Clathrin-mediated endocytosis in AP-2–depleted cells

Alison Motley, Nicholas A. Bright, Matthew N.J. Seaman, and Margaret S. Robinson

University of Cambridge, Department of Clinical Biochemistry, Cambridge Institute for Medical Research (CIMR), Cambridge CB2 2XY, UK

We have used RNA interference to knock down the AP-2 μ2 subunit and clathrin heavy chain to undetectable levels in HeLaM cells. Clathrin-coated pits associated with the plasma membrane were still present in the AP-2–depleted cells, but they were 12-fold less abundant than in control cells. No clathrin-coated pits or vesicles could be detected in the clathrin-depleted cells, and post-Golgi membrane compartments were swollen. Receptor-mediated endocytosis of transferrin was severely inhibited in both clathrin- and AP-2–depleted cells. Endocytosis of EGF, and of an LDL receptor chimera, were also inhibited in the clathrin-depleted cells; however, both were internalized as efficiently in the AP-2–depleted cells as in control cells. These results indicate that AP-2 is not essential for clathrin-coated vesicle formation at the plasma membrane, but that it is one of several endocytic adaptors required for the uptake of certain cargo proteins including the transferrin receptor. Uptake of the EGF and LDL receptors may be facilitated by alternative adaptors.

Introduction

The AP-2 adaptor complex has long been assumed to be essential for clathrin-mediated endocytosis. It is a major component of purified clathrin-coated vesicles, second in abundance only to clathrin itself in most coated vesicle preparations. It has been shown to have binding sites not only for clathrin, but also for cargo proteins and for phospholipids. The prevailing view is that clathrin-mediated endocytosis is initiated by the recruitment of AP-2 onto the plasma membrane. Once on the plasma membrane, AP-2 interacts with sorting signals in the cytoplasmic domains of membrane proteins destined to become cargo in the coated vesicle. At the same time, AP-2 recruits clathrin onto the membrane, where it acts as a scaffold for vesicle budding. Recently, a number of accessory proteins have been identified that also participate in clathrin-mediated endocytosis, all of which bind either directly or indirectly to subunits of the AP-2 complex. These proteins together form an interconnected network, with AP-2 believed to be the central component (for review see Conner and Schmid, 2003a).

Although most of the available data are consistent with this model, there are a few discrepancies. First, the budding yeast Saccharomyces cerevisiae has an AP-2–related complex, AP-2R; however, deleting genes encoding AP-2R subunits has no apparent effect on clathrin-mediated endocytosis, or indeed, on any other pathway that has been investigated (Yeung et al., 1999). A caveat here is that AP-2R may not be functionally equivalent to AP-2 in higher cells. Second, some of the accessory proteins that bind to AP-2 have properties suggesting that they may be adaptors in their own right. Epsin, β-arrestin, AP180/CALM, Hip1, Dab2, and ARH have all been shown to bind not only to AP-2, but also to PIP2 and to clathrin, indicating that they may be able to bind to the plasma membrane and to recruit clathrin in an AP-2–independent manner (Gaidarov et al., 1999; Ford et al., 2001, 2002; Mishra et al., 2001, 2002a,b). There is also some evidence that each of these proteins may interact with a specific type of cargo. For instance, epsin has ubiquitin-interacting motifs that may help to facilitate the internalization of plasma membrane proteins like the EGF receptor, which is ubiquitinated in response to ligand binding. Dab2 and ARH are able to bind NPXY motifs, found in members of the LDL receptor family (for review see Bonifacino and Traub, 2003). Thus, an alternative view is that AP-2 may be just one of several endocytic adaptors, and that although it participates in the network of protein–protein interactions and is required for the uptake of certain types of cargo, it is not essential for clathrin-mediated endocytosis.

We asked the question, what would happen if AP-2 were to drop out of the network? Would clathrin and accessory proteins still be recruited onto the plasma membrane? What would happen to receptor-mediated endocytosis? Us-
ing small interfering RNAs (siRNAs), we have been able to knock down the expression of the µ2 subunit of the AP-2 complex to undetectable levels in HeLaM cells. Then, we compared the phenotype of the AP-2–depleted cells with that of cells treated with siRNAs directed against clathrin heavy chain.

Results
Depletion of AP-2 and clathrin by RNA interference
To find the most effective conditions for depleting AP-2 and clathrin, a number of siRNAs were synthesized, and both single and double transfections were performed. We found that siRNAs µ2-2 and chc-2 (see Materials and methods), directed against the AP-2 µ2 subunit and clathrin heavy chain, respectively, produced the most complete disruption, and that in both cases it was best to transfect the cells twice, with a 48-h interval in between. An siRNA directed against the AP-2 α subunit (α-2) also worked well, but not quite as effectively as µ2-2 or chc-2. Fig. 1 shows Western blots of equal protein loadings of control and siRNA-treated cells two days after the second transfection. The signals from both µ2 and clathrin heavy chain are undetectable after knockdown, whereas a weak signal (<5% of control) could be detected in the α-2–treated cells.

