Traffic 2005; **6:** 1014–1026
Blackwell Munksgaard



doi: 10.1111/j.1600-0854.2005.00341.x

# Sorting of Major Cargo Glycoproteins into Clathrin-Coated Vesicles

**Kouki Harasaki, Nienke B. Lubben, Michael Harbour, Marcus J. Taylor and Margaret S. Robinson***

*University of Cambridge, Department of Clinical Biochemistry, Cambridge Institute for Medical Research, Cambridge CB2 2XY, UK*
**Corresponding author: Margaret S. Robinson, msr12@mole.bio.cam.ac.uk*

**The AP-1 and AP-2 complexes are the most abundant adaptors in clathrin-coated vesicles (CCVs), but clathrin-mediated trafficking can still occur in the absence of any detectable AP-1 or AP-2. To find out whether adaptor abundance reflects cargo abundance, we used lectin pull-downs to identify the major membrane glycoproteins in CCVs from human placenta and rat liver. Both preparations contained three prominent high molecular-weight proteins: the cation-independent mannose 6-phosphate receptor (CIMPR), carboxypeptidase D (CPD) and low-density lipoprotein receptor-related protein 1 (LRP1). To investigate how these proteins are sorted, we constructed and stably transfected CD8 chimeras into HeLa cells. CD8-CIMPR localized mainly to early/tubular endosomes, CD8-CPD to the *trans* Golgi network and CD8-LRP1 to late/multivesicular endosomes. All three constructs redistributed to the plasma membrane when clathrin was depleted by siRNA. CD8-CIMPR was also strongly affected by AP-2 depletion. CD8-CPD was moderately affected by AP-2 depletion but strongly affected by depleting AP-1 and AP-2 together. CD8-LRP1 was only slightly affected by AP-2 depletion; however, mutating an NPXY motif in the LRP1 tail caused it to become AP-2 dependent. These results indicate that all three proteins have AP-dependent sorting signals, which may help to explain the relative abundance of AP complexes in CCVs. However, the relatively low abundance of cargo proteins in CCV preparations suggests either that some of the APs may be empty or that the preparations may be dominated by empty coats.**

**Key words: AP-1, AP-2, endosome, RNA interference, TGN**

**Received 26 April 2005, revised and accepted for publication 8 August 2005, published on-line 16 September 2005**

Cargo proteins are sorted and packaged into clathrin-coated vesicles (CCVs) by interacting with adaptors, which bind to specific sorting signals in the cytoplasmic tails of the cargo proteins and link them to clathrin. There are two highly abundant adaptors in mammalian CCVs: AP-1 (for adaptor protein complex 1), which is involved in clathrin-mediated intracellular trafficking, and AP-2, which is involved in clathrin-mediated endocytosis. Both AP-1 and AP-2 are heterotetramers, consisting of two large subunits (γ and β1 in AP-1, α and β2 in AP-2), a medium-sized (μ) subunit and a small (σ) subunit (1,2). The μ subunits bind to sorting signals with the consensus sequence YXXΦ (3,4), and the γ-type large subunits together with the σ subunits bind to sorting signals with the consensus sequence [D/E]XXXL[L/I] (5). Clathrin-coated vesicle preparations from different tissues contain different ratios of AP-1 to AP-2 (e.g. in mammary gland, there is more AP-1, whereas in brain there is more AP-2) (6), but invariably, the AP complexes are major components of the CCVs, second in abundance only to clathrin itself.

Over the last 2 years, others and we have been investigating the functions of the AP-1 and AP-2 complexes in tissue culture cells using siRNA knockdowns. Surprisingly, we have found that even when we deplete AP-2 to undetectable levels, some cargo proteins are still endocytosed normally in a clathrin-dependent manner. Thus, the transferrin receptor, which has a YXXΦ-sorting signal, and a chimeric construct containing a [D/E]XXXL[L/I]-sorting signal, are both strongly affected by AP-2 knockdown (7,8). However, a low-density lipoprotein (LDL) receptor chimera (CD8-LDLR), which has an NPXY sorting signal, and the EGF receptor, which may use ubiquitin as a sorting signal (9; see also 10,11), are still internalized at normal rates in AP-2-depleted cells. On the basis of these observations, we proposed that proteins with YXXΦ and [D/E]XXXL[L/I] signals need AP-2 for their internalization, but that other proteins, including the LDL and EGF receptors, may make use of alternative endocytic adaptors, such as Dab2 and epsin, which bind to NPXY and ubiquitin, respectively (7). Although less is known about the AP-1 pathway, we have found that CCVs isolated from AP-1-depleted cells still contain cargo proteins that traffic between intracellular membranes, indicating that there are also alternative adaptors for intracellular trafficking (12).

If some cargo proteins do not need APs to be packaged into CCVs, why then do CCVs contain so much more AP-1 and AP-2 than any of the other adaptors? One possibility is that the CCVs also contain much more AP-dependent cargo than AP-independent cargo. So far, the only study to address the question of cargo abundance is a recent proteomics analysis of rat brain CCVs by McPherson and co-workers (13). They found that the majority of the cargo proteins in their preparations were synaptic vesicle components, which had presumably been captured by the CCVs during membrane retrieval after neurotransmitter

release. However, most of these proteins are expressed only in neurons and neuroendocrine cells, and so far, very little is known about their sorting signals.

To investigate how more representative cargo proteins are sorted, we have isolated CCVs from human placenta and rat liver and used lectin pulldowns to identify the most abundant membrane glycoproteins. We have then transplanted the cytoplasmic tails of these proteins onto a CD8 reporter and stably transfected the constructs into HeLa cells, so that we could follow their trafficking in control and siRNA-treated cells.

## Results

### *Major membrane glycoproteins in CCVs from placenta and liver*

Clathrin-coated vesicles were isolated from human placenta and rat liver, and their purity was monitored using electron microscopy (EM) and SDS–PAGE followed by mass spectrometry. Both preparations were found to consist mainly of coated structures (Figure 1A), including some coats that clearly contain vesicles (arrows) as well as smaller coats that may be empty. By SDS–PAGE, we found clathrin and AP subunits to be the major gel bands in both preparations (Figure 1B). However, there are clear differences in the protein composition of the two preparations. Visual inspection of some of the bands identified using mass spectrometry indicates that the AP-2 complex is more abundant in placenta CCVs, while in liver CCVs there is more AP-1 (e.g. note the relative intensities of the μ1 and μ2 subunits in the two lanes).

None of the major gel bands in either preparation corresponded to CCV cargo proteins. To enrich for such proteins, we took advantage of the observation that most of the CCV cargo proteins that have been identified, including cell surface receptors and lysosomal membrane proteins, are glycosylated. Thus, we extracted the preparations with 1 M Tris – HCl and 1% Triton-X-100, to solubilize coat and membrane proteins, respectively, then pulled down glycoproteins using lectin affinity matrices. We tried a number of different lectins (see *Materials and Methods*) and found that wheat germ agglutinin (WGA), which binds *N*-acetylglucosamine and sialic acid, gave the most clear-cut results. Concanavalin A, which binds mannose, produced a similar pattern by SDS–PAGE (unpublished observations), indicating that the WGA pulldown enriches for all glycoproteins that contain *N*-acetylglucosamine and/or sialic acid and not just those that are particularly rich in those two sugars. Figure 1C shows a WGA pulldown from human placenta CCVs, next to a crude membrane fraction from an earlier stage in the preparation, which was treated in an identical manner. The CCV lane has a much simpler pattern, with several prominent bands. The bands labeled 1–5 were excised and analyzed using mass spectrometry. The three high molecular-weight bands, 1, 2 and 3, were found to be LDL receptor-related protein 1 (LRP1), the cation-independent mannose 6-phosphate receptor (CIMPR) and carboxypeptidase D (CPD), respectively. Band 4 contained several proteins, including the EGF receptor and oxytocinase, which is the human placental ortholog of the insulin-regulated aminopeptidase found in rat adipocytes. Band 5 was matched with the transferrin receptor. All of these are proteins that cycle between endosomes, the plasma membrane and/or the *trans* Golgi network (TGN), so their enrichment in CCVs is consistent with what is known about their intracellular trafficking. However, we could not have predicted that these would be the most abundant proteins in the lectin pulldowns (e.g. the LDL receptor, which was the first CCV cargo protein to be characterized, was not detectable in the pulldowns, whereas CPD has never before been shown to be associated with clathrin).

To find out whether CCVs from liver also contain these glycoproteins, we carried out a similar pulldown using a Tris/Triton extract of a rat liver preparation. Again, we found three high molecular-weight bands, which by mass spectrometry were shown to be LRP1, CIMPR and CPD (Figure 1D). Thus, even though placenta and liver carry out different functions and differ in their relative abundance of AP-1 and AP-2, their CCVs are dominated by the same three major cargo glycoproteins.

### *Localization of CD8 chimeras*

LRP1, CIMPR and CPD are all type I membrane proteins, and there is evidence that most or all of the targeting information for the three proteins resides in their cytoplasmic tails (14–18). Therefore, we were able to investigate their sorting in HeLa cells using a CD8 chimera system. Figure 2 shows the three constructs diagrammatically, together with the sequences of their cytoplasmic tails. Known or suspected sorting signals for clathrin-mediated trafficking are indicated in the sequences in bold. All three chimeras were transfected into HeLa cells using an IRES vector, and stably expressing cell lines were cloned.

