes, whereas 40% of AP-3 label was not clearly associated with either endosomes or TGN (indicated as cytoplasmic). The majority of the cytoplasmic membranes is probably of endosomal origin, but we cannot exclude that they include peripheral TGN profiles as well (Puertollano et al., 2003; Waguri et al., 2003). Therefore, it remains possible that AP-3 does play a minor role in the trafficking of cargo molecules from the TGN, as was also suggested by others (Nishimura et al., 2002; Rous et al., 2002).

Another issue we addressed was the potential interaction of the AP-3 complex with clathrin. The association of AP-3 with clathrin has been shown previously (Dell’Angelica et al., 1998), but we provide here the first quantitative analysis in direct comparison with a well-known clathrin-binding adaptor, AP-1. We found in HepG2 cells 46% of AP-3–positive membranes, and 91% of AP-1–positive membranes were coated for clathrin as well. A previous work in neuroendocrine PC12 cells revealed that 65% of the AP-3–positive membranes labeled for clathrin (Dell’Angelica et al., 1998), which is possibly explained by the fact that in these cells, AP-3 is involved in clathrin-dependent formation of synaptic vesicles from endosomes (Faundez et al., 1998). Together, the data in PC12 and HepG2 cells indicate that AP-3 uses clathrin in vivo, but that at steady state its association with clathrin is significantly lower than that of AP-1. Possibly, the interaction between AP-3 and clathrin is weaker than that found with the other adaptor complexes. This would also explain why the AP-3 complex is not present in clathrin-coated vesicle preparations and why clathrin is not readily immunoprecipitated with antibodies against AP-3 (Simpson et al., 1996; unpublished data). Alternatively or additionally, the assembly of an AP-3 clathrin coat may be a relatively slow process, resulting in lower steady-state levels of association. A third possible explanation is that clathrin might play a facilitating rather than obligatory role. Clearly, further analyses are required to distinguish between these possibilities.
Materials and methods

Antibody reagents

Mouse mAbs against the AP-3 β subunit were generated by immunizing female BALB/c mice with a GST fusion protein corresponding to the hinge domain of the subunit (aa 608-800 of human β-adaptin). Fusion of the spleen cells with myeloma cells and screening of the resulting antibodies was performed as described by Bock et al. (1997). Clones that yielded an immunofluorescence pattern identical to that of an anti-β polyclonal (Simpson et al., 1997; a gift from Dr. M.S. Robinson, University of Cambridge, Cambridge, UK) were further characterized by immuno-EM. The clone SA4 gave the most intense labeling, so it was used for all the immuno-EM experiments performed in this paper. The SA4 cell line and resultant antibodies are available from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). The following polyclonal antibodies were generously donated to us: anti-AP-1 (Dr. M. Robinson, University of Cambridge, Cambridge, UK); antibody 9311 against human LAMP-1 and antibody 9321 against human LAMP-2 (Dr. M. Fukuda, La Jolla Cancer Research Foundation, La Jolla, CA); anti-mouse IgG1/2bLAMP-1 (Dr. I. Mellman, Yale University, New Haven, CT); anti-C1-MPR (Dr. K. von Figura, University of Göttingen, Göttingen, Germany); antibody K1 against the ASGPR (Dr. A. Schwartz, Washington University, St. Louis, MO); and anti-clathrin (Dr. S. Convera, University of Massachusetts Medical School, Worcester, MA). Anti-biotin 100-4198 was obtained from Rockland Immunochemicals. To compare the degree of colocalization of AP-3 and clathrin with that of AP-1 and clathrin, an mAb from Dr. E. Ungewickell (Medical University Hannover, Hannover, Germany) was used instead of the polyclonal anti-AP-1. As a bridging step between mouse mAbs and protein A–gold, we used rabbit anti–mouse (DakoCytomation). The hybridoma cell lines 1D4B (rat anti–mouse LAMP-1; Hughes and August, 1981) and H5C6 (mouse anti–human CD63) were obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa (Department of Biological Sciences, Iowa City, IA). The cell line TIB219 (rat anti–mouse TIR; Lesley et al., 1984) was obtained from the American Type Culture Collection. All three antibodies were purified from the culture media using an anti–rat or anti–mouse affinity column, dialyzed, and eluted, and conjugated to Alexa Fluor® 488 or 594 using a protein-labeling kit according to the manufacturer’s instructions (Molecular Probes, Inc.).