Although the µ2 and α siRNAs only directly affect the expression of the subunit against which they were targeted, Fig. 1 b shows that levels of the other subunit were also reduced. In contrast, depleting AP-2 subunits has no effect on the amount of clathrin in the cell, and clathrin depletion does not affect the levels of AP-2 (Fig. 1 b). We also investigated whether depleting µ2 affects the proportion of soluble versus membrane-associated α, by centrifuging cell homogenates at high speed and then probing Western blots of the supernatant and pellet. Fig. 1 c shows that normally, most of the α is in the pellet, but depleting µ2 causes some of the protein to shift into the supernatant. These results are consistent with analyses of the AP-1 and AP-3 complexes, which show that deleting any one of the subunits decreases the stability of the remaining subunits, and that the partial complexes that form in the absence of a particular subunit are inactive and fail to localize to membranes (Meyer et al., 2000; Peden et al., 2002).

Both AP-2 and clathrin depletion were found to slow down the growth of the cells, but we did not see any apparent increase in apoptosis (unpublished data), although clathrin loss has been reported to cause apoptosis in a chicken B cell line (Wetten et al., 2002). By phase-contrast microscopy, the µ2-2–treated cells look essentially normal (compare Fig. 1 e with the control cells in Fig. 1 d). However, many of the chc-2–treated cells were found to be heavily vacuolated, and nearly half of the cells had two or more nuclei (Fig. 1 f), indicating that cytokinesis is blocked in clathrin-depleted cells. Interestingly, a similar phenotype has been reported in clathrin-deficient Dictyostelium (Niswonger and O’Halloran, 1997).

Fig. 1 (g–n) shows the appearance of the cells by immunofluorescence. In g–k, either control cells (g) or AP-2 knockdown cells (h–k) were labeled with an antibody against the AP-2 α subunit. After a single transfection, the α-adaptin labeling was found to be very patchy in both α-2–treated (h) and µ2–2–treated (i) cells; individual spots were of approximately equal intensity to those in control cells, but much fewer in number and usually occurring in clusters. After two transfections (j and k), membrane-associated labeling was essentially undetectable in the µ2-2–treated cells, although cytosolic labeling of partial complexes containing the α subunit could be seen (k). The α-2 knockdown (j) was not quite so complete in that some of the cells still had residual spots after the second transfection. Clathrin knockdowns (l–n) caused a more uniform loss of signal. After a single transfection (m), the number of clathrin-positive spots per cell was similar to controls (l), but the intensity of the spots was reduced. After two transfections, clathrin labeling was essentially undetectable (n). In all three cases, virtually 100% of the cells were affected by the end of the full course of treatment.

Localization of other proteins in the siRNA-treated cells
To find out what happens to other coat proteins in cells where one of them has been depleted, we performed triple labeling on cells treated with both µ2-2 and chc-2 siRNAs. For these experiments, we plated out equal numbers of control and siRNA-treated cells onto microscope slides so the two types of cells could be viewed together in the same field, and then labeled the cells with antibodies against α-adaptin (blue), clathrin heavy chain (red), and epsin 1 (green).

Fig. 2 shows that normally, the three proteins have overlapping distributions at the plasma membrane, with additional intracellular structures labeled with the clathrin antibody. Knocking down µ2 causes a reduction in the number of epsin spots (c). These spots do not contain any detectable α-adaptin, which is now diffuse and cytosolic (a). However, many of the epsin-positive spots in these cells are also positive for clathrin (b; insets), suggesting that the two proteins are able to co-assemble on the plasma membrane even in the absence of AP-2.

In cells where clathrin had been depleted, AP-2 labeling was not markedly different from control cells (d). There were still multiple spots associated with the plasma membrane, often occurring in rows. These spots were also positive for epsin (f). However, the epsin labeling was consistently brighter in clathrin-depleted cells, indicating that more of the protein was associated with the plasma membrane. This was confirmed by Western blotting of cell homogenates centrifuged at high speed; in control and AP-2–depleted cells, epsin partitioned approximately equally between supernatant and pellet, whereas in clathrin-depleted cells, it was found almost exclusively in the pellet (unpublished data).