The steady-state distribution of the constructs is shown in Figure 3A–F. Unlike wild-type CD8, which is localized mainly at the plasma membrane in transfected HeLa cells (19, and our own unpublished observations), all three of the chimeras have a predominantly intracellular distribution. However, each of the constructs has a distinct localization pattern. Double labeling for various marker proteins showed that much of the CIMPR chimera (Figure 3A) is endosomal, co-localizing with internalized WGA, especially at early time points (Figure 3B shows WGA internalized for 15 min). There is also a more perinuclear pool of CD8-CIMPR, which is likely to reside in the TGN (16). The CPD chimera (Figure 3C) has near-perfect co-localization with TGN46 (Figure 3D). The LRP1 chimera (Figure 3E), like the CIMPR chimera, localizes to

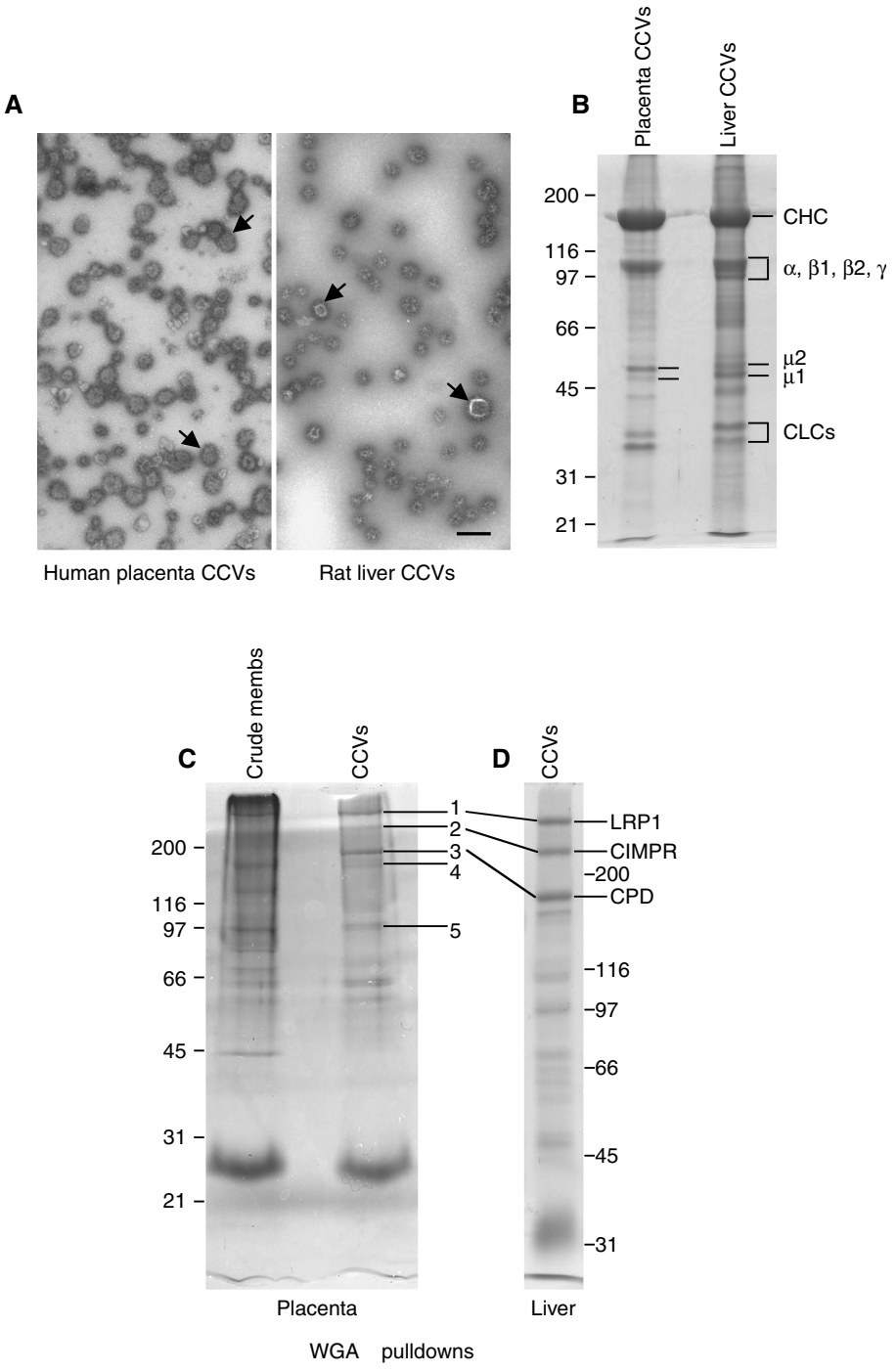
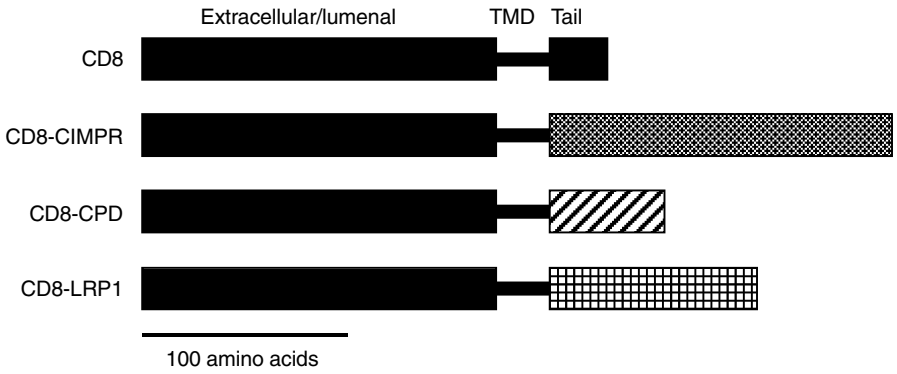



**Figure 1: Micrographs and gels of clathrin-coated vesicles (CCVs) purified from human placenta and rat liver.** A) CCVs were negatively stained and examined by electron microscopy. Both preparations appear to be purified to near homogeneity. Some of the coats that clearly contain vesicles are indicated with arrows. Scale bar: 200 nm; B) SDS–PAGE of the two preparations. Each lane contains approximately 3.5 μg protein. Gel bands were identified using mass spectrometry. CHC: clathrin heavy chain; CLCs: clathrin light chains. C) and D) Crude membranes or CCVs (containing 350 μg protein) were extracted with 1 M Tris–HCl and 1% Triton-X-100, and glycoproteins were pulled down using a wheat germ agglutinin (WGA) affinity matrix and analyzed using mass spectrometry. The indicated bands correspond to low-density lipoprotein receptor-related protein 1 (LRP1) (band 1), the cation-independent mannose 6-phosphate receptor (CIMPR) (band 2), carboxypeptidase D (CPD) (band 3), the EGF receptor and oxytocinase (band 4) and the transferrin receptor (band 5). Each lane contains half of the total eluate; thus, approximately 50 times the amount of CCV protein shown in (B) was used to produce each of the two CCV pulldown lanes.

endosomes, but it shows better overlap with internalized EGF than with internalized WGA, especially at later time points (Figure 3F shows EGF internalized for 30 min). This indicates that the LRP1 chimera resides in a later endosomal compartment than the CIMPR chimera.

To examine the localization of the three chimeras at the ultrastructural level, we labeled frozen thin sections of the cells with anti-CD8 followed by protein A coupled to 15 nm gold. CD8-CIMPR was found to be associated with tubulovesicular membranes scattered throughout the cell (Figure 4A). CD8-CPD also had a tubular appearance, but with a much more restricted distribution, nearly always positioned on one side of a Golgi stack (Figure 4B). CD8-LRP1 had the lowest density of labeling, but some of the multivesicular bodies were positive for this construct, both on the limiting membrane and on internal vesicles (Figure 4C). Thus, the EM localization data are consistent with the immunofluorescence results and confirm that CD8-CIMPR is associated with early (tubular) endosomes,




CD8
KRLKRRRVCKCPRPVVKSGDKPSLSARYV

CIMPR
KKERREMVMSRLTNCCRRSANVSYK**YSKV**NKEEEADENETEWLMEEIQPPAPRPGKEGQENGHVAAKSVRAADTLSA
LHGDEQDSEDEVLTLPEVKVRPPGRAPGAEGGPPLRPLPRKAPPPLRADDRVGLVRGEPARRGRPRAAATPISTFHD
DS**DEDLL**HV

CPD
KSNRHKDG**FHRL**RGHHDEYE**DEIRMM**STG**SKKSLL**SH**EFQDETDTEET**LYSSKH

LRP1
KRRVQGAKGFQHQRMTNGAMNVEIG**NPTY**KMYEGGEPD**DVGGLL**DADFALDPDKPTNFT**NPVYATL**YMGGHGSRHSL
ASTD**EKRELL**GRGPEDEIGDPLA

**Figure 2: Diagram of the CD8 constructs and sequences of their cytoplasmic tails.** Potential clathrin-dependent sorting signals are indicated in bold. The tyrosine in the lipoprotein receptor-related protein 1 (LRP1) tail that contributes both to the second NPXY motif and to the YXXΦ motif is underlined.

CD8-CPD with the TGN and CD8-LRP1 with later (multivesicular) endosomes.

### *Enrichment of the chimeras in CCVs*

Although all three chimeras accumulate in compartments that can be accessed by clathrin-mediated trafficking, we did not see much co-localization with clathrin at either the light or the electron microscope level. However, the association of cargo proteins with CCVs is known to be very transient, so that at any one time most of the protein will be elsewhere in the cell. To investigate whether the three constructs are getting packaged into CCVs, we isolated CCVs from each of the cell lines. We have previously shown that CCV preparations from HeLa cells are less clean than preparations from tissues like placenta or liver, so we have developed a control for specificity (12). Cells are depleted of clathrin heavy chain using siRNA, then a CCV preparation is carried out in the usual manner. Preparations from the clathrin-depleted cells still contain all of the contaminants found in control preparations, such as ribosomes and smooth membrane vesicles, but they are devoid of CCVs. By comparing the levels of a particular protein in preparations from control and clathrin-depleted cells, we can determine whether or not it is a *bona fide* CCV component.