Flow cytometry

A quantitative assay for AP-3–dependent sorting was used as described by Peden et al. (2002), with the modifications listed below. Cells were trypsinized, washed in complete medium, and incubated with Alexa® 488–conjugated anti-LAMP-1 and anti-CD63 antibodies (2 μg/ml for 30 min at 4°C. After shifting to 37°C for various times, the antibody binding was stopped by pelleting the cells and resuspending them in 3% formaldehyde. To determine the amount of recycling of LAMP-1, CD63, and TIR, cells were either allowed to internalize labeled antibodies for 20 min, after which excess antibodies were washed away with ice-cold DMEM. The cells were then incubated with 24 μg/ml quenching antibody (anti-Alexa® 488; Molecular Probes, Inc.) in DME at 37°C for various times. To ensure complete quenching, the cells were placed on ice for 30 min before being fixed in 3% formaldehyde. The cells were analyzed using a flow cytometer (Epics-XI; Beckman Coulter).

Retroviral infections

The AP-3-β subunit–deficient mocha cell line (CRL-2709; American Type Culture Collection), previously described by Peden et al. (2002), was infected with virus expressing human CD63, followed by infection with the wild-type β subunit of the AP-3 complex or empty virus as described by Swift et al. (1999). The virus used was a version of pBMN modified such that the hygromycin selectable marker was expressed on the same transcript as the Vpr gene. All pictures were taken in the same experiment as the rescued mochas yielded 74 gold particles, representing non–specific background. After subtracting the background label over the distinct compartments from the specific label, the percentage of total label over a given compartment in rescued mocha cells was calculated (Table II). All tubulo-vesicular membranes within 200 nm from an endosomal vacuole were designated as endosomal compartments, whereas membranes within 500 nm of the trans-side of a Golgi complex were designated TGN. Tubulo-vesicular membranes that in the plane of the section were not associated with Golgi or endosomes were designated as cytoplasmic. All pictures were taken on a transmission electron microscope (model 1010; JEOL USA, Inc.).

Immunofluorescence

Cells were cultured on multwell slides (ICN Biomedicals), fixed for 15 min with 3% PFA in PBS, quenched for 10 min with 0.1 M glycine in PBS, permeabilized with 0.4% saponin in PBS for 10 min, and then subjected to the primary antibody for 20 min. After incubation with secondary antibody (conjugated to Alexa® 488 or 594 for 20 min, coverslips were mounted onto the cells with Vectashield® (Vector Laboratories). Images were acquired using a microscope (Axiovert 200; Carl Zeiss MicroImaging, Inc.) fitted with a Plan-Apochromat 1.4 NA 63× objective, a camera (AxioCam; Carl Zeiss MicroImaging, Inc.), and a Quad pass filter set (Chroma Technology Corp.), all controlled by AxioVision 3.1 software (Carl Zeiss MicroImaging, Inc.). The brightness and contrast of acquired images was adjusted using Adobe Photoshop® v6.0.

Online supplemental material

Fig. S1 shows the characterization of mAb SA4 against the β subunit of AP-3 that was used in this work. Fig. S2 shows the fluorescence staining of LAMP-1 and CD63 in mock-transfected and rescued mocha cells. Fig. S3 shows the colocalization of AP-3 and LAMP-1 in endosomal tubules of rescued mocha cells.
cued mocha cells. Fig. S4 and Fig. S5 show the triplicates of the antibody binding and recycling assays described in Fig. 6 and Fig. 7, respectively. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200311064/DC1.

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References

Akasaki, K., M. Fukuzawa, H. Kinoshita, K. Furuno, and H. Tsuji. 1993. Cycling of two endogenous lysosomal membrane proteins, lamp-2 and acid phosphatase, between the cell surface and lysosomes in cultured rat hepatocytes. J. Biochem. (Tokyo). 114:598–604.

