

# Assembly and function of AP-3 complexes in cells expressing mutant subunits

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The mouse mutants *mocha* and *pearl* are deficient in the AP-3  $\delta$  and  $\beta$ 3A subunits, respectively. We have used cells from these mice to investigate both the assembly of AP-3 complexes and AP-3 function. In *mocha* cells, the  $\beta$ 3 and  $\mu$ 3 subunits coassemble into a heterodimer, whereas the  $\sigma$ 3 subunit remains monomeric. In *pearl* cells, the  $\delta$  and  $\sigma$ 3 subunits coassemble into a heterodimer, whereas  $\mu$ 3 gets destroyed. The yeast two hybrid system was used to confirm these interactions, and also to demonstrate that the A (ubiquitous) and B (neuronal-specific) isoforms of  $\beta$ 3 and  $\mu$ 3 can interact with each other. Pearl cell lines

were generated that express  $\beta$ 3A,  $\beta$ 3B, a  $\beta$ 3A $\beta$ 2 chimera, two  $\beta$ 3A deletion mutants, and a  $\beta$ 3A point mutant lacking a functional clathrin binding site. All six constructs assembled into complexes and were recruited onto membranes. However, only  $\beta$ 3A,  $\beta$ 3B, and the point mutant gave full functional rescue, as assayed by LAMP-1 sorting. The  $\beta$ 3A $\beta$ 2 chimera and the  $\beta$ 3A short deletion mutant gave partial functional rescue, whereas the  $\beta$ 3A truncation mutant gave no functional rescue. These results indicate that the hinge and/or ear domains of  $\beta$ 3 are important for function, but the clathrin binding site is not needed.

## Introduction

Adaptor proteins (APs)\* are heterotetrameric complexes that facilitate cargo selection and coated vesicle budding from different membrane compartments. Four such complexes have been identified in mammals, all of which consist of two large subunits: a  $\beta$  subunit and a more divergent, complex-specific subunit ( $\gamma$ ,  $\alpha$ ,  $\delta$ , or  $\epsilon$ ); a medium-sized or  $\mu$  subunit; and a small or  $\sigma$  subunit. The subunits are assembled into a structure resembling Mickey Mouse, with a brick-like "head," consisting of the NH<sub>2</sub>-terminal domains of the two large subunits, the medium subunit, and the small subunit, out of which protrude the COOH-terminal "ear" domains of the large subunits, connected by flexible hinges. The AP-1 and AP-2 complexes were identified first as abundant components of clathrin-coated vesicles. More recently, the AP-3 and AP-4 complexes were identified, primarily by finding homologues of the AP-1 and AP-2 subunits in the EST

database, raising antibodies against recombinant proteins, and showing by coimmunoprecipitation experiments that the proteins were assembled into two novel complexes (Robinson and Bonifacino, 2001).

Although the AP-3 complex was identified relatively recently, it has been much more amenable to genetic analysis than the AP-1 or AP-2 complexes. When the  $\delta$  subunit of the complex was first cloned, it was found to be the mammalian homologue of the protein encoded in *Drosophila* by the *garnet* gene, one of the classical eye color genes (Ooi et al., 1997; Simpson et al., 1997). *garnet* mutants have defective pigment granules, and because of similarities between pigment granules and lysosomes, this suggested a role for the AP-3 complex in the trafficking of proteins destined for lysosomes and related organelles. Subsequent studies in yeast showed that deleting any of the four AP-3 subunit genes causes missorting of a subset of proteins normally destined for the vacuole, the yeast equivalent of the lysosome (Cowles et al., 1997; Stepp et al., 1997). Two naturally occurring mouse mutants have also been identified with mutations in AP-3 subunits. The *mocha* (*mb*) mouse has a null mutation in the  $\delta$  subunit of the complex (Kantheti et al., 1998), whereas the *pearl* (*pe*) mouse has effectively a null mutation in the  $\beta$ 3A subunit (small amounts of mRNA can be detected which encode a truncated protein, missing the last 125 amino acids, but the protein has not been detected on Western blots) (Feng et al., 1999; Zhen et al., 1999). The mouse mutants have a similar phenotype to the human

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\*Abbreviations used in this paper: AP, adaptor proteins; HPS, Hermansky Pudlak syndrome.

Key words: coated vesicles; adaptors; clathrin; *mocha*; *pearl*

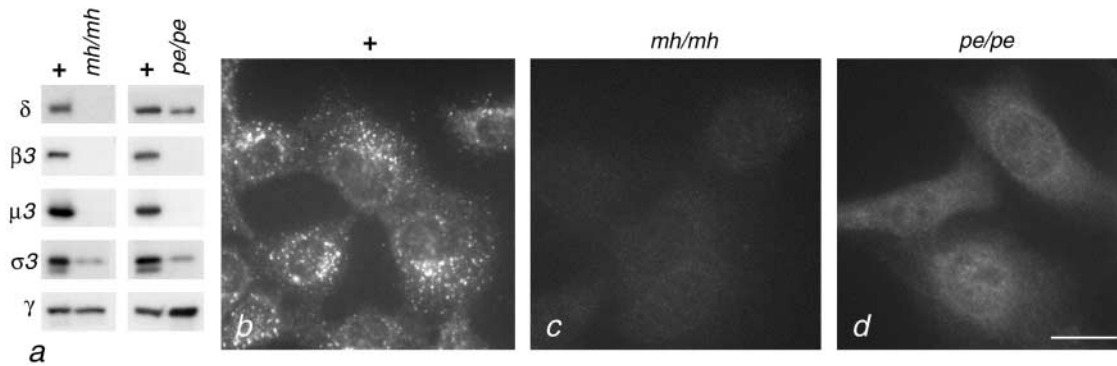


Figure 1. **AP-3 subunits in *mh* and *pe* cells.** (a) Western blots of lysates from the *mh* and *pe* cells, as well as a wild-type control cell line (melan-a cells), were probed with antibodies against all four AP-3 subunits and with an antibody against the AP-1  $\gamma$  subunit as a control. Only the  $\sigma 3$  subunit is detectable in the *mh* cells under these conditions, while only the  $\delta$  and  $\sigma 3$  subunits are detectable in the *pe* cells. (b–d) The same cells were labeled for immunofluorescence with an antibody against the AP-3  $\delta$  subunit. Control cells (b) have the typical punctate pattern, *mh* cells (c) show no labeling, and *pe* cells (d) have diffuse cytoplasmic labeling, indicating that although the  $\delta$  subunit is detectable, it is cytosolic rather than membrane-associated. Bar, 20  $\mu$ m.

genetic disorder Hermansky Pudlak syndrome (HPS), and indeed a subset of HPS patients has been identified with mutations in  $\beta 3A$  (Dell'Angelica et al., 1999). In both the mice and the humans, AP-3 deficiency results in hypopigmentation because of abnormalities in melanosomes, prolonged bleeding because of deficiencies in platelet dense granules, and reduced secretion of lysosomal enzymes into the urine. AP-3-deficient fibroblasts appear relatively normal, but several studies have revealed that lysosomal membrane proteins in these cells show increased trafficking via the plasma membrane (Dell'Angelica et al., 1999, 2000; Le Borgne et al., 1998). The *mh* mouse also has neurological defects, although the *pe* mouse and the  $\beta 3A$ -deficient humans are neurologically normal. This is presumably because there is a second  $\beta 3$  isoform,  $\beta 3B$ , which is specifically expressed in neurons and neuroendocrine cells (Kantheti et al., 1998). Together, these findings indicate that the AP-3 complex facilitates the trafficking of certain types of cargo to lysosomes and lysosome-related organelles.