Figure 5 shows equal protein loadings of homogenates and CCV preparations from our three chimera-expressing

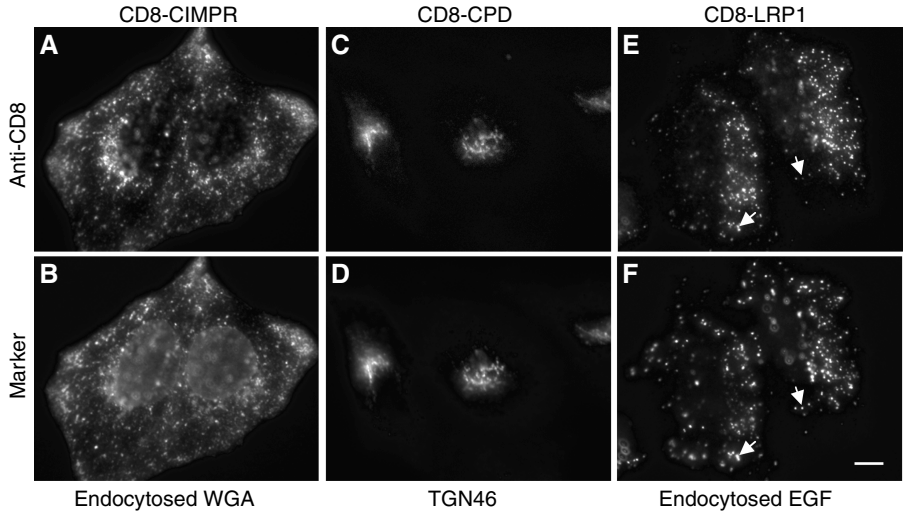



**Figure 3: Steady-state distribution of the three chimeras.** CD8-CIMPR (A) shows significant co-localization with wheat germ agglutinin endocytosed for 15 min (B). CD8-CPD (C) strongly co-localizes with TGN 46 (D). CD8-LRP1 (E) shows partial co-localization with EGF endocytosed for 30 min (F) (some of the areas of overlap are indicated with arrows). Scale bar: 10 μm.

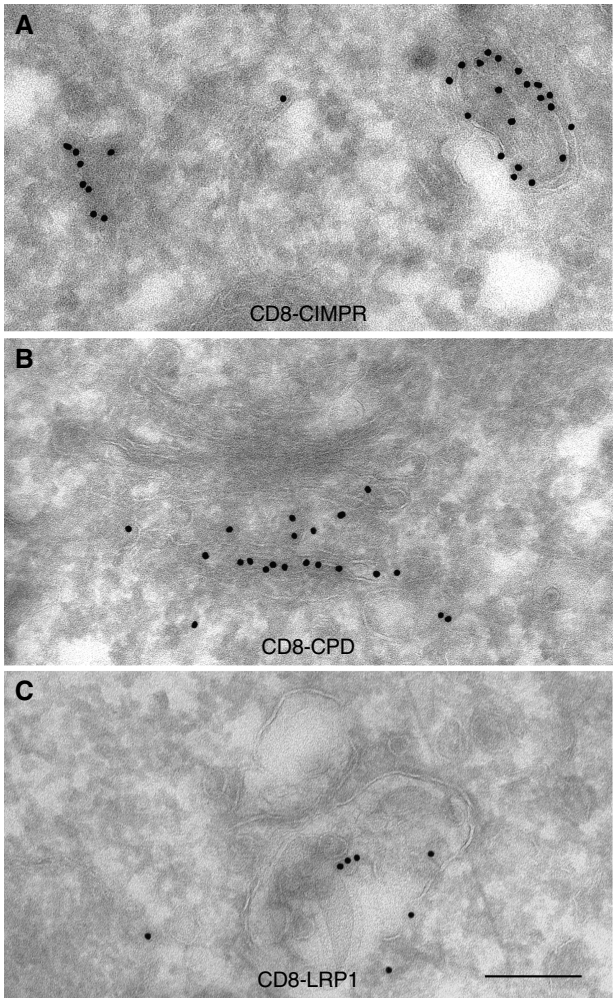



**Figure 4: Localization of the three chimeras using electron microscopy.** Frozen thin sections were labeled with anti-CD8 followed by protein A coupled to 15 nm gold. A) The CD8-CIMPR chimera localizes to tubulovesicular membranes. B) The CD8-CPD chimera is highly concentrated in TGN membranes. C) The CD8-LRP1 chimera localizes to multivesicular bodies, both the limiting membrane and the internal vesicles. Scale bar: 200 nm.

cell lines and from a CD8-expressing cell line, probed with antibodies against CD8, clathrin heavy chain and (as a loading control) EF-2, a ribosome-associated protein. In the cells expressing CD8 on its own, it is clear that the construct is not enriched in CCVs. In fact, there is less CD8 in the CCV preparation than in the whole cell homogenate and slightly more CD8 in the CCV preparation when clathrin is depleted. This indicates that the CD8 is not actually in the CCVs but in contaminating membranes. In contrast, both the CIMPR chimera and the CPD chimera are strongly enriched in the CCV preparation over whole cell homogenate, and in both cases, most of the signal is lost from the CCV preparation when the cells are depleted of clathrin. The blot of the LRP1 chimera is somewhat harder to interpret because of the relatively low expression of the construct, which goes up dramatically when clathrin is knocked down (compare the signals in the homogenates of control and clathrin-depleted cells). Leupeptin also causes a dramatic increase in the expression of the LRP1 chimera (unpublished observations), indicating that much of the construct may be trafficking to lysosomes and getting degraded and that this pathway may be impaired when clathrin is depleted. Although similar amounts of CD8-LRP1 are present in the CCV preparations from control and clathrin-depleted cells, this is not a fair comparison because there is more LRP1 chimera to start with in the clathrin-depleted cells. A better comparison can be made between the homogenate and CCV lanes of the control cells and between the homogenate and CCV lanes of the clathrin-depleted cells. Here, it can be seen that the LRP1 chimera is strongly enriched in the CCV preparation from the control cells but not in the preparation from the clathrin-depleted cells. Thus, all three of the chimeras, but not CD8 alone, are packaged as cargo into CCVs.

### *Surface expression of the chimeras*

If the three chimeras are being internalized by clathrin-mediated endocytosis, then knocking down clathrin should cause them to accumulate on the cell surface, and if the internalization is AP-2 dependent, then knocking down AP-2 should have a similar effect. Therefore, we treated each of the three cell lines with siRNAs, then quantified the amount of construct on the cell surface by incubating the cells at 4 °C with anti-CD8 followed by $^{125}$I-protein A.

Figure 6A shows the total amount of label bound to the cells under control conditions and after either AP-2 (μ2) or clathrin knockdown. There is a great deal of variability in surface labeling in the untreated cells: a significant amount of both the CIMPR and the CPD chimera is at the plasma membrane, while the signal from the cells expressing the LRP1 chimera is only approximately twofold the signal we get when we label control HeLa cells, which do not express any CD8. In Figure 6B, we have normalized the results by dividing the signal after knockdown by the signal before knockdown. Knocking down AP-2 causes the amount of CD8-CIMPR at the cell surface to go up approximately threefold, and knocking down clathrin produces approximately fourfold increase. Knocking down AP-2 also causes approximately threefold increase in the amount of CD8-CPD at the cell surface, while knocking down clathrin produces approximately 15-fold increase. The CD8-LRP1-expressing cells are the least affected by AP-2 knockdown, with only approximately 40% increase in the amount of construct at the cell surface, but they are the most strongly affected by clathrin knockdown, with approximately 25-fold increase. Thus, although all three constructs are affected to some extent by both clathrin and AP-2 knockdown, the relative effects are very different.

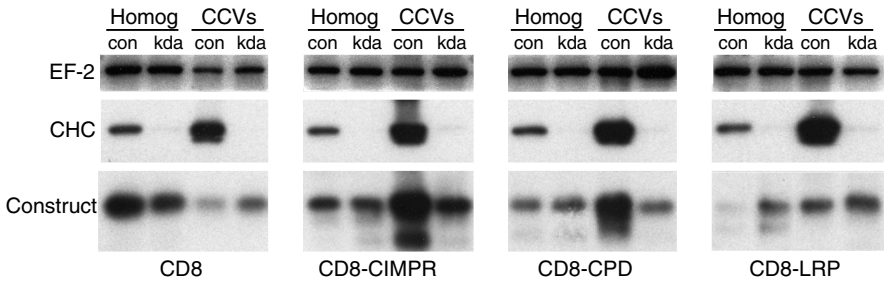



**Figure 5: Enrichment of the constructs in clathrin-coated vesicles.** Equal protein loadings of whole cell homogenates and CCV preparations from control (con) and clathrin-depleted (kDa) cells were analyzed using SDS–PAGE followed by Western blotting. EF-2, a ribosome-associated protein, was used as a loading control. CD8 is not enriched in CCVs over whole cell homogenate, and it is not lost from the CCV preparation when clathrin is depleted. CD8-CIMPR and CD8-CPD are both strongly enriched in the CCV preparation, and the signal is greatly reduced in preparations from clathrin-depleted cells. CD8-LRP1 is also enriched in the CCV preparation when compared with whole cell homogenate, although the increased stability of the construct in clathrin-depleted cells means that the difference is not apparent when CCV preparations from control and siRNA-treated cells are compared.