Ultrastructure of the cells
To observe the phenotype of AP-2– and clathrin-depleted cells at the ultrastructural level, EM was performed. Fig. 3 (a and b) shows that control cells contain numerous clathrin-coated pits associated with the plasma membrane, indicated by the large arrowheads. A morphometric analysis (Fig. 4) showed that in these cells, 0.6% of the cell surface is occupied by clathrin-coated pits. In the AP-2–depleted cells (c–e), clathrin-coated pits were found to be 12-fold less abundant, occupying only 0.05% of the cell surface. The morphology of the coat appears to be identical in control...
Figure 1. Effects of depleting AP-2 and clathrin heavy chain from HeLaM cells. (a) Equal protein loadings of homogenates of either control cells or cells treated with siRNAs directed against clathrin heavy chain, α-adaptin, or μ2 were subjected to SDS-PAGE, and Western blots were probed with antibodies against the indicated protein. Clathrin heavy chain and the AP-2 μ2 subunit were both undetectable after knockdown, whereas a weak signal (<5% of control) was detected after α knockdown. (b) Equal protein loadings of homogenates from control and siRNA-treated cells were subjected to SDS-PAGE, and Western blots were cut in four and probed with the indicated antibody. Anti-actin was included as a loading control. As well as affecting its target, the α siRNA causes a depletion in μ2, and the μ2 siRNA causes a depletion in α. (c) Homogenates of control and μ2-depleted cells were centrifuged at high speed, and supernatants and pellets were probed with anti-α. Knocking down μ2 increases the percentage of α in the supernatant. (d–f) Phase-contrast micrographs of cells treated with a control (nonfunctional) siRNA (d), cells treated with μ2 siRNA (e), and cells treated with clathrin heavy chain siRNA (f). The μ2 siRNA-treated cells look essentially normal. However, many of the clathrin heavy chain siRNA-treated cells are vacuolated, and nearly half are multinucleated. (g–k) Control cells (g), cells treated once with α (h) and μ2 (i) siRNAs, and cells treated twice with α (j) and μ2 (k) siRNAs were labeled with an antibody against the AP-2 α subunit. The labeling becomes patchy after one hit, and after both hits there is little or no label associated with the plasma membrane. (l–n) Control cells (l) and cells treated once (m) or twice (n) with a clathrin heavy chain siRNA were labeled with an antibody against clathrin. The signal disappears more uniformly than the AP-2 signal, again becoming undetectable on membranes after two hits. Bars, 20 μm.
and AP-2–depleted cells; however, the coated pits tend to be smaller in the AP-2–depleted cells. In the clathrin-depleted cells, clathrin-coated pits were undetectable, and nearly all of the budding profiles that could be observed at the plasma membrane had the characteristic appearance of caveolae.

We also examined intracellular membranes in the control and knockdown cells. Fig. 3f shows the Golgi region of an AP-2–depleted cell, with the Golgi stack indicated (G). A clathrin-coated vesicle (arrow) can be seen budding from a membrane on the trans side of the stack, presumably the TGN. Control cells have a similar Golgi morphology (unpublished data). However, in clathrin-depleted cells, there is a striking amount of swelling of post-Golgi compartments (Fig. 3g), presumably because membrane is unable to leave by the clathrin-coated vesicle route.

**Effects on clathrin-mediated endocytosis: transferrin receptor**

It has been well documented that AP-2 is required for the uptake of cargo proteins with YXXΦ-type sorting signals, such as
the transferrin receptor. This motif has been shown to bind to hydrophobic pockets in the \( \mu 2 \) subunit (Ohno et al., 1995; Owen and Evans, 1998). Mutating the signal in the transferrin receptor prevents the receptor from entering the cell by clathrin-mediated endocytosis, causing it to be internalized 5–10 times more slowly (Jing et al., 1990). Similarly, mutating the \( \mu 2 \) subunit so that it can no longer bind the motif, and then overexpressing it in cells so that most of the AP-2 complexes contain mutant rather than wild-type \( \mu 2 \), also strongly inhibits uptake of the transferrin receptor (Nesterov et al., 1999).

Fig. 5a shows the effects of AP-2 and clathrin knockdown on transferrin receptor endocytosis. For these experiments, the cells were incubated at 4°C with \(^{125}\)I-labeled transferrin to allow binding (but not internalization) to occur, and then were warmed to 37°C for various lengths of time. At the end of the incubation, the medium was harvested, surface-bound ligand was stripped off at low pH, the cells were solubilized in 1 M NaOH, and the label in all three fractions was quantified. The percentage of counts in the NaOH extract (i.e., intracellular counts) is shown in the graph. In control cells, \( >50\% \) of the prebound transferrin is internalized within 5 min after warm-up, but after 10 min, the amount of intracellular transferrin starts to go down, as it gets recycled back into the medium. Knocking down both AP-2 and clathrin

---

**Figure 3. Electron micrographs of control and siRNA-treated cells.**

The cells in these experiments were incubated with an antibody against the transferrin receptor coupled to 8-nm gold before fixation to monitor the efficiency of transferrin receptor endocytosis. (a and b) Control cells have abundant clathrin-coated pits (large arrowheads) associated with the plasma membrane. Gold particles (small arrowheads) can be seen in endosomes. (c–f) Cells were treated with \( \mu 2 \)-2 siRNA. These cells still have clathrin-coated pits associated with the plasma membrane, but when the pits were quantified by morphometry in control and AP-2–depleted cells, they were found to be 12-fold less abundant in the AP-2–depleted cells. Morphologically, the coated pits are similar to those in control cells, but they tend to be smaller. Gold particles (small arrowheads) remain on the cell surface. A clathrin-coated bud or vesicle in the Golgi region (G) is indicated with the arrow in f. (g) Clathrin-depleted cells have no recognizable clathrin-coated pits or vesicles, either at the plasma membrane or on intracellular membranes, although COP-coated vesicles can still be seen on the cis side of the Golgi stack. In addition, membranes on the trans side of the Golgi stack are swollen. Bar, 500 nm.
causes a profound inhibition of transferrin uptake. In both cases, the transferrin never accumulates inside the cells, but remains primarily on the cell surface, where it slowly dissociates into the medium. Even after 30 min, <10% of the transferrin is recovered in the intracellular fraction. Thus, as expected, both AP-2 and clathrin are required for efficient internalization of the transferrin receptor.