Whether or not AP-3 is associated with clathrin is still somewhat controversial. Unlike AP-1 and AP-2, AP-3 is not enriched in purified clathrin-coated vesicles (Newman et al., 1995; Simpson et al., 1996). However, a clathrin-binding consensus sequence, L[L,I][D,E,N][L,F][D,E], has been identified in the hinge domains of  $\beta 1$  and  $\beta 2$ , and similar sequences are found in  $\beta 3A$  and  $\beta 3B$  which can interact with clathrin in vitro (Dell'Angelica et al., 1998; ter Haar et al., 2000). AP-3 is also able to coassemble with clathrin in vitro and to support clathrin recruitment onto liposomes (Dell'Angelica et al., 1998; Drake et al., 2000). On the other hand, an in vitro system for the budding of synaptic-like microvesicles from endosomes has been shown to require AP-3 but not clathrin (Shi et al., 1998), indicating that the two can function independently. In addition, gene deletion studies in yeast indicate that AP-3 and clathrin function on different pathways; however, yeast  $\beta 3$  does not contain a clathrin binding motif. Immunolocalization studies, at both the light and the electron microscope level, have yielded conflicting results (Simpson et al., 1996, 1997; Dell'Angelica et al., 1998). Thus, the question of whether or not there is an

obligatory coupling between AP-3 and clathrin in mammalian cells has not yet been resolved.

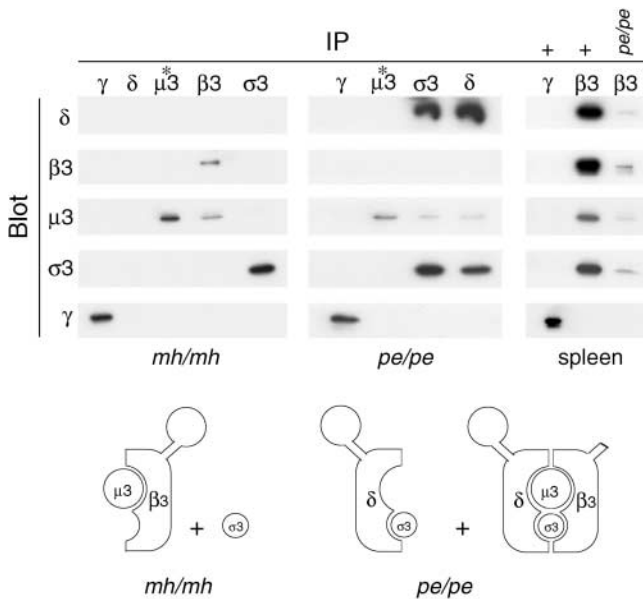
Here we have taken advantage of the mouse mutants to address two questions. First, what happens to the other AP-3 subunits if one of them is missing? And second, to what extent can we rescue the phenotype of mocha and pearl cells by transfecting them with either a wild-type copy of the missing subunit, or alternatively with one that has been altered in some manner? In particular, are  $\beta 3A$  subunits that no longer have the clathrin binding motif still functional? These studies provide insights into the assembly and function not only of the AP-3 complex, but of the other AP complexes as well.

## Results

### Assembly of AP-3 complexes in *mh* and *pe* cells

To investigate the mocha (*mh*) and pearl (*pe*) phenotypes at the cellular level, we have established an *mh* cell line and made use of a previously established *pe* cell line. Fig. 1 a shows Western blots of extracts from these cells probed for the four AP-3 subunits:  $\delta$ ,  $\beta 3$ ,  $\mu 3$ , and  $\sigma 3$ . In the cells from the mocha homozygote (*mh/mh*),  $\delta$ ,  $\beta 3$ , and  $\mu 3$  are all undetectable, while  $\sigma 3$  is detectable but at reduced levels. In the pearl cells (*pe/pe*),  $\beta 3$  and  $\mu 3$  are both undetectable, while  $\delta$  and  $\sigma 3$  are both detectable at reduced levels. When the cells are labeled for immunofluorescence using a  $\delta$ -specific antibody, the typical punctate pattern can be seen in control cells (+), the *mh* cells are unlabeled, and the *pe* cells show diffuse cytoplasmic labeling, indicating that although  $\delta$  is present in *pe* cells, it is unable to associate with membranes in the absence of the  $\beta 3$  and/or  $\mu 3$  subunits (Fig. 1, b–d).

Previous studies have shown that in *mh* and *pe* mice, mRNAs encoding the three nonmutated subunits are expressed normally, indicating that the subunits are synthesized at wild-type levels but then get degraded because they are unstable (Kantheti et al., 1998; Feng et al., 1999). Thus, trace amounts of the nonmutated subunits may be present in the *mh* and *pe* cells, which are below the limit of detection on whole cell Western blots. To try to concentrate the subunits,



**Figure 2. Immunoprecipitates and Western blots of AP-3 subunits from *mh* and *pe* cells.** Extracts of cultured *mh* cells (left), cultured *pe* cells (middle), and spleens from both *pe* and control mice (right) were immunoprecipitated with the indicated antibody. Most immunoprecipitations were performed under nondenaturing conditions, with the exception of the anti- $\mu 3$  immunoprecipitations, which were performed in the presence of SDS. The gels were then blotted onto nitrocellulose and probed with the indicated antibody. An antibody against the AP-1  $\gamma$  subunit was included as a control. The *mh* cells can be seen to express trace amounts of  $\beta 3$  and  $\mu 3$ , which form a dimer, whereas the  $\sigma 3$  subunit appears to be monomeric. The  $\delta$  and  $\sigma 3$  expressed in the *pe* cells also form a dimer, and in addition a small amount of complete heterotetramer is made. The various subcomplexes that form in the *mh* and *pe* cells are indicated diagrammatically.

and to determine whether they are associated with each other, we immunoprecipitated cell extracts with antibodies against the  $\delta$ ,  $\beta 3$ , and  $\sigma 3$  subunits under nondenaturing conditions, and with anti- $\mu 3$ , which only recognizes the denatured protein, in samples that had been denatured by boiling in SDS. Western blots of the immunoprecipitates were then probed with antibodies against all four of the AP-3 subunits, as well as with an antibody against the  $\gamma$  subunit of AP-1 as a control.