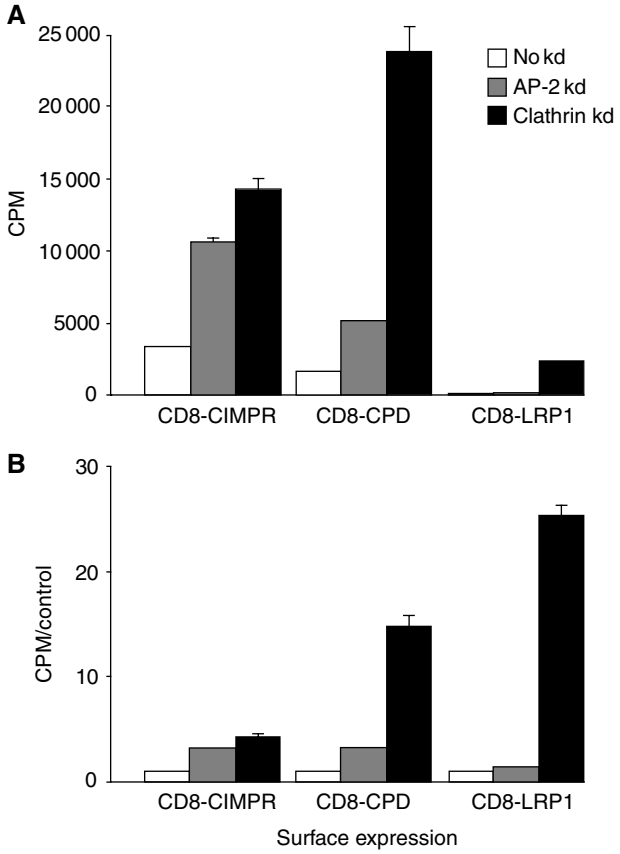



**Figure 6: Surface expression of CD8 chimeras in control and siRNA-treated cells.** A) Cells were incubated at 4 °C with anti-CD8 followed by $^{125}$I-protein A, and total counts per minute per mg cell protein (CPM) were plotted. B) Surface expression was normalized for each of the cell lines by dividing the CPM in the siRNA-treated cells by the CPM in the control cells. Both clathrin and AP-2 knockdowns increase the surface expression of all three chimeras, but the relative effects are very different.

### *The CD8-CIMPR chimera*

The increase in the surface expression of the various chimeras after clathrin knockdown is not necessarily caused by a block in clathrin-mediated endocytosis; it could also be due to a disruption in clathrin-mediated intracellular trafficking. For instance, the CIMPR has sorting signals in its cytoplasmic tail that bind to AP-1 and Golgi-localized γ-ear-containg, ARF-binding proteins (GGAs), both of which are involved in trafficking between the TGN and endosomes, and abolishing these pathways could cause the CD8-CIMPR construct to accumulate on the cell surface. To look specifically at endocytosis of the CIMPR chimera, we used an antibody uptake assay that we developed for our previous studies on the CD8-LDLR chimera (7).

Cells were incubated with anti-CD8 followed by $^{125}$I-protein A at 4 °C, then warmed to 37 °C for various lengths of time, after which the protein A remaining on the cell surface was removed by acid stripping. Figure 7A shows the percentage of prebound label that has been internalized, averaging results from three separate experiments carried out on different days. The rate of uptake of antibody in the untreated cells and the decrease in antibody uptake in the clathrin-depleted cells are virtually identical to what we found in our earlier study on the LDLR chimera (7). However, whereas knocking down AP-2 has no effect on the rate of internalization of CD8-LDLR, it has nearly as strong an effect on the rate of internalization of CD8-CIMPR as knocking down clathrin. Thus, this experiment provides further evidence that AP-2 is required for efficient endocytosis of the CD8-CIMPR chimera.

### *The CD8-CPD chimera*

We used the same antibody uptake assay to monitor the rate of internalization of the CD8-CPD chimera in control and siRNA-treated cells. Figure 7B shows that in control cells, the construct is taken up efficiently, although not

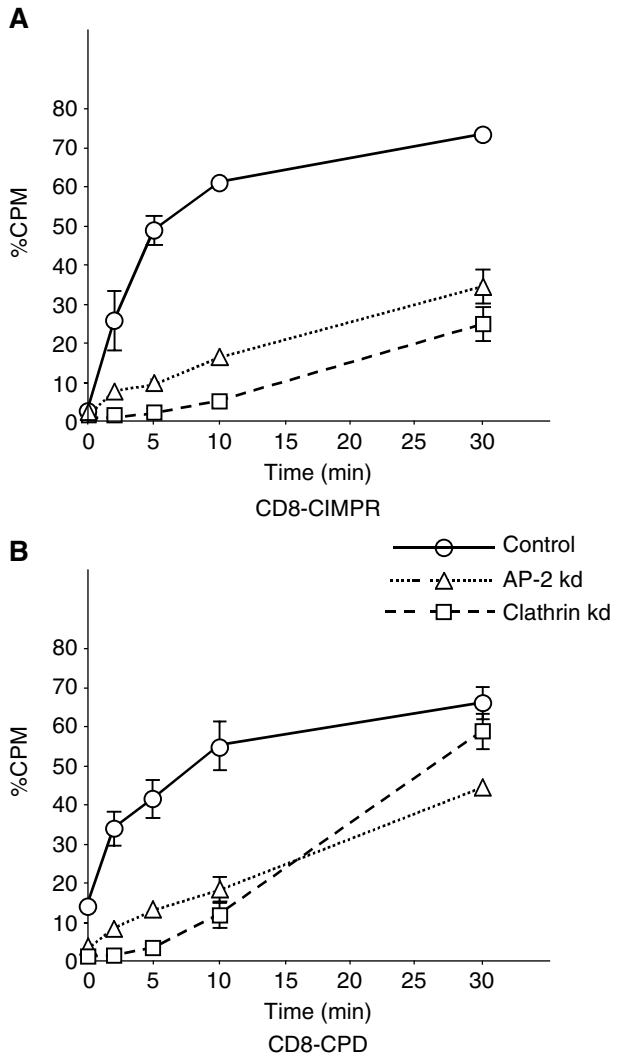



**Figure 7: Internalization kinetics of the CIMPR and CPD chimeras.** Control and siRNA-treated cells expressing either CD8-CIMPR (A) or CD8-CPD (B) were incubated at 4 °C with anti-CD8 followed by $^{125}$I-protein A, then warmed to 37 °C for various lengths of time, after which label remaining on the cell surface was removed by acid stripping. The graph shows the percentage of total counts that are still cell-associated. Both clathrin and AP-2 depletion strongly affect the rate of uptake of both constructs.

quite as rapidly as the CIMPR chimera. Knocking down clathrin strongly inhibits the internalization of the construct at early time points, and knocking down AP-2 also has a strong inhibitory effect. However, for reasons that are not clear, at later time points (e.g. 30 min) there is much more uptake of the CPD chimera than of the CIMPR chimera in clathrin- and AP-2-depleted cells. Nevertheless, the results clearly show that the CPD chimera requires both clathrin and AP-2 for rapid and efficient internalization.

Because clathrin depletion causes approximately 15-fold increase in the amount of CD8-CPD at the cell surface, while AP-2 depletion only causes approximately threefold increase (see Figure 6), it is likely that clathrin is involved in the trafficking of the construct not only at the plasma membrane but also at intracellular locations. The presence of an acidic cluster in the cytoplasmic tail of CPD suggests that its trafficking may be dependent on phosphofurin acidic cluster sorting protein 1 (PACS-1) (20). PACS-1 was originally identified in a yeast two-hybrid library screen as a binding partner for furin, another acidic cluster-containing protein that resides mainly in the TGN. PACS-1 is believed to provide a link between acidic cluster-containing cargo proteins and AP-1, allowing the cargo proteins to be retrieved from endosomes and recycled back to the TGN in a clathrin-dependent manner (21). Therefore, we used two new siRNAs to deplete PACS-1 and a previously characterized siRNA to deplete AP-1, then quantified the amount of CD8-CPD at the cell surface. Figure 8A shows that both of the PACS-1 siRNAs are effective at depleting their target protein.

In the AP-1-depleted cells, there is approximately sixfold increase in surface labeling for CD8-CPD (Figure 8B). This is consistent with AP-1 contributing to clathrin-mediated sorting of CPD. Surprisingly however, knocking down PACS-1 has no effect, suggesting either that the acidic cluster is not the dominant sorting signal or alternatively that the acidic cluster does not need PACS-1 for its sorting. We also investigated the effect of knocking down AP-1 and AP-2 together. Interestingly, like the clathrin knockdown, the combined knockdown causes approximately 15-fold increase in the amount of CD8-CPD at the cell surface. These results indicate that AP-1 and AP-2 act synergistically to maintain the steady-state distribution of CPD, with AP-1 facilitating packaging into intracellular CCVs and AP-2 facilitating packaging into endocytic CCVs.

Because there may be changes in the intracellular distribution of CD8-CPD that do not lead to changes in the amount on the cell surface, we also used immunofluorescence to compare the steady-state localization of the chimera in cells treated with the various siRNAs. Figure 9 shows that knocking down either clathrin, AP-1 or AP-1 and AP-2 together causes the construct to move away from the TGN into a more peripheral location. However, knocking down AP-2 does not have a very strong effect on the intracellular distribution of CD8-CPD, and PACS-1-depleted cells are not noticeably different from controls.