Akasaki, K., A. Michihara, K. Mibuka, Y. Fujiwara, and H. Tsuji. 1995. Biosynthetic transport of a major lysosomal membrane glycoprotein, lamp-1: convergence of biosynthetic and endocytic pathways occurs at three distinctive points. Exp. Cell Res. 220:464–473.

Akasaki, K., A. Michihara, Y. Fujiwara, K. Mibuka, and H. Tsuji. 1996. Biosynthetic transport of a major lysosome-associated membrane glycoprotein 2, lamp-2: a significant fraction of newly synthesized lamp-2 is delivered to lysosomes by way of early endosomes. J. Biochem. (Tokyo). 120:1088–1094.

Bock, J.B., J. Klumperman, S. Davanger, and R.H. Scheller. 1997. Syntaxin 6 functions in trans-Golgi network vesicle trafficking. Mol. Biol. Cell. 8:1261–1271.

Clark, R.H., J.C. Stinchcombe, A. Day, E. Blott, S. Booth, G. Bossi, T. Hamblin, E.G. Davies, and G.M. Griffiths. 2003. Adaptor protein 3-dependent microtubule-mediated movement of lytic granules to the immunological synapse. Nat. Immunol. 4:1111–1120.

Cowles, C.R., G. Odorizzi, G.S. Payne, and S.D. Emr. 1997. The AP-3 adaptor complex is essential for cargo-selective transport to the yeast vacuole. Cell. 91:109–118.

Dautry-Varsat, A., A. Ciechanover, and H.F. Lodish. 1983. pH and the recycling of asialoglycoprotein receptor-ligand uncoupling: double-label autoradiography. J. Cell Biol. 109:2703–2720.

Doray, B., P. Ghosh, J. Griffith, H.J. Geuze, and S. Kornfeld. 2002. Cooperation of two endogenous lysosomal membrane proteins, lamp-2 and acid phosphatase, on the trans-Golgi network. J. Cell Biol. 159:2321–2330.

Drake, M.T., Y. Zhu, and S. Kornfeld. 2000. The assembly of AP-3 adaptor complex with clathrin. Science. 280:431–434.

Dell’Angelica, E.C., V. Rouille, and B. Hoflack. 1998. The mammalian AP-3 adaptor-like complex mediates the intracellular transport of lysosomal membrane glycoproteins. J. Biol. Chem. 273:29451–29461.

Dunn, K.W., T.E. McGraw, and F.R. Maxfield. 1989. Iterative fractionation of recycling receptors from lysosomally destined ligands in an early sorting endosome. J. Cell Biol. 109:3303–3314.

Faundez, V., J.T. Horng, and R.B. Kelly. 1998. A function for the AP3 coat complex in synaptic vesicle formation from endosomes. Cell. 93:423–432.

Geuze, H.J. 1998. The role of endosomes and lysosomes in MHC class II functioning. Immunol. Today. 19:282–287.

Geuze, H.J., J.W. Slot, G.J. Strous, A. Haslik, and K. von Figura. 1985. Possible pathways for lysosomal enzyme delivery. J. Cell Biol. 101:2253–2262.

Harter, C., and I. Mellman. 1992. Transport of the lysosomal membrane glycoprotein Igp120 (IgpA) to lysosomes does not require appearance on the plasma membrane. J. Cell Biol. 117:311–325.

Heuser, J.E., and J. Keen. 1988. Deep-etch visualization of proteins involved in clathrin assembly. J. Cell Biol. 107:877–886.

Hopkins, C.R., A. Gibson, M. Shipman, D.K. Strickland, and I.S. Trowbridge. 1994. In migrating fibroblasts, recycling receptors are concentrated in narrow tubules in the pericentriolar area, and then routed to the plasma membrane of the leading lamella. J. Cell Biol. 125:1265–1274.

Hughes, E.N., and J.T. August. 1981. Characterization of plasma membrane protein microdomains identified by monoclonal antibodies. J. Biol. Chem. 256:664–671.