Fig. 2 (left) shows that under these conditions both  $\beta 3$  and  $\mu 3$  can be detected in the *mh* cells, and furthermore that they assemble into a heterodimer since both subunits come down with anti- $\beta 3$ . In contrast, the  $\sigma 3$  in these cells appears to exist as a monomer, since it does not coimmunoprecipitate with any of the other subunits. In the *pe* cells (Fig. 2, middle),  $\delta$  and  $\sigma 3$  form heterodimers which come down with antibodies against both subunits, and in addition, a small amount of  $\mu 3$  can be detected which coprecipitates with the  $\delta/\sigma 3$  dimers. This suggests either that the  $\mu 3$  may be assembling with the  $\delta$  and  $\sigma 3$  subunits into a heterotrimer, or alternatively, since the *pe* mutation is not a true null, that there are small amounts of truncated  $\beta 3A$  expressed in these cells which can form heterotetramers. To distinguish between these two possibilities, we immunoprecipitated ex-

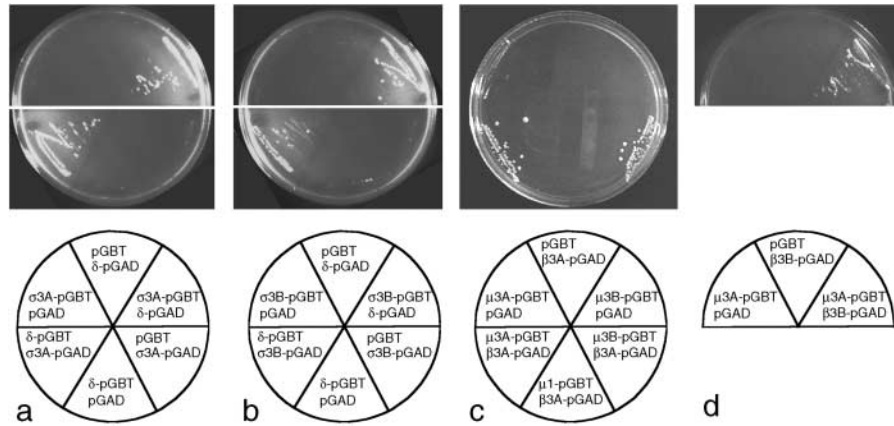
tracts from *pe* spleen under nondenaturing conditions with a  $\beta 3A$ -specific antibody (the reason for using spleen for this experiment is that it is possible to get a more highly concentrated extract from tissues than from cultured cells, and AP-3 is known to be expressed at particularly high levels in spleen). Fig. 2 (right) demonstrates that there are indeed small amounts of truncated  $\beta 3A$  made in *pe* cells which can coassemble with the other three subunits to form a heterotetramer, although we cannot rule out the possibility that some of the  $\mu 3$  may also be assembled into a heterotrimer. Together, these studies indicate that  $\beta 3$  and  $\mu 3$  can interact with each other, forming heterodimers in *mh* cells, and that  $\delta$  and  $\sigma 3$  also interact with each other, forming heterodimers in *pe* cells. The interactions that we can detect in the mutant cells are shown diagrammatically in the bottom part of Fig. 2.

### Subunit interactions

To investigate interactions between the AP-3 subunits further, and also to determine whether the A and B isoforms of the various subunits can associate with each other, we cloned the cDNAs into the vectors pGBT9 and pGAD424 for analysis using the yeast two-hybrid system. Fig. 3, a and b, shows that  $\delta$  interacts under these conditions with  $\sigma 3A$  and  $\sigma 3B$ , both of which are expressed ubiquitously. In addition,  $\beta 3A$  interacts both with the ubiquitously expressed  $\mu 3A$  and with the neuronal-specific  $\mu 3B$  (Fig. 3 c), and  $\beta 3B$  interacts with  $\mu 3A$  (Fig. 3 d). For reasons that are not clear, we were unable to detect any interaction between  $\beta 3B$  and  $\mu 3B$  (unpublished data). However, the observations that  $\beta 3A$  can interact with  $\mu 3B$  and that  $\beta 3B$  can interact with  $\mu 3A$  suggest that, instead of having a neuronal-specific complex and a nonneuronal-specific complex, the subunits can “mix and match” if they are coexpressed in the same cell. The interactions involving the AP-3 subunits appear to be complex-specific, as we could detect no interactions between  $\beta 3$  and  $\mu 1$  (Fig. 3 c), or between  $\delta$  and  $\sigma 1$  (unpublished data). Other pairs of subunits were also tested, but the only interactions we were able to detect were those shown in Fig. 3.

### Sorting of lysosomal membrane proteins

Previous studies have shown that the steady-state distribution of lysosomal membrane proteins, such as LAMP-1, LIMP-2, and CD63, appears normal in AP-3-deficient cells, but that they follow different trafficking routes to the lysosome. Le Borgne et al. (1998) demonstrated that cells that had been depleted of  $\mu 3$  by incubation with antisense oligonucleotides showed increased endocytosis of antibodies against LAMP-1 and LIMP-2 that had been added to the culture medium, indicating that these proteins had been rerouted to the cell surface. Dell’Angelica et al. (1999, 2000) reported similar findings on primary cultures of fibroblasts from  $\beta 3A$ -deficient patients and from *mh* and *pe* mice. We have also found that antibodies against LAMP-1 are readily endocytosed by both the *mh* and the *pe* cell lines (see Figs. 4 and 8). However, because all cell lines are different from each other, we needed to confirm that the antibody endocytosis was a direct result of the AP-3 deficiency. Thus, we transiently transfected the cells with a wild-type copy of the



**Figure 3. Subunit interactions between AP-3 subunits detected using the yeast two-hybrid system.** Yeast cells were transformed with cDNAs in either pGBT9 or pGAD424, as indicated, and interactions were assayed by growth in the absence of histidine. Panels a and b show that the  $\delta$  subunit interacts with both  $\sigma$ 3A and  $\sigma$ 3B, panel c shows that the  $\beta$ 3A subunit interacts with both  $\mu$ 3A and  $\mu$ 3B, and panel d shows that the  $\beta$ 3B subunit interacts with  $\mu$ 3A.

defective subunit. Cells transfected with both the  $\delta$  and the  $\beta$ 3A constructs could be identified with a  $\delta$ -specific antibody: the nontransfected *mh* cells were unlabeled, whereas the nontransfected *pe* cells gave diffuse rather than punctate labeling with the anti- $\delta$  antibody (see Fig. 1). Fig. 4 shows transiently transfected *mh* cells labeled for  $\delta$  (a), endocytosed anti-LAMP-1 (b), and endocytosed WGA (c). The cell expressing  $\delta$  has taken up significantly less anti-LAMP-1 than its nontransfected neighbors, while all of the cells have taken up similar amounts of WGA, indicating that endocytosis in general is not impaired in the  $\delta$ -expressing cell. Similar results were obtained with *pe* cells transfected with  $\beta$ 3A (see Fig. 8 a).

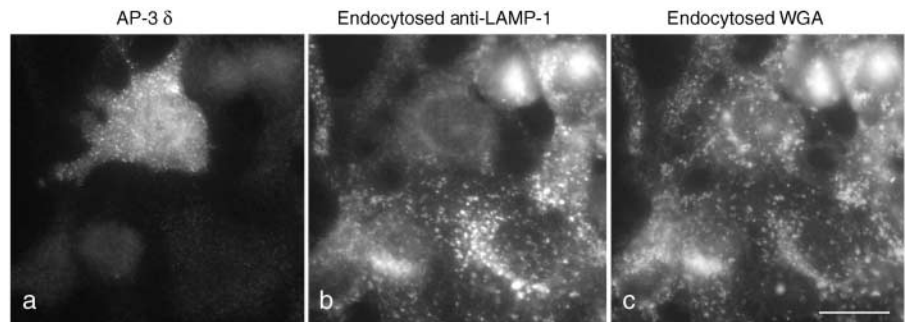
**Introduction of altered subunits**

The availability of an assay for AP-3-mediated sorting means that we can now test whether not only wild-type subunits, but also subunits that have been altered in some way, are functional. Because some such subunits might be expected to give intermediate rescue, we developed a quantitative assay based on FACS analysis for use on stably transfected cells (see below). Unfortunately, we have so far been unable to obtain any *mh* cell lines that were homogeneously expressing  $\delta$ ; thus, we decided to concentrate on *pe* cells. In addition to cells expressing wild-type  $\beta$ 3A, we have established *pe* cell lines expressing  $\beta$ 3B, a  $\beta$ 3A- $\beta$ 2 chimera containing the  $\beta$ 3A NH<sub>2</sub>-terminal domain (amino acids 1–686)

but the  $\beta$ 2 hinge and ear domains ( $\beta$ 3A $\beta$ 2); two  $\beta$ 3A deletion mutants ( $\beta$ 3A $\Delta$ 807–831 and  $\beta$ 3A807 stop); and a  $\beta$ 3A point mutant ( $\beta$ 3A817AAA). The  $\beta$ 3A $\Delta$ 807–831 mutant is missing a fragment of the distal part of the hinge, including the clathrin binding domain (amino acids 817–822), whereas the  $\beta$ 3A807stop mutant is missing the distal part of the hinge, including the clathrin binding domain, and all of the ear. In the  $\beta$ 3A817AAA mutant, the clathrin binding motif has been mutated from SLLDLD to AAADLD. This same mutation has been shown to abolish clathrin binding in vitro (Dell’Angelica et al., 1998; unpublished data). Schematic diagrams of the constructs are shown in Fig. 5 a.