### *The CD8-LRP1 chimera*

LRP1 is a receptor for a number of different extracellular ligands (22), so it was surprising that there was so little of the chimera on the cell surface. This could be either because the construct normally appears only briefly at the plasma membrane and then is rapidly internalized or alternatively because it rarely arrives at the plasma membrane. We initially attempted to investigate the endocytosis of the LRP1 chimera using the same 4 °C binding, 37 °C warm-up assay that we used for the CIMPR and CPD chimeras. However, the signal was so low that it was

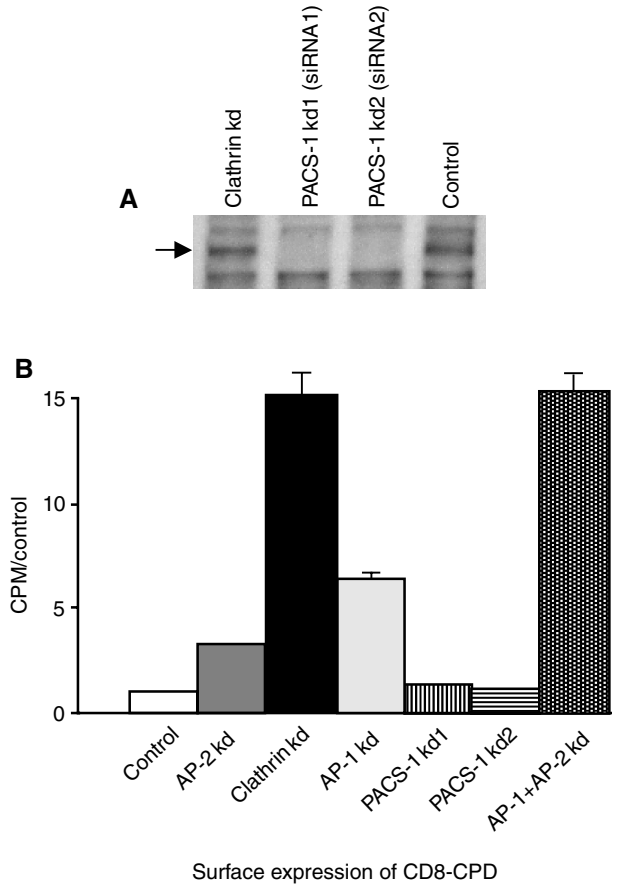



**Figure 8: Surface expression of CD8-CPD in control and siRNA-treated cells.** A) The effectiveness of the two PACS-1 siRNAs was tested by Western blotting. Although the antibody cross-reacts with other proteins, the band with the expected size for PACS-1 (arrow) disappears when cells are treated with either of the two siRNAs. B) Cells were incubated at 4 °C with anti-CD8 followed by $^{125}$I-protein A, and surface expression was normalized by dividing the CPM in the siRNA-treated cells by the CPM in the control cells. AP-2 depletion causes approximately threefold increase in surface expression, clathrin depletion causes approximately 15-fold increase, AP-1 depletion causes approximately sixfold increase and depletion of both AP-1 and AP-2 causes approximately 15-fold increase. However, neither of the PACS-1 siRNAs causes a significant increase in surface expression of CD8-CPD.

difficult to get meaningful data. Therefore, we developed a simple, FACS-based assay for internalization.

Cells were incubated with Cy5-conjugated anti-CD8 either at 4 °C, to allow surface binding but not internalization, or at 37 °C, to allow both binding and internalization, then trypsinized and analyzed by flow cytometry. Figure 10A shows representative histograms of control HeLa cells, CD8-expressing cells and CD8-LRP1-expressing cells. The control HeLa cells bind only a very small amount of antibody at 4 °C (solid gray), which does not increase if the incubation is done at 37 °C (black line). The CD8-expressing cells bind approximately 500-fold more antibody than the control HeLa cells, but the fluorescence intensity at the two temperatures is similar; indeed, there is slightly more antibody bound at 4 °C. In the CD8-LRP1-expressing cells, although there is only about twice as much fluorescence at 4 °C as in the control cells, the signal goes up a further twofold when the cells are incubated at 37 °C, indicating that construct is trafficking to the cell surface, binding antibody and trafficking inside again during the time course of the experiment.

We next investigated the effects of knocking down either AP-2 or clathrin on the fluorescence of the CD8-LRP1-expressing cells at the two temperatures. Figure 10B shows that AP-2 depletion does not affect the 37:4 ratio, indicating that endocytosis of the construct still proceeds normally in the absence of AP-2. However, when clathrin is depleted the fluorescence intensity is the same at 37 and 4 °C, indicating that endocytosis of the construct is impaired. Thus, as predicted by the antibody-binding assay (Figure 6), clathrin depletion prevents the chimera from being internalized, but AP-2 depletion has little or no effect.

The cytoplasmic tail of LRP1 contains a number of potential clathrin-dependent sorting signals, both AP-2 dependent and AP-2 independent: NPTY, DVGGLL, NPVY, YATL and EKRELL (see Figure 2). Of the two NPXY-type motifs, only the second is preceded by a phenylalanine in the −2 position, and there is evidence that [F/Y]XNPXY is the optimal motif for internalization (9,23). This sequence overlaps with the YATL motif, with the same tyrosine contributing to both. Therefore, we mutated this tyrosine to an alanine, expecting to see a strong effect on the trafficking and/or steady-state distribution of the construct.

Surprisingly, the mutant construct has the same apparent steady-state distribution as the wild-type construct (Figure 11A; see also Figure 3), showing partial overlap with internalized EGF (Figure 11B). Knocking down clathrin increases the surface expression of the mutant construct >30-fold (Figure 11C), which is similar to the effect of clathrin knockdown on the wild-type construct (see Figure 6). However, unlike the wild-type construct, the surface expression of the mutant construct is also strongly affected by AP-2 knockdown (Figure 11C; see also Figure 6). We also used the flow cytometry assay to monitor endocytosis of the construct and found approximately twofold increase in fluorescence intensity when control cells were incubated at 37 °C, which was abolished by clathrin knockdown (Figure 11D). This again is similar to our data on cells expressing the wild-type construct (see Figure 10B). However, AP-2 knockdown is nearly as effective as clathrin knockdown in blocking the internalization of the mutant construct. These results suggest that the FXNPXY motif is the dominant sorting signal in the chimera with the wild-type LRP1 tail, and that when it is abolished, another sorting signal(s) – presumably one or both of the [D/E]XXXL[L/I] motifs – takes over.

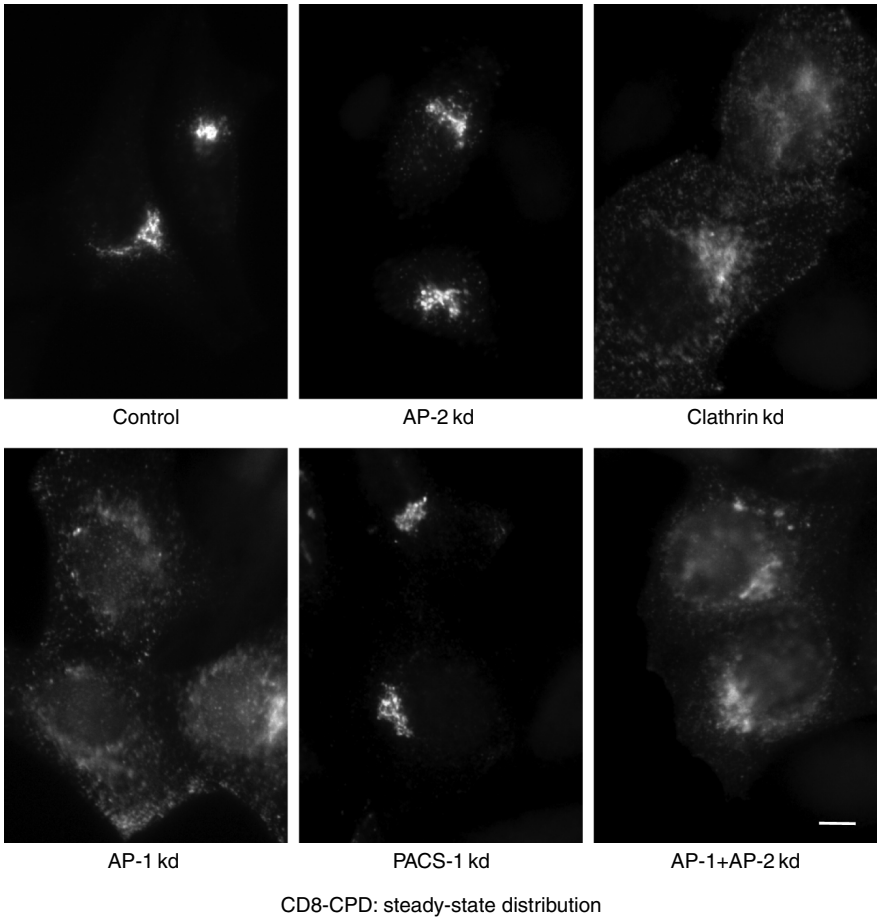



**Figure 9: Steady-state distribution of CD8-CPD in control and siRNA-treated cells.** Knocking down clathrin and AP-1 (either alone or in combination with AP-2) causes the construct to move away from the TGN into more peripheral membranes. Knocking down AP-2 on its own has at most a weak effect, and knocking down PACS-1 has no discernible effect. Scale bar: 10 μm.