**EGF receptor**

The behavior of the EGF receptor was less predictable. This receptor has a YXXΦ consensus sequence in its cytoplasmic tail, which binds to μ2 in vitro (Owen and Evans, 1998), and which acts as an internalization signal when transplanted onto other proteins (Chang et al., 1993). Surprisingly, however, mutating this sequence does not change the internalization rate of the receptor (Nesterov et al., 1995; Sorkin et al., 1996). In addition, EGF receptor endocytosis was found to proceed normally in cells expressing mutant μ2 (Nesterov et al., 1999). These observations indicate that the receptor must have another signal (or signals) for endocytosis, and there is increasing evidence that ubiquitin may be that signal (Haglund et al., 2003). However, it has not yet been tested whether EGF internalization is really independent of AP-2, because the cells expressing mutant μ2 still contained normal levels of AP-2, which was recruited normally onto the plasma membrane (Nesterov et al., 1999). Thus, it is possible that the EGF receptor may bind to another part of AP-2, and/or that AP-2 may be necessary for coat assembly.

Fig. 5 b shows a similar experiment to the one shown in Fig. 5 a, but using 125I-labeled EGF as the ligand. In control cells, EGF is internalized slightly more slowly than transferrin, but by 10 min, >50% of the label is intracellular.
Knocking down clathrin strongly inhibits the receptor-mediated endocytosis of EGF. Strikingly, however, knocking down AP-2 has no significant effect on EGF uptake. The rate of accumulation of intracellular EGF is virtually identical in control and AP-2–depleted cells.

**LDL receptor**

There is currently some controversy as to how the LDL receptor’s internalization signal actually works. This signal has been reported to bind to several coat components, including the μ2 subunit of AP-2 (Boll et al., 2002), clathrin (Kibbey et al., 1998), and proteins with phosphotyrosine-binding domains like Dab2 and ARH (Mishra et al., 2002a,b). Initially, we attempted to monitor receptor internalization using 125I-labeled LDL, but these experiments were complicated by the very rapid dissociation of the ligand from its receptor in HeLa cells (see Materials and methods). Therefore, we stably transfected the cells with a construct consisting of the extracellular and transmembrane domains of CD8 fused to the LDL receptor tail, then assayed for internalization following a similar protocol to the one just mentioned, but with anti-CD8 followed by 125I-labeled protein A as the ligand. Fig. 5c shows that in control cells, the LDL receptor chimera is efficiently internalized, and that knocking down clathrin strongly inhibits uptake. However, knocking down AP-2 again has no effect on the rate of endocytosis of the protein. Thus, out of the three transmembrane proteins that we have analyzed in siRNA-treated HeLa cells, two are internalized in an AP-2–independent manner.

**Discussion**

In this work, we have knocked down both clathrin heavy chain and the AP-2 μ2 subunit to undetectable levels using siRNAs. Remarkably, we find that clathrin-mediated endocytosis can still occur in the absence of any detectable AP-2 at the plasma membrane. Although we cannot rule out the possibility that trace amounts of AP-2 may contribute to endocytosis in the siRNA-treated cells, it is clear that AP-2 is not an essential structural component of the coat, as had always been assumed. A similar conclusion has been reached by Conner and Schmid (2003b, in this issue), who used a different approach to inactivate AP-2, and also found that effects on clathrin-mediated endocytosis were cargo-specific. This means that there must be other proteins in the cell that can fulfill the role that had always been assigned to AP-2. These proteins cannot completely replace AP-2, because the number of coated pits per cell is reduced by 12-fold in AP-2–depleted cells, and clathrin-mediated endocytosis of the transferrin receptor is abolished. In addition, others have shown that mutations in the AP-2 μ subunit in *Drosophila* cause either embryonic lethality or neurological defects, depending on the allele (Gonzalez-Gaitan and Jackle, 1997). However, we find that both the EGF receptor and the LDL receptor chimera are internalized just as efficiently in AP-2–depleted cells as in control cells, presumably by making use of alternative adaptors.

Over the last several years, a number of reports have been published that challenge the view that all cargo proteins are endocytosed by the same molecular mechanism. Marks et al. (1996) showed that overexpression of constructs with either YXXΦ or dileucine internalization signals causes endogenous proteins with the same signal to accumulate on the cell surface, but endogenous proteins with the other signal are still endocytosed normally. They concluded that the two signals were competing for two distinct components of the endocytic machinery. However, they could not rule out the possibility that both of these components were part of the AP-2 complex, and indeed, subsequent reports showed that dileucine signals can also bind to AP complexes, most likely through their β subunits (for review see Bonifacino and Traub, 2003). Warren et al. (1998) used a similar approach to show that the transferrin, EGF, and LDL receptors do not compete with each other for internalization. They suggested that although AP-2 interactions might be necessary for the internalization of some cargo proteins, other cargo proteins could be interacting with other components of the coat. However, until now, it has not been possible to test these ideas by specifically depleting different coat components from the cell.