We first investigated whether the constructs were capable of assembling into AP-3 complexes by immunoprecipitating cell extracts with an antibody against the  $\delta$  subunit, then probing Western blots of the immunoprecipitates with antibodies against the other three subunits. Fig. 5 b shows that in the nontransfected cells, only  $\sigma$ 3 coprecipitated with  $\delta$  at detectable levels (see also Figs. 1 and 2), while in cells transfected with  $\beta$ 3A, all three of the other subunits were detectable, indicating that the  $\beta$ 3A had assembled into complexes together with  $\mu$ 3, thus protecting the  $\mu$ 3 subunit from degradation. Similarly, in cells transfected with the other five constructs, the construct itself and the  $\mu$ 3 subunit came down with the  $\delta$  and  $\sigma$ 3 subunits. These results are consistent with studies on the AP-1 and AP-2 complexes, which indicate that the NH<sub>2</sub>-terminal domains of the  $\beta$  subunits are incorporated into the

**Figure 4. Phenotype of rescued and nonrescued *mh* cells.** *mh* cells were transiently transfected with wild-type  $\delta$ , then incubated with a mixture of rat anti-LAMP-1(b) and fluorescent WGA (c) for 6 h. Triple labeling shows that the cell in the field that is expressing  $\delta$  has taken up less anti-LAMP-1 than its neighbors, indicating that there is less misrouting of LAMP-1 to the plasma membrane in this cell. Uptake of WGA is similar in all of the cells. Bar, 20  $\mu$ m.



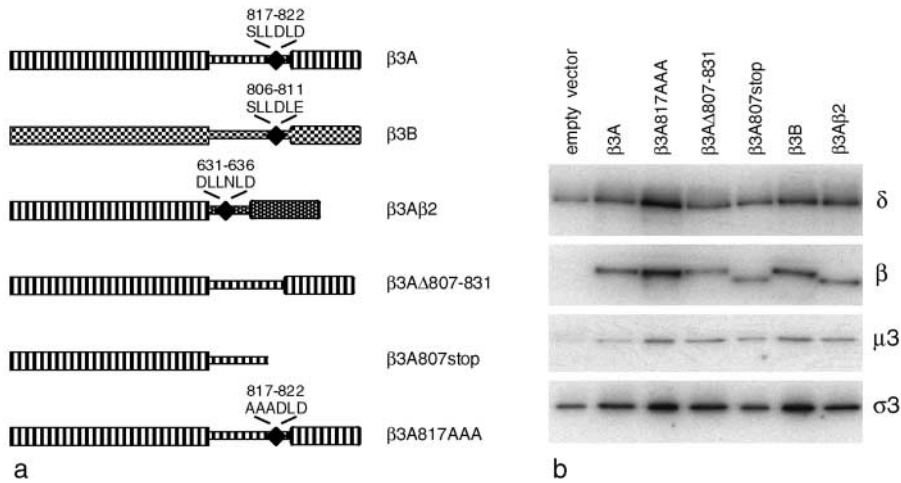


Figure 5. (a) Schematic diagrams of six  $\beta 3$ -based constructs that were stably transfected into *pe* cells. (b) Extracts from cell lines expressing each of the six constructs, as well as a cell line stably transfected with empty vector, were immunoprecipitated with anti- $\delta$ , and blots were probed with antibodies against  $\delta$ , the construct itself (in most cases anti- $\beta 3A$ , although the last two lanes were probed with antibodies against  $\beta 3B$  and  $\beta 2$ , respectively),  $\mu 3$ , and  $\sigma 3$ . Only  $\delta$  and  $\sigma 3$  can be detected in the immunoprecipitate from the cell line transfected with the empty vector, whereas all four subunits can be detected in the cell lines expressing the six constructs, indicating that complete heterotetramers have been formed.

“head,” where they can interact with the other subunits, while the hinge and ear domains have other functions. The  $\mu 3$  subunit in all of the immunoprecipitates is presumably  $\mu 3A$ , since  $\mu 3B$  is only expressed in neurons and neuroendocrine cells. Thus, this result is consistent with results from the two-hybrid experiments, which indicate that although  $\beta 3B$  is normally only expressed in the same cells that express  $\mu 3B$ , it can interact with the ubiquitously expressed  $\mu 3A$ .

To determine whether the complexes could be recruited onto membranes, cells expressing each of the six constructs were labeled for immunofluorescence with anti- $\delta$ . In non-transfected *pe* cells,  $\delta$  has a diffuse cytoplasmic distribution (Fig. 1 d); however, in cells expressing each of the five constructs, the  $\delta$  labeling was punctate, indicating that it was associated with membranes (Fig. 6). We also compared the distribution of AP-3 in cells expressing the various constructs with that of clathrin, using a confocal microscope (Fig. 7; AP-3

is green and clathrin is red). In cells expressing wild-type  $\beta 3A$ , AP-3 and clathrin were found to have distinct distributions: (a) they are often in the same general vicinity, and in some cases there may be overlap (yellow), but much of the AP-3 labeling is negative for clathrin. In contrast, when cells are double labeled for the AP-1 adaptor complex and clathrin (b), essentially all of the structures that are positive for AP-1

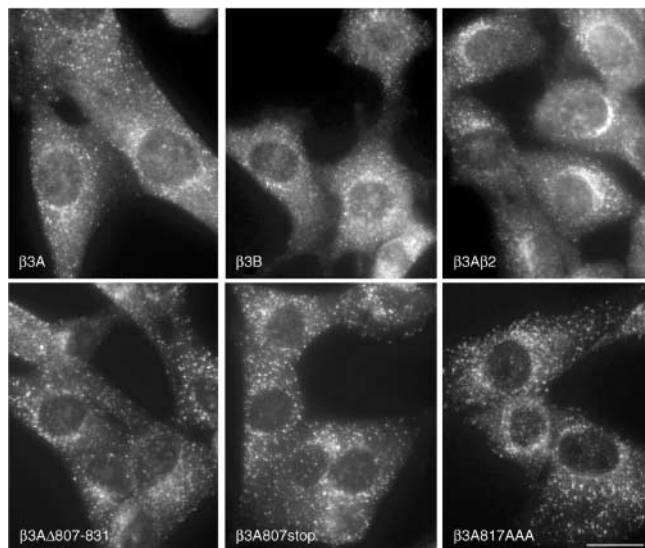


Figure 6. Immunofluorescence of cell lines expressing each of the six constructs labeled with anti- $\delta$ . All of the cells show punctate labeling with some concentration in the perinuclear region, indicating that the AP-3 complexes assembled from all six constructs are recruited onto membranes. Bar, 20  $\mu m$ .