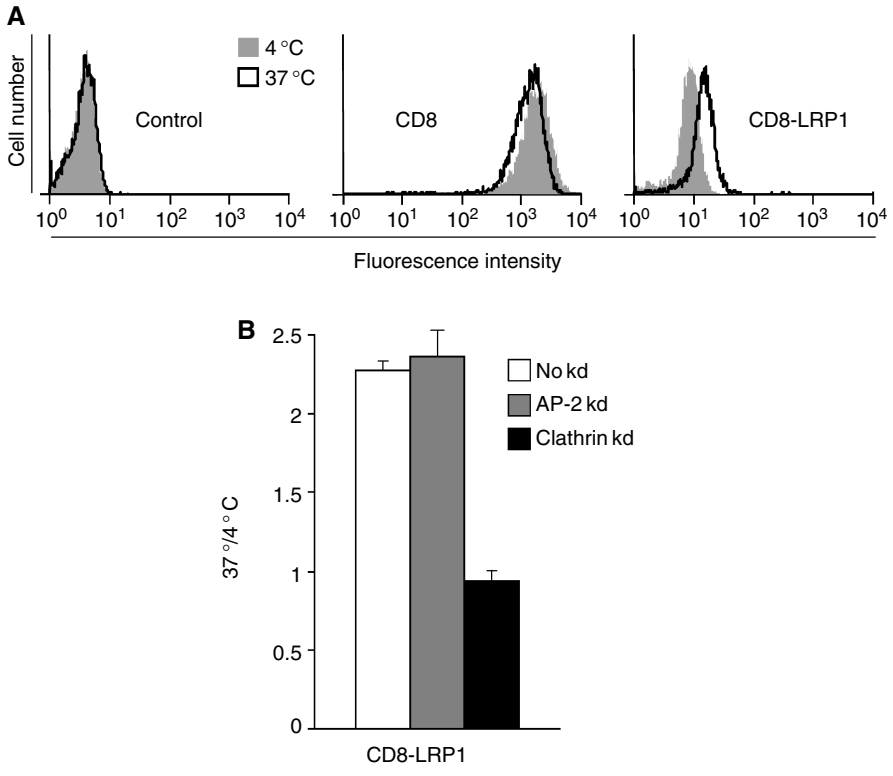



**Figure 10: Endocytosis of CD8-LRP1 determined by flow cytometry.** A) Cells were incubated with Cy5-conjugated anti-CD8 at either 4 or 37 °C, and the signal was quantified by flow cytometry. Representative histograms of control (i.e. nontransfected), CD8-expressing and CD8-LRP1-expressing cells are shown. The increase in signal at 37 °C in the CD8-LRP1-expressing cells indicates that the chimera is trafficking to the cell surface and getting internalized. B) Data pooled from three experiments on untreated and siRNA-treated CD8-LRP1-expressing cells using the assay shown in (A). There is approximately twofold increase in signal at 37 °C in both untreated and AP-2-depleted cells, indicating that AP-2 depletion does not inhibit internalization of the chimera. In the clathrin-depleted cells, the signal is the same at the two temperatures, indicating that internalization is blocked.

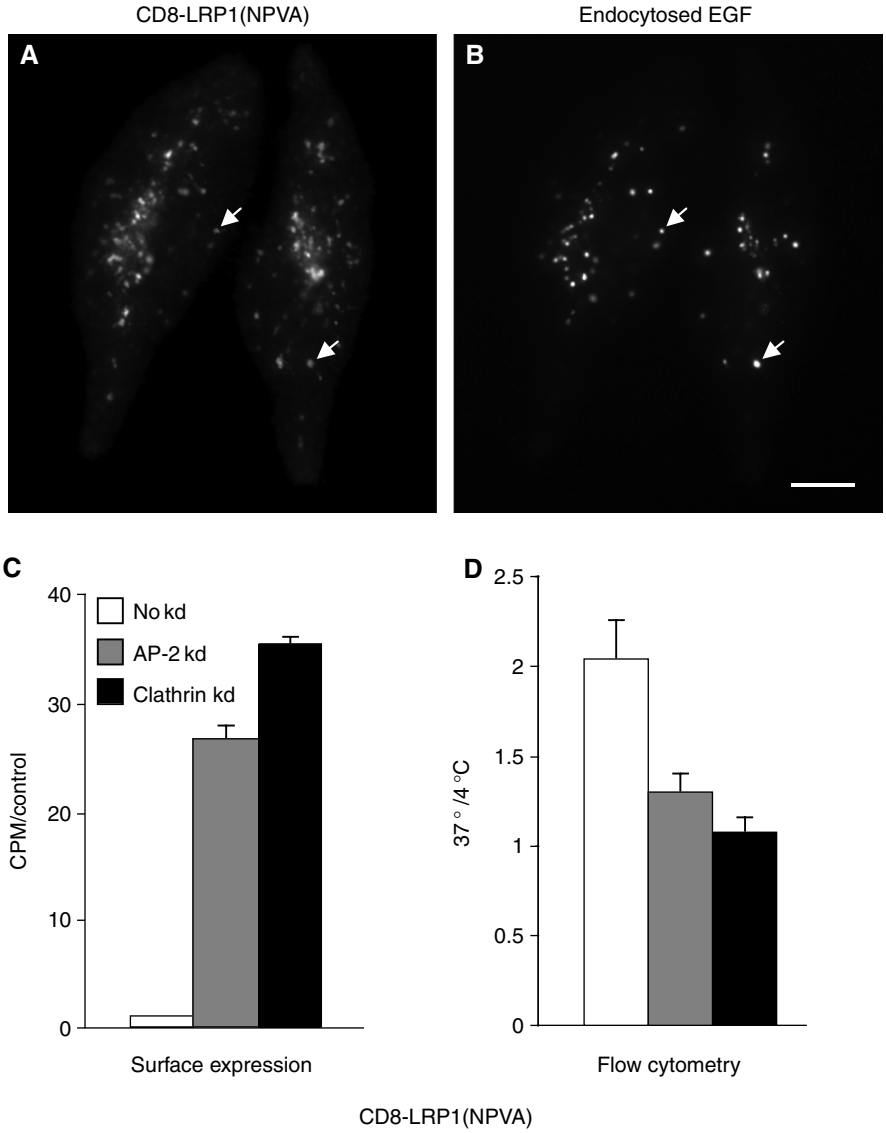



**Figure 11: Steady-state distribution and behavior of a CD8-LRP1 chimera with a mutation in the NPVY motif.** A) and B) Cells were stably transfected with a construct in which the tyrosine had been mutated to an alanine [CD8-LRP1(NPVA)]. The construct (A) still shows partial co-localization with EGF internalized for 30 min (B) (some of the areas of overlap are indicated with the arrows). Scale bar: 10 μm; C) The surface expression of CD8-LRP1 (NPVA) was determined by incubating cells at 4 °C with anti-CD8 followed by $^{125}$I-protein A and normalized by dividing the CPM in the siRNA-treated cells by the CPM in the control cells. Both clathrin and AP-2 depletion strongly increase the surface expression of the NPVA mutant. D) Internalization of CD8-LRP1 (NPVA) was assayed by incubating cells with Cy5-conjugated anti-CD8 at either 4 or 37 °C and quantifying the signal using flow cytometry. Both clathrin and AP-2 depletion decrease the signal at 37 °C relative to 4 °C, indicating that both knockdowns inhibit internalization of the NPVA mutant.

## Discussion

A number of different sorting signals have been identified for clathrin-mediated trafficking (9), only two of which, the YXXΦ motif and the [D/E]XXXL[L/I] motif, have been definitively shown to bind to AP complexes. The other motifs may function by binding to other adaptors, such as Dab2 and epsin at the plasma membrane, and GGAs and epsinR on intracellular membranes. However, at least in mammalian CCVs, there is much more AP-1 and AP-2 than any of the other adaptors. This led us to speculate that the major cargo proteins in CCVs might depend upon AP complexes for their sorting.

Although many cargo proteins have been shown to be enriched in CCVs by Western blotting, so far, little is known about the relative amounts of these proteins and how they are packaged. To identify the major membrane glycoproteins in CCVs, we carried out WGA pulldowns on extracts of CCVs purified from human placenta and rat liver. In both cases, we found three prominent high molecular-weight bands: CIMPR, CPD and LRP1. The transferrin receptor, which is probably the best-characterized CCV cargo protein, was also visible as a Coomassie Blue-stained band in the human placenta pulldown. Because CIMPR, CPD and LRP1 are all type I membrane proteins, we were able to transplant their cytoplasmic tails onto a CD8 reporter and investigate the sorting of all three constructs using antibodies against CD8. In addition to carrying out immunolocalization and surface-binding assays, we have made use of a new method we recently developed, which involves isolating CCVs from control and siRNA-treated cells (12). We find that all three constructs are highly enriched in CCVs from control cells but not from clathrin-depleted cells, demonstrating that this method can be used to investigate the trafficking not only of

endogenous cargo proteins but also of engineered proteins that have been transfected into the cells.

Of the three CCV cargo proteins, the CIMPR has been the most extensively studied, and our results are largely in agreement with previous findings. The CD8-CIMPR chimera, like endogenous CIMPR, localizes primarily to endosomes and the TGN, and is efficiently packaged into CCVs. There is also a substantial amount of the construct at the plasma membrane, and perhaps as a consequence, clathrin knockdowns have a less dramatic effect on the surface expression of CD8-CIMPR than either CD8-CPD or CD8-LRP1, causing only a fourfold increase. AP-2 knockdowns also cause an increase in surface expression of CD8-CIMPR, although not quite as much as clathrin knockdowns, possibly because knocking down clathrin affects intracellular trafficking as well as endocytosis. For instance, the CIMPR has a DXXLL motif, which binds to GGAs (24), and this motif has been shown to be important for the sorting of lysosomal enzymes bound to the CIMPR (25). The YXXΦ motif is essential for rapid endocytosis of the CIMPR (26), and consistent with this observation, we find that AP-2 knockdowns affect the rate of internalization of CD8-CIMPR nearly as much as clathrin knockdowns do.

Much less is known about the trafficking of CPD, and its abundance in CCVs purified from both placenta and liver was unexpected. Like the CD8-CPD chimera, endogenous CPD has been shown to reside mainly in the TGN, but a fraction of the protein is at the plasma membrane where it is efficiently endocytosed (27). Mutagenesis studies on CPD suggest that it contains more than one sorting signal (17,18). Most prominent is an acidic cluster containing potential phosphorylation sites for casein kinase II, which is believed to function by interacting with PACS-1. However, we found that PACS-1 knockdowns did not affect the steady-state distribution of CD8-CPD. Other potential sorting signals in the CPD tail include FHRL, which resembles a YXXΦ motif, and two [D/E]XXXL[L/I]-like sequences, DEIRMM and SKKSLL (17,18). Although the relative importance of these sequences is not yet known, our results clearly show that trafficking of the CD8-CPD chimera is clathrin-dependent. In addition, we find that knocking down AP-1 and AP-2 together produces a strikingly similar phenotype to knocking down clathrin, indicating that CPD depends on both APs for its normal steady-state distribution. However, at present, we cannot say whether AP-1 and AP-2 bind directly or indirectly to the CPD tail or which of the potential sorting signals they recognize.