Hunziker, W., and H.J. Geuze. 1996. Intracellular trafficking of lysosomal membrane glycoproteins. Bioessays. 18:379–389.

Jing, S.Q., T. Spencer, K. Miller, C. Hopkins, and I.S. Trowbridge. 1990. Role of the human transferrin receptor cytoplasmic domain in endocytosis: localization of a specific signal sequence for internalization. J. Cell Biol. 110:283–294.

Klausner, R.D., G. Ashwell, J. van Renswoude, J.B. Harford, and K.R. Bridges. 1983. Binding of apotransferrin to K562 cells: explanation of the transferrin cycle. Proc. Natl. Acad. Sci. USA. 80:2263–2266.

Klumperman, J., A. Hille, T. Veenendaal, V. Oortschoot, W. Stoorvogel, K. von Figura, and H.J. Geuze. 1993. Differences in the endosomal distributions of the two mannose 6-phosphate receptors. J. Cell Biol. 121:997–1010.

Kornfeld, S., and I. Mellman. 1989. The biogenesis of lysosomes. Annu. Rev. Cell Biol. 5:483–525.

Le Borgne, R., A. Alconada, U. Bauer, and B. Hoflack. 1998. The mammalian AP-3 adaptor-like complex mediates the intracellular transport of lysosomal membrane glycoproteins. J. Biol. Chem. 273:29451–29461.

Lesley, J., R. Hyman, R. Schulte, and J. Trotter. 1984. Expression of transferrin receptor on murine hematopoietic progenitors. Cell. Immunol. 83:14–25.

Liou, W., H.J. Geuze, and J.W. Slot. 1996. Improving structural integrity of cyto- sosomes for immunogold labeling. Histochem. Cell Biol. 106:41–58.

Lippincott-Schwartz, J., and D.M. Fambrough. 1987. Cycling of the integral membrane glycoprotein, LEP100, between plasma membrane and lysosomes: kinetic and morphological analysis. Cell. 49:669–677.

Mallard, F., C. Antony, D. Tenza, J. Salamero, B. Goud, and L. Johannes. 1998. Direct pathway from early/recycling endosomes to the Golgi apparatus revealed through the study of shiga toxin B-fragment transport. J. Cell Biol. 143:973–990.

Marsh, M., G. Griffiths, G.E. Dean, I. Mellman, and A. Helenius. 1986. Three-dimensional structure of endosomes in BHK-21 cells. Proc. Natl. Acad. Sci. USA. 83:2899–2903.

Mayor, S., J.F. Presley, and F.R. Maxfield. 1993. Sorting of membrane compo- nents from endosomes and subsequent recycling to the cell surface occurs by a bulk flow process. J. Cell Biol. 121:1257–1269.

Meyer, C., D. Ziaioli, S. Laumann, E.L. Eskelin, J. Hamann, P. Safig, K. von Figura, and P. Schu. 2000. μ1A adaptin-deficient mice: lethality, loss of AP-1 binding and rerouting of mannose 6-phosphate receptors. EMBO J. 19:2193–2203.

Nishimura, N., H. Plutner, K. Hahn, and W.E. Balch. 2002. The shc subunit of AP-3 is required for efficient transport of VSV-G from the trans-Golgi net- work to the cell surface. Proc. Natl. Acad. Sci. USA. 99:6755–6760.

Odorizzi, G., C.R. Cowles, and S.D. Emr. 1998. The AP-3 complex: a coat of many colours. Trends Cell Biol. 8:282–288.

Peden, A.A., R.E. Rudge, W.W. Lui, and M.S. Robinson. 2002. Assembly and function of AP-3 complexes in cells expressing mutant-subunits. J. Cell Biol. 156:327–336.

Peters, C., and K. von Figura. 1994. Biogenesis of lysosomal membrane organelles. FEBS Lett. 346:108–114.

Perrimon, J., M. Bakkali, and J. Stempel. 1993. Morphology and dynamics of clathrin/GGA1-coated carriers budding from the trans-Golgi network. Mol. Biol. Cell. 14:1549–1557.