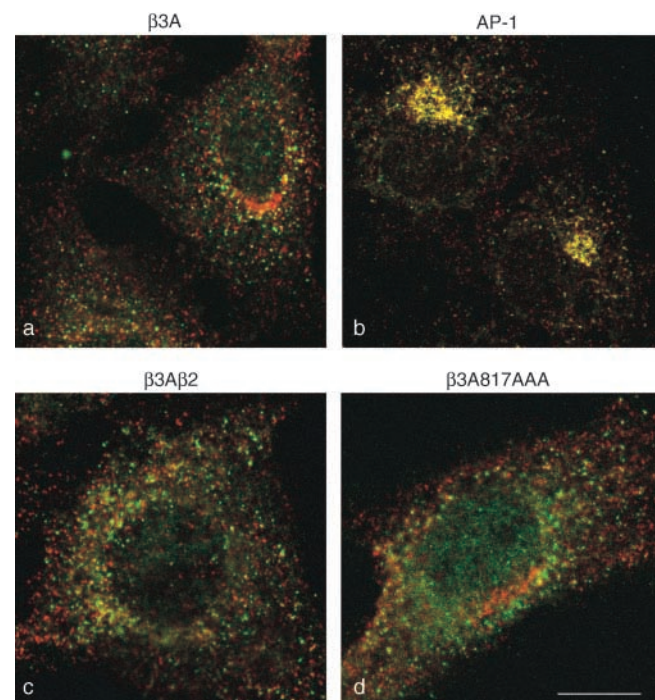
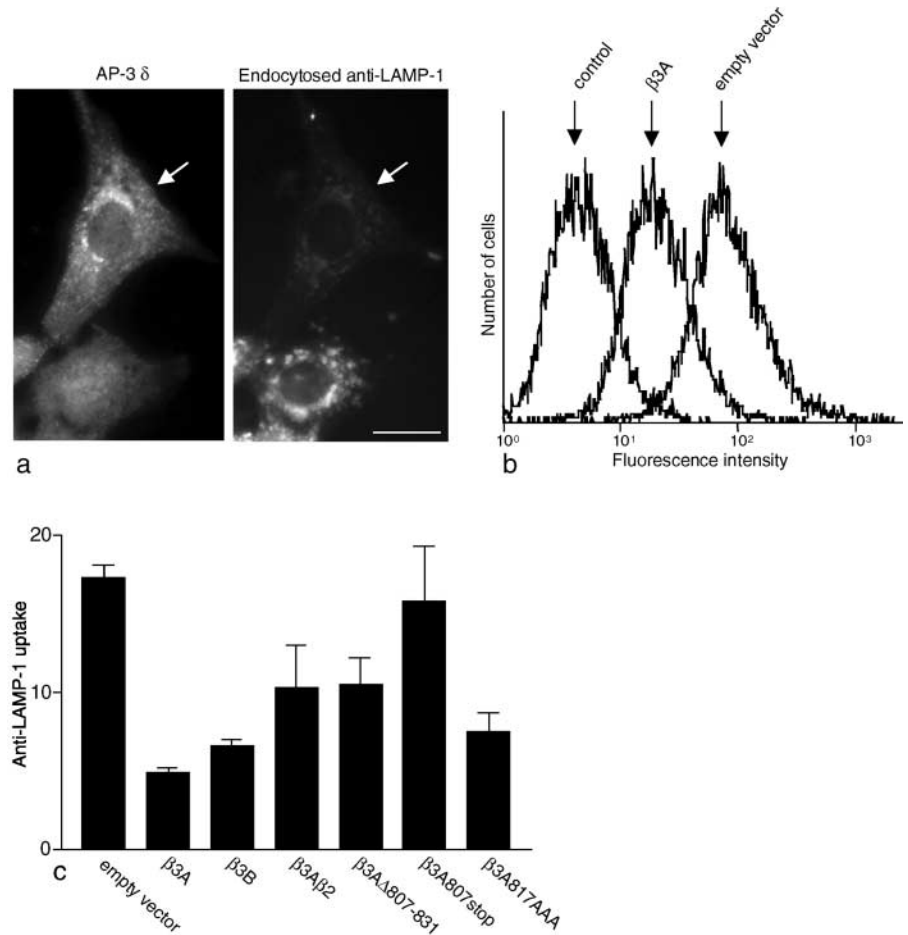


Figure 7. Confocal micrographs showing double labeling for AP complexes and clathrin. *pe* cells expressing  $\beta 3A$  (a),  $\beta 3A\beta 2$  (c), or  $\beta 3A817AAA$  were double labeled with anti- $\delta$  (green) and anti-clathrin (red), and COS cells were double labeled with anti- $\gamma$  (green) and anti-clathrin (red). Essentially all of the anti- $\gamma$  labeling is also positive for clathrin (note how there are no green dots in b, only yellow). In contrast, in all three cell lines expressing  $\beta 3$  constructs, much of the AP-3 labeling is negative for clathrin. (Transfected *pe* cells could not be double labeled with anti- $\gamma$  and anti-clathrin because the anti- $\gamma$  mAb only recognizes the protein in nonrodent cells.) Bar: (a and b) 10  $\mu m$ ; (c and d) 20  $\mu m$ .

**Figure 8. Functional rescue of *pe* cells by the various  $\beta 3$  constructs.**

(a) A mixture of cells transfected with empty vector and cells expressing  $\beta 3A$  were incubated 6 h with anti-LAMP-1 directly conjugated to Alexa Fluor 488, then double labeled with anti- $\delta$ .  $\beta 3A$ -expressing cells (arrow), which could be identified by the punctate, perinuclear distribution of  $\delta$ , took up less anti-LAMP-1 than cells transfected with empty vector. (b) Anti-LAMP-1 uptake analyzed by flow cytometry. The control peak shows the fluorescence intensity of a  $\beta 3A$ -expressing cell line incubated with a control antibody (anti-KLH) conjugated to Alexa Fluor 488. The empty-vector peak shows the level of fluorescence intensity when a nonexpressing *pe* cell line was incubated with the anti-LAMP-1 antibody, whereas the  $\beta 3A$  peak shows the level of fluorescence intensity when the *pe* cell line expressing  $\beta 3A$  was incubated with the anti-LAMP-1 antibody. Anti-LAMP-1 uptake in the  $\beta 3A$ -expressing cell line was 27% that of the empty vector-transfected line. (c) Graph of the results obtained by flow cytometry. In each case three different cell lines, stably expressing each of the six constructs were analyzed, as well as three cell lines transfected with empty vector. In each experiment, 10,000 cells were counted and the experiments were repeated on different days and the results for each cell line were averaged. The values were obtained by dividing the geometric mean fluorescent intensity of cells incubated with the anti-LAMP-1 antibody by that of cells incubated with the control antibody. Error bars show the standard deviation from the mean.  $\beta 3A$ ,  $\beta 3B$ , and  $\beta 3A817AAA$  constructs all gave good rescue ( $P \leq 0.0001$ );  $\beta 3A\beta 2$  and  $\beta 3A\Delta 807-831$  both gave partial rescue ( $P = 0.0059$  and  $P = 0.0015$ , respectively); and  $\beta 3A807$  stop gave no significant rescue ( $P = 0.2523$ ).  $P$  values were obtained using a one-tailed unpaired  $t$  test. Bar, 20  $\mu m$ .