The approach that we have used in the present paper should be widely applicable for looking at the internalization of other transmembrane proteins, to determine whether or not their endocytosis is dependent on AP-2. So far, most functional analyses of the roles of various proteins in clathrin-mediated endocytosis have made use of dominant-negative mutants (for review see Conner and Schmid, 2003a), but these may lead to indirect effects (e.g., overexpressing a truncated version of a particular protein may impede interactions involving other components of the coated pit). RNA interference is a much cleaner way of testing the function of a protein because it removes the protein instead of altering it and adding it back in excess amounts. The CD8 chimera system may be especially useful, because theoretically, one could transplant the cytoplasmic tail from any type I membrane protein, or an artificial tail designed to test a potential internalization signal, onto the CD8 reporter, and then assay for uptake using reagents that are commercially available.

Fig. 6 is a schematic diagram summarizing our results and how we interpret them. Normally, clathrin, AP-2, and alternative adaptors all co-assemble at the plasma membrane, bringing cargo with different types of internalization signals into the coated pit. In the absence of AP-2, alternative adaptors are still recruited onto the plasma membrane, where they interact with a subset of the cargo proteins, including the EGF and LDL receptors, and co-assemble with clathrin. These cargo proteins are still efficiently internalized, but cargo proteins like the transferrin receptor, which can only interact with AP-2, stay on the cell surface. In addition, because AP-2 is the major clathrin adaptor at the plasma membrane, there is less clathrin recruited onto the membrane and fewer (and perhaps smaller) coated pits per cell. In the absence of clathrin, both AP-2 and alternative adaptors are still recruited onto the plasma membrane, where they interact with each other and most likely with potential cargo proteins as well, but there are no morphologically recognizable coated pits, and all of the cargo proteins are internalized much more slowly.

Several recent papers provide insights into the identity of these alternative adaptors. In particular, Traub and col-
leagues have shown that a number of the endocytic accessory proteins, including epsin, Hip1, Dab2, and ARH, can bind to plasma membrane lipids and to clathrin, and they have proposed that these proteins may, in fact, be cargo-selective clathrin adaptors (Mishra et al., 2001, 2002a,b). However, they hypothesize that the role of these proteins is to “potentiate AP-2-driven coat formation” (Mishra et al., 2002a), whereas the present paper suggests that alternative adaptors may, in fact, be just as capable of driving coat formation as AP-2. This makes mammalian cells much more similar to yeast in this regard than had previously been suspected. *S. cerevisiae* can still carry out most of its clathrin-mediated trafficking even when all three of its AP complexes are absent (Huang et al., 1999; Yeung et al., 1999), presumably because most of its cargo proteins do not depend on YXXΦ motifs. Ubiquitin appears to be the major endocytic signal in yeast, and there is some evidence that it is recognized by the epsin orthologues Ent1p and Ent2p (Shih et al., 2002; Aguilera et al., 2003). Mammalian epsins may play a similar role, capturing ubiquitinated cargo proteins like the activated EGF receptor. ARH and Dab2 are both good candidates for cargo-specific adaptors for the LDL receptor, and may be functionally redundant because mutations in either gene alone do not block LDL uptake (Mishra et al., 2002b). By using RNA interference to deplete these and other candidate adaptors from cells (either alone or in combination) and then monitoring the internalization of different membrane proteins, it should be possible to match each of the alternative adaptors with its respective cargo.

**Materials and methods**

**siRNA knockdowns**

HeLaM cells (Tiwari et al., 1987) were seeded at a density of 10⁶ cells per 9-cm dish. At least 2 h after seeding the cells, the first transfection was performed. For each 9-cm dish, 50 µl OligofectAMINE™ (Invitrogen) was added to 100 µl Opti-MEM® I (Life Technologies), and the solution was incubated at RT for 5–10 min. This was then added to a second solution or 800 µl Opti-MEM® I plus 50 µl 20 µM siRNA, and the mixture was incubated at RT for 15–20 min. Next, 4 ml Opti-MEM® I was added to the siRNA mixture to make a final volume of 5 ml, and this was added to the cells after rinsing them once with Opti-MEM® I. The transfection mixture was left for 4 h on the cells, after which 5 ml DME containing 20% FCS without antibiotics was added, and the cells were left in this mixture until they were trypsinized the following day. Two transfections were performed, on d0 and d2. The cells were trypsinized 24 h after each transfection and were seeded into two 9-cm dishes for the second transfection, whereas after the second transfection, they were plated onto coverslips or 35-mm dishes for uptake assays. For each experiment, an extra 35-mm dish was used to assay efficiency of knockdown.