The LRP1 tail is particularly rich in potential sorting signals, including two NPXY motifs, two [D/E]XXXL[L/I] motifs and a YXXΦ motif. Two previous studies led us to suspect that trafficking of the CD8-LRP1 chimera would be AP-2 dependent. First, Bu and co-workers (14) engineered mutations into a tagged LRP1 'minireceptor' (i.e. missing part of the extracellular domain) and came to the conclusion that the YXXΦ motif was the most important sorting signal for endocytosis. Second, Conner and Schmid (28) used a dominant negative approach to block AP-2 function and showed that internalization of both transferrin and RAP (a ligand for LRP1) was inhibited, but internalization of EGF still proceeded normally. However, the CD8-LRP1 chimera behaves somewhat differently. The steady-state distribution and the rate of internalization of the chimera are both strongly affected by clathrin depletion, but they are affected either very little or not at all by AP-2 depletion. Interestingly, mutating the tyrosine residue that contributes both to the YXXF motif and to the second NPXY motif makes the construct much more dependent upon AP-2. Thus, for reasons that are still not clear, in the context of a CD8 chimera, the FXNPXY motif appears to be the dominant sorting signal in the LRP1 tail rather than the YXXΦ motif, and the construct can still be correctly sorted in the absence of any detectable AP-2. However, a role for AP-2 in the sorting of the CD8-LRP1 chimera is unmasked when a single amino acid substitution is made, indicating that AP-2 contributes to the sorting of the chimera even though it is not absolutely required.

Thus, all three of the major glycoproteins that we identified in our CCV preparations use (or can use) AP complexes for their trafficking. Although this is consistent with the hypothesis that AP complexes are more abundant than other adaptors because AP-dependent cargo proteins are more abundant than AP-independent cargo proteins, there are two caveats. First, LRP1 may be equally or more dependent on other adaptors that recognize the FXNPXY motif. Second, it is clear from the gels shown in Figure 1 that in both placenta and liver CCV preparations, the AP complexes are in vast molar excess over their cargo proteins; indeed, in order to detect the cargo proteins at all, we had to use lectin pulldowns of CCV extracts. One possible explanation for the high abundance of AP complexes relative to cargo is that APs and other adaptors may function mainly to promote clathrin assembly onto membranes, and cargo binding may be an incidental event; thus, many of the adaptors would be 'empty'. However, a recent live cell-imaging study suggests that in fact cargo capture may play a key role in CCV formation, stabilizing nascent clathrin-coated pits and committing them to become coated vesicles (29). An alternative explanation is that many of the CCVs in our preparations may themselves be empty, without any vesicles inside. Normally, clathrin and adaptors are thought to assemble only onto membranes, but during the time it takes to prepare tissues for CCV isolation, there may be anomalous assembly of some of the soluble clathrin and adaptors into empty coats [indeed, the abundance of 'empties' in CCV preparations was first remarked upon by Pearse (30)]. Interestingly, in CCV preparations from HeLa cells, which can be processed more quickly than tissues like placenta or liver, we find that the CIMPR and transferrin receptor are among the major bands that are depleted when

clathrin is knocked down (unpublished observations). Thus, the cargo to adaptor ratio in CCVs isolated from placenta and liver may be misleadingly low.

What are the sorting signals on the CIMPR, CPD and LRP1 that bind to AP complexes and direct them into CCVs? The CIMPR and LRP1 most likely use YXXΦ and/or [D/E]XXXL[L/I] motifs. The CPD tail also contains sequences that are related to these two motifs; however, none of them quite fits the consensus sequences. Thus, further studies will be necessary to determine precisely how CPD is sorted by AP complexes. It is possible that CPD – and other cargo proteins as well – may use novel mechanisms to bind to APs. The precedent of the COPII complex, which has multiple binding sites on its surface for different cargo proteins (31), supports the possibility that there may be additional sorting signals that bind to AP complexes, which have yet to be characterized.

## Materials and Methods

### *Protein chemistry*

Unless otherwise specified, reagents were purchased from Sigma. Clathrin-coated vesicles were isolated from rat liver using a previously published method (32) and from human placenta using the method described by Manfredi and Bazari (33) for brain CCVs, followed by a sucrose step gradient using the method described by Pilch (32) *et al.* for liver CCVs. To isolate membrane glycoproteins, we incubated 100–200 μL of a rat liver or human placenta CCV sample (approximately 2 mg/mL) or a similar amount of resuspended pellet from the first high-speed centrifugation step (i.e. crude membranes) with an equal volume of extraction solution (2 M Tris pH 7.0, 2% Triton-X-100, 2 mM EDTA, 0.04% $NaN_3$ and 0.2% β-mercaptoethanol) for 60 min at room temperature, then centrifuged in a Beckman TLA 100.2 rotor at 100,000 ×g for 15 min at 4 °C. The supernatant was diluted with 10 volumes of Buffer A (0.1 M MES, 0.2 mM EGTA, 0.5 mM $MgCl_2$, 0.02% $NaN_3$ and 0.2 mM AEBSF, pH 6.5) and 25 μL (1:1 v/v) of lectin coupled to agarose was added and incubated on a rocker at 4 °C for 45 min. The lectins included WGA, concanavalin A, *Ricin communis* agglutinin I, *Dolichos biflorus* agglutinin, peanut agglutinin and soybean agglutinin, all purchased from Vector Laboratories (Burlingame, CA, USA). The lectin-agarose together with any bound proteins was then pelleted and washed three times with cold Buffer A, protein was eluted by boiling in 30 μL of ×2 SDS–PAGE sample buffer and the samples were subjected to SDS–PAGE. Coomassie Blue-stained gel bands were excised, washed, in-gel digested with trypsin and subjected to MALDI-TOF mass spectrometry (34) using either a Voyager-DE STR mass spectrometer (Perseptive Biosystems, League City, TX, USA) or a TofSpec 2E (Micromass UK). Database searches using peptide masses were performed with the Mascot program (http://www.matrixscience.com).

Clathrin-coated vesicles were also isolated from HeLa cells, using the method of Hirst *et al.* (12). Western blots of whole cell homogenates and of isolated CCVs were probed with antibodies against EF-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), clathrin heavy chain (35), PACS-1 (a kind gift from Colin Crump and Gary Thomas, Vollum Institute) and CD8 (Santa Cruz Biotechnology), followed by a rabbit antibody linker if appropriate and then by $^{125}$I-protein A, as previously described (36).

### *Construction and localization of the constructs*

EST cDNA clones encoding CPD (Clone ID 2303562) and LRP1 (Clone ID 2324562) were obtained from the IMAGE Consortium. Primers were designed to polymerase chain reaction (PCR) through the region encoding the cytoplasmic tails of the respective proteins, incorporating an AflII site at the 5′ end. PCR products were ligated to the AflII site at the end of the transmembrane domain coding sequence of CD8 in pBluescript (a kind gift from Gudrun Ihrke, University of Cambridge) (37), and the resulting chimeras were cloned into pIRESNeo2 (BD Bioscience, Oxford, UK). The constructs were sequenced to confirm that a correct in-frame fusion had been achieved, then transfected into HeLa cells. Stably expressing cells were selected and maintained in the presence of 500 μg/mL G418 (Invitrogen, Paisley, UK). The CD8-expressing and CD8-CIMPR-expressing HeLa cell lines were generous gifts from Matthew Seaman (Cambridge Institute for Medical Research, Cambridge, UK) (16). Point mutations were made using a QuikChange II Site-Directed Mutagenesis Kit (Stratagene Corporation, La Jolla, CA, USA).

The constructs were localized by immunofluorescence and immunogold EM. Antibodies used for immunofluorescence included affinity-purified rabbit anticlathrin heavy chain (35), mouse monoclonal anti-CD8 (Ancell Corporation, Bayport, MN, USA) and sheep anti-TGN46 (Serotec Inc., Oxford, UK). Secondary antibodies were purchased from Molecular Probes. For some experiments, endosomes were labeled using ligand uptake assays. Cells were incubated either with rhodamine-conjugated WGA (Vector Laboratories), diluted 1:5000 for 15 min at 37 °C, or with Texas Red-conjugated EGF (Molecular Probes, Inc.), diluted 1:250 for 30 min at 37 °C. Cells were viewed using a Zeiss Axiophot fluorescence microscope equipped with a CCD camera (Princeton Scientific Instruments, Monmouth Junction, NJ, USA), and photographs were recorded using IP Laboratories software and then moved into Adobe Photoshop. For immunogold localization of the CD8 chimeras, cells were fixed for 1 h with 4% paraformaldehyde, 0.1% glutaraldehyde, 2% acryline in 0.1 M sodium cacodylate, pH 7.2, at room temperature, pelleted and embedded in gelatin. The cells were then prepared for ultrastructural immunocytochemistry as previously described (36), using rabbit anti-CD8 (a kind gift from Stefano Bonatti, Universita' di Napoli Federico II, Naples, Italy) (38) followed by protein A coupled to 15 nm gold. Sections were observed in a Philips CM100 transmission electron microscope.