(green) are also positive for clathrin (red; note that there is extensive yellow labeling and very little green). In cells expressing the various constructs, the extent of overlap between AP-3 and clathrin was very similar to that in cells expressing wild-type  $\beta 3A$ . This is in spite of the fact that the  $\beta 3A\beta 2$  chimera (c) has more potential clathrin binding sites than wild-type  $\beta 3A$  (not only the clathrin-binding consensus sequence LLNLD in its hinge domain, but also three copies of another motif implicated in clathrin binding, DLL [Morgan et al., 2000], as well as clathrin binding activity in its ear domain [Owen et al., 2000]). Constructs lacking a clathrin binding domain, like  $\beta 3A817AAA$  (d), did not look appreciably different from the other constructs: again, there were some yellow structures, as well as red and green. However, at the light microscope level, it is impossible to resolve two structures that are less than  $\sim 0.2 \mu m$  apart from each other, so to quantify the true extent of overlap between AP-3 and clathrin in cells expressing the various constructs, it will be necessary to go to the electron microscope level.

**Functional rescue of *pe* cells**

Having established that all six constructs are able to coassemble with the other AP-3 subunits and be recruited onto

membranes, we went on to investigate their ability to rescue the LAMP-1 missorting phenotype. For these experiments, a rat monoclonal antibody against LAMP-1 was directly conjugated to Alexa Fluor 488. Double-labeling immunofluorescence performed on a mixture of *pe* cells transfected with empty vector and *pe* cells expressing wild-type  $\beta 3A$ , incubated with the antibody for 6 h, showed that the nonexpressing cells took up significantly more antibody than the expressing cells (Fig. 8 a).

For quantitative studies, each stably transfected cell line was incubated for 6 h either with the anti-LAMP-1 antibody or with an isotype control antibody (anti-KLH), which also had been conjugated to Alexa Fluor 488. The cells were then trypsinized, fixed, and analyzed by flow cytometry. Fig. 8 b shows the results obtained with one of the cell lines expressing wild-type  $\beta 3A$ . The peak marked "control" shows the fluorescence intensity of the  $\beta 3A$ -expressing cells incubated with the control antibody, which is likely to be taken up by fluid phase endocytosis only. The peaks marked " $\beta 3A$ " and "empty vector" show the levels of fluorescence intensity observed when the  $\beta 3A$ -expressing line and one of the cell lines transfected with empty vector were incubated with the anti-LAMP-1 antibody. In both cases, the fluores-

cence intensity is higher than that observed with the control antibody, indicating that the anti-LAMP-1 has been taken up by receptor-mediated endocytosis as well as fluid-phase endocytosis. However, the fluorescence intensity of the  $\beta$ 3A-expressing line is 4.41 times that of the control. In contrast, the fluorescence intensity of the cell line transfected with empty vector is 16.61 times that of its own control (unpublished data, but similar results were obtained with all of the cell lines incubated with the control antibody). Thus, the amount of specific uptake of anti-LAMP-1 in the  $\beta$ 3A-expressing line is 27% of that in the empty vector-transfected line. This is comparable to results obtained by others who have investigated anti-LAMP-1 uptake in normal and AP-3-deficient primary fibroblasts from both mice and humans: wild-type cells were reported to internalize between 20 and 45% as much antibody as mutant cells (Dell'Angelica et al., 1999, 2000).

Fig. 8 c shows the extent of functional rescue obtained with all six constructs. In each case, three different cell lines stably expressing each of the six constructs were analyzed, as well as three cell lines transfected with empty vector. The experiments were repeated on different days and the results for each cell line were averaged.  $\beta$ 3A can be seen to give the best rescue, with anti-LAMP-1 uptake  $4.89 \pm 0.237$  (SD), as compared with  $17.25 \pm 0.821$  in cells transfected with empty vector. The  $\beta$ 3B construct also rescued well, with anti-LAMP-1 uptake  $6.58 \pm 0.421$ . Whether the difference between the  $\beta$ 3A- and  $\beta$ 3B-expressing cells reflects functional differences between the two  $\beta$ 3 isoforms, or whether it reflects other differences between the various cell lines (since wild-type cell lines can vary at least this much in their anti-LAMP-1 uptake), is not at present clear. The  $\beta$ 3A $\beta$ 2 chimera and the  $\beta$ 3A $\Delta$ 807–831 deletion mutant both partially rescued the missorting phenotype, with anti-LAMP-1 uptake  $10.30 \pm 2.620$  and  $10.48 \pm 1.635$  respectively. However, there was no significant difference in antibody uptake between cells expressing the truncation mutant,  $\beta$ 3A807stop ( $15.73 \pm 3.512$ ), and cells transfected with empty vector. Thus, the distal hinge and/or ear domain of the  $\beta$ 3 subunit appears to be required for AP-3 function, even though the construct lacking these domains is still able to assemble into AP-3 complexes and be recruited onto membranes.

There are at least two possible explanations for the intermediate level of functional rescue that we observed with the  $\beta$ 3A $\Delta$ 807–831 mutant. One possibility is that the clathrin-binding consensus sequence, which is missing from this construct, is required for full functional rescue. Alternatively, the construct might be defective in some other way: for instance, the ear might not be correctly folded. The point mutant  $\beta$ 3A817AAA enables us to distinguish between these two possibilities. Instead of a 25-amino acid deletion, this construct has only 3 amino acid substitutions, but (at least in vitro) it is unable to bind clathrin (Dell'Angelica et al., 1998). Fig. 8 c shows that when this construct is expressed in living cells, anti-LAMP-1 uptake is  $7.49 \pm 1.128$ , which is only slightly more than in  $\beta$ 3A-expressing cells and not significantly different from  $\beta$ 3B-expressing cells. Thus, although the hinge and/or ear domains of  $\beta$ 3A are needed for protein function, the clathrin-binding consensus sequence appears to be dispensable.

## Discussion

The availability of mice with null, or effectively null, mutations in AP-3 subunits has enabled us to study AP-3 assembly and function in mammalian cells. In the past we and others have transfected mammalian cells with mutant AP-1 and AP-2 subunits for studies on both assembly and function, but interpretation of these studies has been complicated by the presence of endogenous wild-type subunits (Robinson, 1993; Page and Robinson, 1995). In the present study, we have been able to introduce mutant subunits into cells with little or no background.

We first investigated whether partial AP-3 complexes were able to assemble in the absence of a particular subunit. We found that *mb* cells, missing the  $\delta$  subunit, assembled heterodimers from the  $\beta$ 3 and  $\mu$ 3 subunits, whereas the  $\sigma$ 3 subunit remained monomeric. The *pe* cells, which express very low levels of truncated  $\beta$ 3A, mainly assembled heterodimers consisting of  $\delta$  and  $\sigma$ 3 subunits, as well as a very small amount of complete heterotetramer. All of the subunits were destabilized to some extent by the absence of either  $\delta$  or  $\beta$ 3, but this was particularly striking in the case of the  $\mu$ 3 subunit, which seemed to exist only as a dimer with the  $\beta$ 3 subunit, even when the  $\beta$ 3 subunit was barely detectable.