Two independent siRNAs were used to investigate the effects of knockdown of each target. For AP-2, the targets were the α and µ2 subunits of the complex. The α-2 siRNA target sequence was AAGAGCAUGUG-CAGCUGGCCCA and the µ-2 target sequence was AAGUGGGAUCCCGGUACUCGU (other sequences, designated α-1 and µ-2–1, were ineffective). The clathrin heavy chain target sequences were AAG-CUGGGAAAAACUCUUCAGA (chc-1) and UAAUCCAAUUGAGAGACC-CAUU (chc-2). The control siRNA was a nonfunctional oligo, µ2–1, originally designed to knock down the µ2 subunit, target sequence AACAG-CAACCUCUCUUACGG. All siRNAs were designed according to the manufacturer’s instructions and were synthesized as Option C siRNAs by Dharmacon, Inc.

**Immunofluorescence and Western blotting**

Antibodies used for immunofluorescence included an affinity-purified rabbit polyclonal antiserum directed against clathrin heavy chain (Simpson et al., 1996); a mouse monoclonal anti-α-adaptin, AP-6, provided by Frances Brodsky (University of California, San Francisco, San Francisco, CA); and a commercially available goat anti-epsin 1 (Santa Cruz Biotechnology, Inc.). Secondary antibodies were all purchased from Molecular Probes, Inc. For Western blotting, α and µ2 subunits of AP-2 were detected using mouse monoclonal antibodies from Transduction Laboratories, clathrin heavy chain was detected using the same rabbit antibody that was used for immunofluorescence, and actin was detected using a rabbit antibody from Sigma-Aldrich. Incubations with the mouse antibodies were followed by an incubation with rabbit anti–mouse immunoglobulin (DakoCytomation). The blots were then probed with 125I-labeled protein A (Amersham Biosciences).

**EM**

Cells to be used for EM were first fed with mouse anti-transferrin receptor B3/25 conjugated to 8-nm gold, a gift from Colin Hopkins and Liz Alichin (Imperial College London, London, UK). The cells were incubated with the antibody–gold conjugate for 30 min at 4°C, followed by a 10-min chase at 37°C. They were then fixed in tissue culture dishes by adding double-strength fixative (5% glutaraldehyde and 4% PFA in 0.2 M sodium cacodylate, pH 7.2, containing 6 mM CaCl₂) to an equal volume of tissue culture.
medium for 2 min at 37°C. This solution was aspirated off and replaced by 2.5% glutaraldehyde and 2% PFA in 0.1 M sodium cacodylate buffer, pH 7.2, containing 3 mM CaCl₂. The cells were fixed for a further 3 h at RT, washed with 0.1 M sodium cacodylate buffer, pH 7.2, and post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2, for 1 h. The cells were scraped from the dish and pelleted, and the pellet was washed with 0.05 M sodium maleate buffer, pH 5.2, and en bloc stained with 0.5% uranyl acetate in 0.05 M sodium maleate buffer. The cell pellets were dehydrated in ethanol, exchanged into 1:2-epoxy propane, and embedded in Araldite CY212 epoxy resin (Agar Scientific).

50-nm ultrathin sections were cut using a diamond knife mounted on an ultramicrotome (Reichert Ultratut 5; Leica), collected onto formvar-carbon-coated EM grids, and stained with uranyl acetate and Reynolds lead citrate (Reynolds, 1963). The sections were observed in a transmission electron microscope (model CM 100; Philips) at an operating voltage of 80 kV.

For quantification of the percentage of plasma membrane occupied by coated pits, cell pellets were randomly sectioned and oriented in the electron microscope. Grids were systematically scanned at a magnification of 13,300, and 128 images of each condition were captured using a camera (Megaview II TEM; Soft Imaging System). A 500-nm lattice overlay was used to score intersections with the plasma membrane and coated pits (Weibel, 1979; Griffiths, 1993) using analySIS® 3.0 image analytical software.

Internalization assays

Ligand uptake assays were performed on cells seeded onto 35-mm dishes on the day preceding the experiment. On the day of the experiment, the dishes were placed on ice and were washed briefly with prechilled serum-free medium (SFM; DME and 20 mM Hepes containing 1% BSA). The cells were then incubated on a rocker for 30 min at 4°C in 0.6 ml SFM containing either 125I-labeled EGF or 125I-labeled transferrin (both obtained from PerkinElmer) at a concentration of 500 nCi/ml. The dishes were washed six times with ice-cold SFM, and the surface counts were collected for the zero time point by incubating the cells for 5 min at 4°C in 0.8 ml ice-cold acid wash (0.2 M acetic acid and 0.5 M NaCl), followed by a brief rinse with another 0.8-ml acid wash. The other dishes were incubated with 1.5 ml prewarmed medium (DME containing 10% FCS) at 37°C for various times. Endocytosis was stopped by placing the cells on ice, collecting the medium, and rinsing the cells with ice-cold SFM. Label associated with the cell surface was then collected by acid wash, as above. Finally, the intracellular fraction was collected by extracting the cells twice with 0.8 ml 1 M NaOH. The radioactivity in the medium, acid wash, and NaOH extract was quantified using a gamma counter (Nuclear Enterprises).