Rous, B.A., B.J. Reaves, G. Ihrke, J.A. Briggs, S.R. Gray, D.J. Stephens, G.
Banting, and J.P. Luzio. 2002. Role of adaptor complex AP-3 in targeting wild-type and mutated CD63 to lysosomes. *Mol. Biol. Cell.* 13:1071–1082.

Sachse, M., S. Urbe, V. Oorschot, G.J. Strous, and J. Klumperman. 2002. Bilayered clathrin coats on endosomal vacuoles are involved in protein sorting toward lysosomes. *Mol. Biol. Cell.* 13:1313–1328.

Seaman, M.N., C.L. Ball, and M.S. Robinson. 1993. Targeting and mistargeting of plasma membrane adaptors in vitro. *J. Cell Biol.* 123:1093–1105.

Simpson, F., N.A. Bright, M.A. West, L.S. Newman, R.B. Darnell, and M.S. Robinson. 1996. A novel adaptor-related protein complex. *J. Cell Biol.* 133:749–760.

Simpson, F., A.A. Peden, L. Christopoulou, and M.S. Robinson. 1997. Characterization of the adaptor-related protein complex, AP-3. *J. Cell Biol.* 137:835–845.

Slot, J.W., H.J. Geuze, S. Gigengack, G.E. Lienhard, and D.E. James. 1991. Immuno-localization of the insulin regulatable glucose transporter in brown adipose tissue of the rat. *J. Cell Biol.* 113:123–135.

Starcevic, M., R. Nazarian, and E.C. Dell’Angelica. 2002. The molecular machinery for the biogenesis of lysosome-related organelles: lessons from Hermansky-Pudlak syndrome. *Semin. Cell Dev. Biol.* 13:271–278.

Stepp, J.D., K. Huang, and D.E. James. 1997. The yeast adaptor protein complex, AP-3, is essential for the efficient delivery of alkaline phosphatase by the alternate pathway to the vacuole. *J. Cell Biol.* 139:1761–1774.

Stoorvogel, W., H.J. Geuze, and G.J. Strous. 1987. Sorting of endocytosed transferrin and asialoglycoprotein occurs immediately after internalization in HepG2 cells. *J. Cell Biol.* 104:1261–1268.

Sugita, M., X. Cao, G.F. Watts, R.A. Rogers, J.S. Bonifacino, and M.B. Brenner. 2002. Failure of trafficking and antigen presentation by CD1 in AP-3-deficient cells. *Immunity.* 16:697–706.

Swift, S., J.B. Lorenz, P. Achacoso, and G.P. Nolan. 1999. Rapid production of retroviruses for efficient gene delivery to mammalian cells using 293T cell-based systems. In Current Protocols in Immunology. J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, editors. John Wiley & Sons Inc., New York. Suppl. 31.

van Dam, E.M., and W. Stoorvogel. 2002. Dynamin-dependent transferrin receptor recycling by endosome-derived clathrin-coated vesicles. *Mol. Biol. Cell.* 13:169–182.

Verges, M., R.J. Havel, and K.E. Mostov. 1999. A tubular endosomal fraction from rat liver: biochemical evidence of receptor sorting by default. *Proc. Natl. Acad. Sci. USA.* 96:10146–10151.

Waguri, S., F. Dewitte, R. Le Borgne, Y. Rouille, Y. Uchiyama, J.F. Dubremetz, and B. Hoflack. 2003. Visualization of TGN to endosome trafficking through fluorescently labeled MPR and AP-1 in living cells. *Mol. Biol. Cell.* 14:142–155.

Weibel, E.R. 1979. Stereological Methods: Practical Methods for Biological Morphometry. Vol. 1. Academic Press, London/New York. 415 pp.

Wettey, F.R., S.F. Hawkins, A. Stewart, J.P. Luzio, J.C. Howard, and A.P. Jackson. 2002. Controlled elimination of clathrin heavy-chain expression in DT40 lymphocytes. *Science.* 297:1521–1525.