In addition to the naturally occurring AP-3 mouse mutants, knockout mice have been generated which lack the AP-1  $\gamma$  and  $\mu$ 1A subunits. Both mouse strains die during embryogenesis; however, a fibroblast line has been established from the  $\mu$ 1A knockout mice, and these cells were found to assemble a heterotrimer consisting of  $\gamma$ ,  $\beta$ 1, and  $\sigma$ 1 subunits. This complex appears to be fairly stable, although (like the  $\delta/\sigma$ 3 heterodimer) it is unable to be recruited onto membranes (Meyer et al., 2000). The  $\gamma$  knockout mouse dies so early that it has not been possible to establish a  $\gamma$  null cell line, but cells from the heterozygote have been examined and found to express the  $\gamma$  subunit at  $\sim$ 50% normal levels. Both  $\beta$ 1 and  $\mu$ 1 completely comigrate with  $\gamma$  by gel filtration in these cells, indicating that any excess  $\beta$ 1 and  $\mu$ 1 are unstable and get degraded (Zizioli et al., 1999). Similarly, our own studies show that in the absence of the  $\delta$  subunit, the stability of  $\beta$ 3 and  $\mu$ 3 is greatly reduced. However, somewhat different findings have been reported in yeast. When the AP-3  $\delta$ ,  $\beta$ 3, or  $\sigma$ 3 subunits are deleted, the stability of  $\mu$ 3 does not appear to be affected, although it fractionates differently by gel filtration, in particular in the  $\beta$ 3-disrupted strain, where it appears to be monomeric (Panek et al., 1997). In contrast, deleting the  $\alpha$ -like subunit of the AP-2-related complex in yeast does not affect the  $\beta$ 2 subunit, but it has a profound effect on the stability of  $\sigma$ 2 (Yeung et al., 1999). Thus, it is difficult to predict how a particular AP subunit will behave in a particular organism; however, all of these studies consistently show that there are strong interactions between the  $\gamma/\alpha/\delta/\epsilon$  subunits and the  $\sigma$  subunits, and between the  $\beta$  and  $\mu$  subunits.

These same interactions can be detected using the yeast two-hybrid system. The interactions that we report between the  $\delta$  and  $\sigma$ 3 subunits, and between the  $\beta$ 3 and  $\mu$ 3 subunits, are consistent with interactions that we and others have reported between the corresponding pairs of subunits in the AP-1, AP-2, and AP-4 complexes (Page and Robin-

son, 1995; Aguilar et al., 1997; Hirst et al., 1999; Takatsu et al., 2001). In addition, in a previous study using the yeast two-hybrid system, we were able to demonstrate interactions between the two large subunits of the AP-1 and AP-2 complexes,  $\gamma/\alpha$  and  $\beta$  (Page and Robinson, 1995). However, interactions between the large subunits appear to be more difficult to reconstitute, and so far we have been unable to demonstrate any interactions between  $\delta$  and  $\beta 3$ , or between the  $\epsilon$  and  $\beta 4$  subunits of the AP-4 complex (Hirst et al., 1999).  $\epsilon$  and  $\beta 4$  have been shown recently to interact in the presence of  $\sigma 4$  using the yeast three-hybrid system, however (Takatsu et al., 2001), so it is possible that  $\sigma 3$  may promote the interaction between  $\delta$  and  $\beta 3$ . One observation that supports the notion that  $\delta$  and  $\beta 3$  interact in vivo is the finding that *mh* cells have greatly reduced levels of the  $\beta 3$  and  $\mu 3$  subunits. This suggests that  $\beta 3$  may be unstable in the absence of  $\delta$ , which in turn would destabilize  $\mu 3$ .

We also used the yeast two-hybrid system to investigate whether the ubiquitously expressed (A) and neuronal-specific (B)  $\beta 3$  and  $\mu 3$  subunits were capable of interacting with each other. Interactions were detected between  $\beta 3A$  and  $\mu 3A$ , between  $\beta 3A$  and  $\mu 3B$ , and between  $\beta 3B$  and  $\mu 3A$ , although surprisingly not between  $\beta 3B$  and  $\mu 3B$ . The ability of the A and B isoforms to interact under more physiological conditions was demonstrated in our transfection experiments. When *pe* cells were transfected with  $\beta 3B$ , they formed heterotetramers containing the ubiquitously expressed  $\mu 3A$  subunit. Heterotetramers were also assembled from the other five constructs, as expected since they all contained the  $\beta 3A$  NH<sub>2</sub>-terminal domain. Perhaps less predictable was the ability of all the constructs to be recruited equally well onto membranes. Although studies from our own lab and others indicate that the COOH-terminal hinge and ear domains of the adaptor large subunits are not absolutely essential for membrane association, AP complexes containing earless  $\gamma$  or  $\alpha$  are compromised in their ability to bind to their respective membranes (Robinson, 1993); however, the earless  $\beta 3A$  construct,  $\beta 3A807stop$ , showed no increased cytosolic background. We were also interested in the extent to which the various complexes colocalized with clathrin, in particular complexes containing the  $\beta 3A\beta 2$  chimera, since multiple clathrin binding sites have been identified and/or predicted in the  $\beta 2$  COOH-terminal hinge and ear domains (Dell'Angelica et al., 1998; Morgan et al., 2000; Owen et al., 2000). However, this construct, like the others, was at least partially localized to structures that were negative for clathrin. In contrast, AP-1 and AP-2 both show essentially complete colocalization with clathrin (Fig. 7; Robinson, 1987), indicating that the presence of the  $\beta 2$  hinge and ear domains is not enough for a stable association with clathrin, and that there must be additional interactions, possibly involving the  $\gamma$  and  $\alpha$  subunits (Goodman and Keen, 1995; Morgan et al., 2000; Doray and Kornfeld, 2001).

Although all of the constructs assembled into complexes and were recruited onto membranes equally well, they varied widely in their ability to rescue the missorting phenotype of the *pe* cells. Using a quantitative assay for LAMP-1 mislocalization, we were able to show that  $\beta 3B$  gave nearly as good functional rescue as  $\beta 3A$ ; indeed, the differences that we saw

could simply be due to variability from one cell line to another (Dell'Angelica et al., 1999, 2000). However, although  $\beta 3B$  can functionally substitute for  $\beta 3A$ , the opposite may not be true. Synaptic-like microvesicle budding from endosomes has been shown to be dependent upon brain AP-3; liver AP-3 will not suffice (Faundez et al., 1998). This suggests that the neuronal-specific isoform of either  $\beta 3$  or  $\mu 3$ , or both, is required for this activity. Brain AP-3 contains a mixture of A and B isoforms of both  $\beta 3$  and  $\mu 3$ , while the stably transfected cells express only  $\beta 3B$  and  $\mu 3A$ ; thus, it should be possible to test the relative importance of neuronal-specific  $\beta 3$  and  $\mu 3$  by determining whether AP-3 complexes from the transfected cells will substitute for brain AP-3 in the in vitro budding assay.