We also attempted to follow the fate of 125I-labeled LDL, but found that >50% of the prebound ligand dissociated within the first few minutes of warm-up. Therefore, we made use of HeLaM cells expressing a chimera of the extracellular and transmembrane domain of CD8 fused to the tail domain of the LDL receptor. Human CD8 cDNA in pBluescript was a gift from Dr. Elizabeth VerBerkmoes (University of Cambridge, UK; Ihle et al., 2001). The cytoplasmic tail of the mouse LDL receptor (from the arginine residue at position 813) was amplified by PCR from an EST (Clone ID 2581960; GenBank/EMBL/DDBJ accession no. gi6519196) obtained from the I.M.A.G.E. Consortium, incorporating an AflII site into the 5'UTR of the gene. The construct was sequenced in yeast (Megaview II TEM; Soft Imaging System). A 500-nm lattice overlay was used to score intersections with the plasma membrane and coated pits (Weibel, 1979; Griffiths, 1993) using analySIS® 3.0 image analytical software.

This work was supported by grants from the Wellcome Trust (grant 053316/Z/98 to M.S. Robinson) and the Medical Research Council (grant G9310915 to M.S. Robinson and J.P. Luzio).

Accepted: 30 May 2003
Submitted: 24 July 2003

References

Ahlgren, R.C., H.A. Watson, and B. Wendland. 2003. The yeast Epsin ENV1 is required for membranes through multiple independent interactions. J. Biol. Chem. 278:10757–10763.

Boll, W., J. Rapoport, C. Brunner, Y. Modis, S. Prehn, and T. Kirchhausen. 2002. The mu2 subunit of the clathrin adaptor AP-2 binds to FDNPVPY and YppΦ sorting signals at distinct sites. Traffic. 3:590–600.

Bonifacino, J.S., and L.M. Traub. 2003. Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu. Rev. Biochem. In press.

Chang, C.P., C.S. Lazar, B.J. Walsh, M. Komuro, J.F. Collawn, L.A. Kuhn, J.A. Tainer, I.S. Trowbridge, M.G. Farquhar, M.G. Rosenfeld, et al. 1993. Ligand-induced internalization of the epidermal growth factor receptor is mediated by multiple endocytic codes analogous to the tyrosine motif found in constitutively internalized receptors. J. Biol. Chem. 268:19312–19320.

Conner, S.D., and S.L. Schmid. 2003a. Regulated portals of entry into the cell. Nature. 422:37–44.

Conner, S.D., and S.L. Schmid. 2003b. Differential requirements for AP2 in clathrin-mediated endocytosis. J. Cell Biol. 162:773–779.

Ford, M.G., B.M. Pease, M.K. Higgins, Y. Vallis, D.J. Owen, A. Gibson, C.R. Hopkins, P.R. Evans, and H.T. McMahon. 2001. Simultaneous binding of LDL and clathrin by AP180 in the nucleation of clathrin lattices on membranes. Science. 291:1051–1055.

Ford, M.G., I.G. Mills, B.J. Peter, Y. Vallis, G.J. Praefcke, P.R. Evans, and H.T. McMahon. 2002. Curvature of clathrin-coated pits driven by epsin. Nature. 419:361–366.

Gaidarov, I., J.G. Krupnick, J.R. Falck, J.L. Benovic, and J.H. Keen. 1999. Arres- tin function in G protein-coupled receptor endocytosis requires phosphoinoside binding. EMBO J. 18:871–881.

González-Gaitán, M., and H. Jackle. 1997. Role of Drosophila alpha-adaptin in pre-synaptic vesicle recycling. Cell. 88:767–776.

Griffiths, G. 1993. Fine Structure Immunocytochemistry. Springer-Verlag, Heidelberg, Germany. 459 pp.

Haglund, K., S. Sigismund, S. Polo, I. Szymkiewicz, P.P. Di Fiore, and I. Dikic. 2003. Multiple monoubiquitination of RTKs is sufficient for their endocyto- sis and degradation. Nat. Cell Biol. 5:461–466.

Huang, K.M., K. D’Hondt, H. Riezman, and S.K. Lemmon. 1999. Clathrin functions in the absence of heterotetrameric adaptors and AP180-related proteins in yeast. EMBO J. 18:3987–3998.

Ihle, G., J.R. Bruns, P.J. Luzio, and O.A. Weiss. 2001. Competing sorting signals guide endolyn along a novel route to lysosomes in MDCK cells. EMBO J. 20:6256–6264.

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

Kibbe, R.G., J. Rizo, L.M. Gierasch, and R.G. Anderson. 1998. The LDL receptor clustering motif interacts with the clathrin terminal domain in a reverse turn conformation. J. Cell Biol. 142:69–77.

Marks, M.S., L. Woodnuff, H. Ohno, and J.S. Bonifacino. 1996. Protein targeting by tyrosine- and dileucine-based signals: evidence for distinct saturable components. J. Cell Biol. 135:341–354.