### *Assays for sorting*

siRNA knockdowns were carried out as previously described (7,12) using oligos μ2-2 for AP-2, chc2-2 for clathrin heavy chain and μ1A for AP-1. Two new siRNAs were synthesized for PACS-1, with the sequences GACGAAGAUCUCCGGAAAG (PACS-1 siRNA1) and AGCAUCCUCAGCACGCCAA (PACS-1 siRNA2). Surface expression of CD8 chimeras was assayed by binding anti-CD8 (Ancell Corporation), followed by $^{125}$I-protein A, to cells incubated at 4 °C, as previously described (39). Internalization of prebound antibody-protein A complex was assayed by warming the cells to 37 °C for various lengths of time, then stripping off surface-bound label by acid washing and quantifying counts in the medium, associated with the cell surface and inside the cells (7). A flow cytometry-based assay was also used to monitor internalization of CD8 chimeras. Cells were incubated with Cy5-labeled monoclonal anti-CD8 (1:20 dilution; Serotec Inc.) either at 4 °C for 45 min or at 37 °C for 15 min, then washed, trypsinized, fixed and analyzed on a FACSCalibur flow cytometer, using FCS Press software.

## Acknowledgments

We thank Matthew Seaman for the cell lines expressing CD8 and CD8-CIMPR, Sew Peak Chew for help with some of the early mass spectrometry, Stefano Bonatti for rabbit anti-CD8, Colin Crump and Gary Thomas for anti-PACS-1, Paul MacAry and Lidia Duncan for help and advice with the flow cytometry, and Paul Luzio, Paul Lehner, John Kilmartin, Matthew Seaman, David Owen and members of the Robinson lab for reading the manuscript and for helpful discussions.

This work was supported by grants from the Wellcome Trust and the Medical Research Council.

## References


1. Owen DJ, Collins BM, Evans PR. Adaptors for clathrin coats: structure and function. Annu Rev Cell Dev Biol 2004;20:153–191.
2. Robinson MS. Adaptable adaptors for coated vesicles. Trends Cell Biol 2004;12:695–704.
3. Ohno H, Stewart J, Fournier MC, Bosshart H, Rhee I, Miyatake S, Saito T, Gallusser A, Kirchhausen T, Bonifacino JS. Interaction of tyrosine-based sorting signals with clathrin-associated proteins. Science 1995;269:1872–1875.
4. Owen DJ, Evans PR. A structural explanation for the recognition of tyrosine-based endocytic signals. Science 1998;282:1327–1332.
5. Janvier K, Kato Y, Boehm M, Rose JR, Martina JA, Kim BY, Venkatesan S, Bonifacino JS. Recognition of dileucine-based sorting signals from HIV-1 Nef and LIMP II by the AP-1 gamma-sigma1 and AP-3 delta-sigma3 hemicomplexes. J Cell Biol 2003;163:1281–1290.
6. Pauloin A, Tooze SA, Michelutti I, Delpal S, Ollivier-Bousquet M. The majority of clathrin coated vesicles from lactating rabbit mammary gland arises from the secretory pathway. J Cell Sci 1999;112:4089–4100.
7. Motley A, Bright NA, Seaman MNJ, Robinson MS. Clathrin-mediated endocytosis in AP-2-depleted cells. J Cell Biol 2003;162:909–918.
8. Höning S, Ricotta D, Krauss M, Späte K, Spolaore B, Motley A, Robinson M, Robinson C, Haucke V, Owen DJ. Phosphatidylinositol-(4,5)-bisphosphate regulates sorting signal recognition by the clathrin–associated adaptor complex AP−2. Mol Cell 2005;18:619–531.
9. Bonifacino JS, Traub LM. Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu Rev Biochem 2003;72:395–447.
10. Chen H, De Camilli P. The association of epsin with ubiquitinated cargo along the endocytic pathway is negatively regulated by its interaction with clathrin. Proc Natl Acad Sci USA 2005;102:2766–2771.
11. Sigismund S, Woelk T, Puri C, Maspero E, Tacchetti C, Transidico P, Di Fiore PP, Polo S. Clathrin-independent endocytosis of ubiquitinated cargos. Proc Natl Acad Sci USA 2005;102:2760–2765.
12. Hirst J, Miller SE, Taylor MJ, von Mollard GF, Robinson MS. EpsinR is an adaptor for the SNARE protein vti1b. Mol Biol Cell 2004;15:5593–5602.
13. Blondeau F, Ritter B, Allaire PD, Wasiak S, Girard M, Hussain NK, Angers A, Legendre-Guillemin V, Roy L, Boismenu D, Kearney RE, Bell AW, Bergeron JJ, McPherson PS. Tandem MS analysis of brain clathrin-coated vesicles reveals their critical involvement in synaptic vesicle recycling. Proc Natl Acad Sci USA 2004;101:3833–3838.
14. Li Y, Marzolo MP, van Kerkhof P, Strous GJ, Bu G. The YXXL motif, but not the two NPXY motifs, serves as the dominant endocytosis signal for low density lipoprotein receptor-related protein. J Biol Chem 2000;275:17187–17194.
15. Dintzis SM, Velculescu VE, Pfeffer SR. Receptor extracellular domains may contain trafficking information. Studies of the 300-kDa mannose 6-phosphate receptor. J Biol Chem 1994;269:12159–12166.
16. Seaman MNJ. Cargo-selective endosomal sorting for retrieval to the Golgi requires retromer. J Cell Biol 2004;165:111–122.
17. Eng FJ, Varlamov O, Fricker LD. Sequences within the cytoplasmic domain of gp180/carboxypeptidase D mediate localization to the trans-Golgi network. Mol Biol Cell 1999;10:35–46.
18. Kalinina E, Varlamov O, Fricker LD. Analysis of the carboxypeptidase D cytoplasmic domain: Implications in intracellular trafficking. J Cell Biochem 2002;85:101–111.
19. Rous BA, Reaves BJ, Ihrke G, Briggs JA, Gray SR, Stephens DJ, Banting G, Luzio JP. Role of adaptor complex AP-3 in targeting wild-type and mutated CD63 to lysosomes. Mol Biol Cell 2002;13:1071–1082.
20. Wan L, Molloy SS, Thomas L, Liu G, Xiang Y, Rybak SL, Thomas G. PACS-1 defines a novel gene family of cytosolic sorting proteins required for trans-Golgi network localization. Cell 1998;94:205–216.
21. Crump CM, Xiang Y, Thomas L, Gu F, Austin C, Tooze SA, Thomas G. PACS-1 binding to adaptors is required for acidic cluster motif-mediated protein traffic. EMBO J 2001;20:2191–2201.
22. Herz J, Strickland DK. LRP: a multifunctional scavenger and signaling receptor. J Clin Invest 2001;108:779–784.
23. Chen WJ, Goldstein JL, Brown MS. NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. J Biol Chem 1990;265:3116–3123.
24. Puertollano R, Aguilar RC, Gorshkova I, Crouch RJ, Bonifacino JS. Sorting of mannose 6-phosphate receptors mediated by the GGAs. Science 2001;292:1663–1665.
25. Chen HJ, Yuan J, Lobel P. Systematic mutational analysis of the cation-independent mannose 6-phosphate/insulin-like growth factor II receptor cytoplasmic domain. An acidic cluster containing a key aspartate is important for function in lysosomal enzyme sorting. J Biol Chem 1997;272:7003–7012.
26. Lobel P, Fujimoto K, Ye RD, Griffiths G, Kornfeld S. Mutations in the cytoplasmic domain of the 275 kd mannose 6-phosphate receptor differentially alter lysosomal enzyme sorting and endocytosis. Cell 1989;57:787–796.
27. Varlamov O, Fricker LD. Intracellular trafficking of metallocarboxypeptidase D in AtT-20 cells: localization to the trans-Golgi network and recycling from the cell surface. J Cell Sci 1998;111:877–885.
28. Conner SD, Schmid SL. Differential requirements for AP-2 in clathrin-mediated endocytosis. J Cell Biol 2003;162:773–779.
29. Ehrlich M, Boll W, Van Oijen A, Hariharan R, Chandran K, Nibert ML, Kirchhausen T. Endocytosis by random initiation and stabilization of clathrin-coated pits. Cell 2004;118:591–605.
30. Pearse BMF. On the structural and functional components of coated vesicles. J Mol Biol 1978;126:803–812.
31. Miller EA, Beilharz TH, Malkus PN, Lee MC, Hamamoto S, Orci L, Schekman R. Multiple cargo binding sites on the COPII subunit Sec24p ensure capture of diverse membrane proteins into transport vesicles. Cell 2003;114:497–509.
32. Pilch PF, Shia MA, Benson RJ, Fine RE. Coated vesicles participate in the receptor-mediated endocytosis of insulin. J Cell Biol 1983;96:133–138.
33. Manfredi JJ, Bazari WL. Purification and characterisation of two distinct complexes of assembly polypeptides from calf brain coated vesicles that differ in their polypeptide composition and kinase activities. J Biol Chem 1987;262:12182–12188.
34. Jensen ON, Podtelejnikov AV, Mann M. Identification of the components of simple protein mixtures by high-accuracy peptide mass mapping and database searching. Anal Chem 1997;69:4741–4750.
35. Simpson F, Bright NA, West MA, Newman LS, Darnell RB, Robinson MS. A novel adaptor-related protein complex. J Cell Biol 1996;133:749–760.
36. Hirst J, Lui WWY, Bright NA, Totty N, Seaman MNJ, Robinson MS. A family of proteins with γ-adaptin and VHS domains that facilitate trafficking between the TGN and the vacuole/lysosome. J Cell Biol 2000;149:67–79.
37. Ihrke G, Bruns JR, Luzio JP, Weisz OA. Competing sorting signals guide endolyn along a novel route to lysosomes in MDCK cells. EMBO J 2001;20:6256–6264.
38. Lotti LV, Mottola G, Torrisi MR, Bonatti S. A different intracellular distribution of a single reporter protein is determined at steady state by KKXX or KDEL retrieval signals. J Biol Chem 1999;274:10413–10420.
39. Hirst J, Borner GHH, Harbour M, Robinson MS. The aftiphilin/p200/gamma-synergin complex. Mol Biol Cell 2005;16:2554–2565.