Two of our constructs gave partial rescue: the  $\beta 3A\beta 2$  chimera and the deletion mutant  $\beta 3A\Delta 807-831$ . In contrast, the premature truncation mutant  $\beta 3A807stop$  was unable to rescue even partially. This finding indicates that the distal hinge and/or ear domains of  $\beta 3$  are essential for function. In the  $\beta 3A\Delta 807-831$  mutant, part of the distal hinge is missing, and this fragment may either contribute directly to  $\beta 3$  function, or alternatively may aid in the folding of the ear. Virtually nothing is known about the ear domain of  $\beta 3$ , although the  $\beta 2$  ear has been shown to bind both clathrin and certain coated vesicle accessory proteins, including AP180, epsin, and Eps15 (Owen et al., 2000). We have shown that AP-3 complexes containing truncated  $\beta 3A$  are recruited onto the membrane, and presumably they are able to select cargo since they contain  $\mu 3A$  (Ohno et al., 1998). Thus, the lack of functional rescue suggests that they fail to produce coated vesicles, consistent with an inability to recruit structural or accessory proteins that might be essential for this event. The ability of the  $\beta 3A\beta 2$  construct to give partial rescue suggests that the  $\beta 3$  and  $\beta 2$  ears may recruit some of the same proteins. However, gels of GST pull-downs using the  $\beta 3A$  ear showed no obvious candidate binding partners, and clathrin could not be detected on Western blots of the pull-downs (D. Owen, personal communication; unpublished data). Thus, the precise function of the  $\beta 3$  ear remains elusive. However, its importance is highlighted by its evolutionary conservation: the  $\beta 3$  ear domains of both *Drosophila* and *C. elegans* are significantly homologous to the ear domains of mammalian  $\beta 3A$  and  $\beta 3B$ , although both *S. cerevisiae* and *S. pombe*  $\beta 3$  subunits appear to lack any ear domain.

Yeast  $\beta 3$  subunits also lack a clathrin-binding consensus sequence, but both *C. elegans* and *Drosophila* have such sequences in their  $\beta 3$  distal hinge domains (LIDVD and LLDDL, respectively). However, we show here that it is possible to mutate this sequence to one that can no longer bind clathrin in vitro (Dell'Angelica et al., 1998), and yet still get functional rescue. This result can now be added to the weight of evidence against an obligatory coupling between AP-3 and clathrin. At present we cannot rule out the possibility that there might be other clathrin binding sites in AP-3, even though there are no obvious candidates (we have tested the hinge and ear domains of the  $\delta$  subunit, but are unable to detect any clathrin in GST pull-downs [unpublished data]). Double labeling for AP-3 and clathrin at the electron microscope level should help to determine whether



there is any colocalization between the two in cells expressing  $\beta 3$  with a mutated clathrin binding domain. However, much of the evidence in support of an association between AP-3 and clathrin comes from studies on the LLDLD sequence, and the present study demonstrates that this sequence is not essential for AP-3 function *in vivo*.

The large subunits of the AP complexes contain a number of other intriguing motifs and domains, including the "KRIGY" and "WIIGY" sequences (Hirst et al., 1999). These motifs are found not only in every member of the  $\gamma/\alpha/\delta/\epsilon$  and  $\beta$  families, but also in the very distantly related  $\beta$ -COP and  $\gamma$ -COP subunits of the coatamer complex. So far nothing is known about their function, but we are currently mutating these motifs in both the  $\delta$  and  $\beta 3$  subunits. By taking advantage of the *mh* and *pe* cell lines, it should be possible to assess the importance of these and other sequences not only in the assembly and localization of coat protein complexes, but also in coated vesicle function.

## Materials and methods

### Cell lines

To generate an *mh* cell line, primary fibroblasts were first cultured by taking muscle from 1–3-d-old mice and mincing the tissue using a scalpel blade, then transferring the tissue to culture dishes containing DME supplemented with 10% fetal calf serum, 50 IU/ml penicillin, 50  $\mu$ g/ml streptomycin, 2 mM L-glutamine, and 52  $\mu$ M mercaptoethanol. After 3–7 d the medium was changed on the cells. Once cells had grown out from the disrupted tissue, they were split and cultured in the above medium supplemented with 200 nM TPA. In some cases the cells changed from slow growth to fast, noncontact-inhibited growth, and one of the fast-growing cell lines was cloned and aliquots were frozen for further experiments.

An immortal *pe* cell line was donated by R. Swank (Roswell Park Cancer Institute, Buffalo, NY). Although this line was generated from skin using methods designed to select for melanocytes, we were unable to detect the melanocyte-specific proteins tyrosinase and TRP-1 in these cells, indicating that they are another type of skin cell, most likely fibroblasts. The mouse melanocyte line, melan-a, was a gift from D. Bennett (St. George's Hospital Medical School, London, UK).

### Antibody-based methods

Immunoprecipitations were performed both on nondenatured proteins and in the presence of SDS, using antibodies against both AP-3 and AP-1 subunits, as described previously (Simpson et al., 1996, 1997). Western blotting and immunofluorescence were also performed using previously published methods (Robinson and Pearse, 1986; Robinson, 1987). The polyclonal rabbit antibodies against AP-3 subunits have already been described (Simpson et al., 1996, 1997); in addition, a mouse monoclonal antibody was raised against the hinge domain (amino acids 608–800) of the AP-3  $\delta$  subunit. The construct was expressed as a GST fusion protein and used to immunize female BALB/c mice. Fusion of the spleen cells with myeloma cells and screening of the resulting antibodies were performed as described by Bock et al. (1997). Ascites fluid was prepared by Joseph Beirao (Josman Laboratories, Napa Valley, CA). The rabbit anticlathrin antibody has been described previously (Simpson et al., 1997). The monoclonal anti- $\gamma$  antibody, mAb 100/3, was purchased from Sigma-Aldrich. The anti-LAMP-1 antibody (1D4B) was initially obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA.

### Yeast two-hybrid analysis

Most molecular biology techniques were performed as described by Sambrook and Russell (2001). To construct the plasmids for yeast two-hybrid analysis, the AP-3 subunits  $\delta$ ,  $\beta 3A$ ,  $\beta 3B$ ,  $\mu 3A$ ,  $\mu 3B$ ,  $\sigma 3A$ , and  $\sigma 3B$  were all cloned into the yeast two-hybrid vectors pGBT9 and pGAD424 (CLONTECH Laboratories, Inc.), using PCR to amplify the coding sequences and to introduce appropriate restriction sites. All constructs that had been made using PCR were sequenced by John Lester (University of Cambridge, Cambridge, UK) to confirm that no errors had been introduced. Interactions were monitored by assaying for growth in the absence of histidine.

### Expression in mammalian cells

All plasmids for expression in mammalian cells were constructed using the vector  $\Delta$ pMEP (Girotti and Banting, 1996). The  $\beta 3A\beta 2$  construct consists of the NH<sub>2</sub>-terminal 686 amino acids of  $\beta 3A$  fused to the last 334 amino acids of  $\beta 2$ . The deletion constructs and the point mutant were made by PCR and sequenced as above to confirm that they were error-free. Cells were transfected using QIAGEN SuperFect transfection reagent and selected in DME plus 10% fetal calf serum with Hygromycin (0.2 mg/ml). At least three cell lines were selected for each construct.

### Flow cytometry

A quantitative assay for AP-3 dependent sorting was developed using flow cytometry. Two rat monoclonal antibodies, anti-LAMP-1 (1D4B) and a control anti-KLH antibody (both IgG2as) were supplied by BD Pharmingen. The antibodies were directly conjugated to Alexa Fluor 488 using the Molecular Probes Alexa 488 protein-labeling kit according to the manufacturer's instructions. Cells were then incubated with either anti-LAMP-1 or control antibody for 6 h at 37° in serum-free media. The cells were rinsed in PBS, trypsinized, fixed in 3% paraformaldehyde, and analyzed on a fluorescence-activated cell scanner (FACSscan; Becton Dickinson). To ensure that differences in anti-LAMP-1 uptake were not due to differences in LAMP-1 expression levels, Western blots of each cell line were probed with the LAMP-1 antibody. Although there was some variability from one line to another, there was no correlation between LAMP-1 expression levels and the amount of anti-LAMP-1 uptake.

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