Meyer, C., D. Ziaioli, S. Lauemann, E.L. Eskelinen, J. Hamann, P. Safirg, K. von Figura, and P. Schu. 2000. mu1A-adaptin-deficient mice: lethality, loss of AP-1 binding and retromer of mannose 6-phosphate receptors. EMBO J. 19:2193–2203.

Mishra, S.K., N.R. Agostinelli, T.J. Brett, I. Mizukami, T.S. Ross, and L.M. Traub. 2001. Clathrin- and AP-2-binding sites in HILP1 uncover a general assembly role for endocytic accessory proteins. J. Biol. Chem. 276:46230–46236.

Mishra, S.K., P.A. Keyel, M.J. Hawryluk, N.R. Agostinelli, S.C. Watkins, and L.M. Traub. 2002a. Disabled-2 exhibits the properties of a cargo-selective endocytic clathrin adaptor. EMBO J. 21:4915–4926.

Mishra, S.K., S.C. Watkins, and L.M. Traub. 2002b. The autosomal recessive hypcholesterolemia (ARH) protein interfaces directly with the clathrin-coat

We thank Mark Marsh (University College London, London, UK) for sharing unpublished data and reagents and Colin Hopkins and Liz Allchin for the B3/2.5-gold conjugate. We also thank Paul Lehner (Cambridge Institute for Medical Research, Cambridge, UK) for the HeLaM cells; Howard Davidson for the chc-1 sirNA sequence, Gudrun Ihle for the CD8 cDNA, and Frances Brodsky for the AP-6 antibody. We are grateful to Paul Luzio, John Kilmartin, David Owen, and members of the Robinson lab for reading the manuscript and for helpful discussions.
machinery. Proc. Natl. Acad. Sci. USA. 99:16099–16104.
Nesterov, A., H.S. Wiley, and G.N. Gill. 1995. Ligand-induced endocytosis of epidermal growth factor receptors that are defective in binding adaptor proteins. Proc. Natl. Acad. Sci. USA. 92:8719–8723.
Nesterov, A., R.E. Carter, T. Sorkina, G.N. Gill, and A. Sorkin. 1999. Inhibition of the receptor-binding function of clathrin adaptor protein AP-2 by dominant-negative mutant mu2 subunit and its effects on endocytosis. EMBO J. 18:2489–2499.
Niswonger, M.L., and T.J. O’Halloran. 1997. A novel role for clathrin in cytokinesis. Proc. Natl. Acad. Sci. USA. 94:8575–8578.
Ohno, H., J. Stewart, M.C. Fournier, H. Bosshart, I. Rhee, S. Miyatake, T. Saito, A. Gallusser, T. Kirchhausen, and J.S. Bonifacino. 1995. Interaction of tyrosine-based sorting signals with clathrin-associated proteins. Science. 269:1872–1875.
Owen, D.J., and P.R. Evans. 1998. A structural explanation for the recognition of tyrosine-based endocytic signals. Science. 282:1327–1332.
Peden, A.A., R.E. Rudge, W.W. Lui, and M.S. Robinson. 2002. Assembly and function of AP-3 complexes in cells expressing mutant subunits. J. Cell Biol. 156:327–336.
Reynolds, E.S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. 17:208–212.
Shih, S.C., D.J. Katzmann, J.D. Schnell, M. Sutanto, S.D. Emr, and L. Hicke. 2002. Epsins and Vps27p/Hrs contain ubiquitin-binding domains that function in receptor endocytosis. Nat. Cell Biol. 4:389–393.
Simpson, F., N.A. Bright, M.A. West, L.S. Newman, R.B. Darnell, and M.S. Robinson. 1996. A novel adaptor-related protein complex. J. Cell Biol. 133:749–760.
Sorkin, A., M. Mazzotti, T. Sorkina, L. Scotto, and L. Beguinot. 1996. Epidermal growth factor receptor interaction with clathrin adaptors is mediated by the Tyr974-containing internalization motif. J. Biol. Chem. 271:13377–13384.
Tiwari, R.K., J. Kusari, and G.C. Sen. 1987. Functional equivalents of interferon-mediated signals needed for induction of an mRNA can be generated by double-stranded RNA and growth factors. EMBO J. 6:3375–3378.
Warren, R.A., F.A. Green, P.E. Stenberg, and C.A. Enns. 1998. Distinct saturable pathways for the endocytosis of different tyrosine motifs. J. Biol. Chem. 273:17056–17063.
Weibel, E.W. 1979. Stereological Methods. Vol 1: Practical Methods for Biological Morphometry. Academic Press, London. 415 pp.
Wettey, F.R., S.F. Hawkins, A. Stewart, J.P. Luzio, J.C. Howard, and A.P. Jackson. 2002. Controlled elimination of clathrin heavy-chain expression in DT40 lymphocytes. Science. 297:1521–1525.
Yeung, B.G., H.L. Phan, and G.S. Payne. 1999. Adaptor complex-independent clathrin function in yeast. Mol. Biol. Cell. 10:3643–3659.