Article

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. Endo-cytosis 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 PIP_2 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-

Abbreviation used in this paper: siRNA, small interfering RNA.

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 a 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 knock-down, 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 (Wettey 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 la-

beling 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 with 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
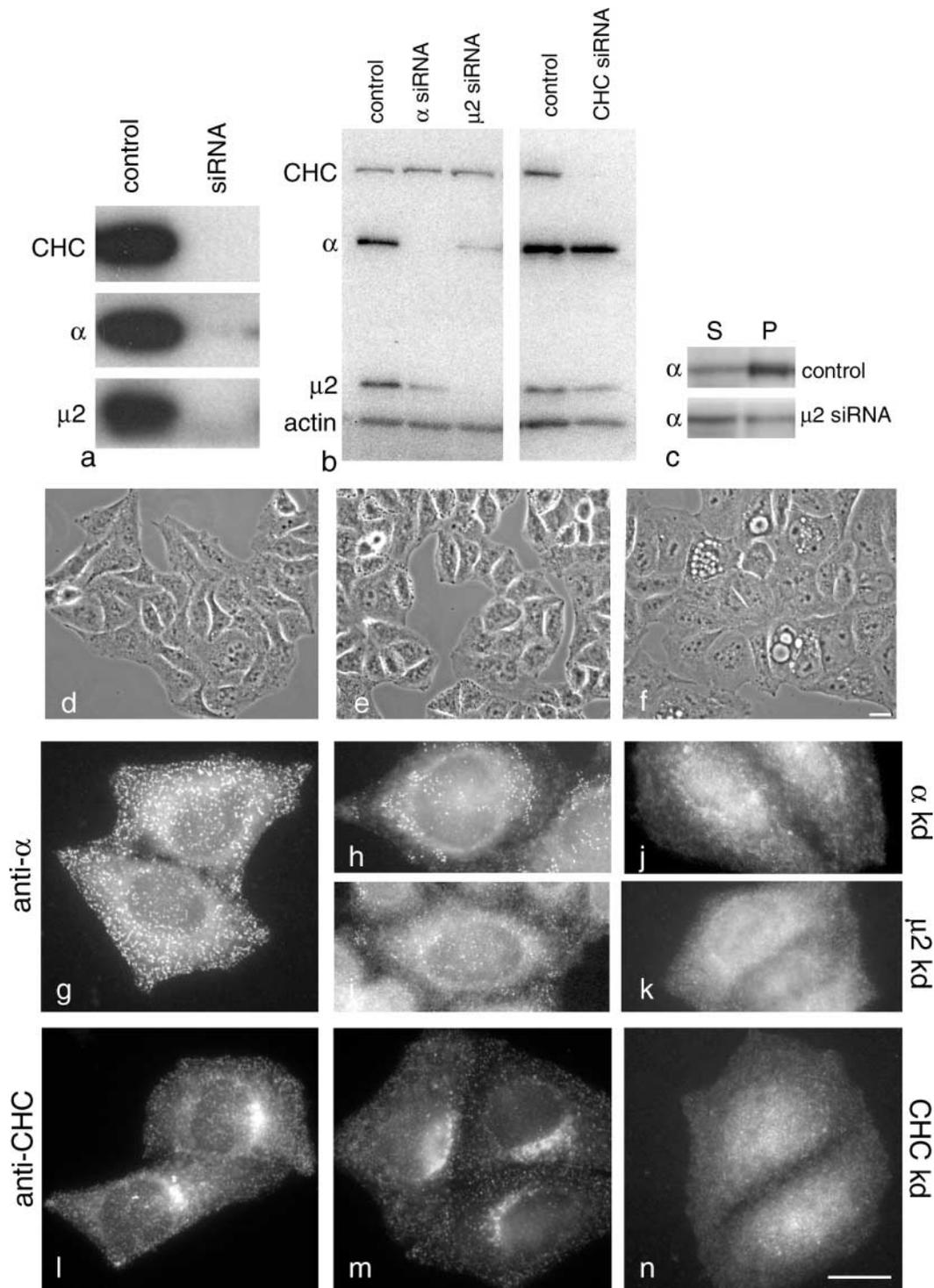
Figure 1. **Effects of depleting AP-2 and clathrin heavy chain from HeLaM cells.** (a) Equal protein loadings of homogenates of either control cells or cells treated with siRNAs directed against clathrin heavy chain, α-adaptin, or $\mu 2$ were subjected to SDS-PAGE, and Western blots were probed with antibodies against the indicated protein. Clathrin heavy chain and the AP-2 $\mu 2$ 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 $\mu 2$, and the $\mu 2$ siRNA causes a depletion in α. (c) Homogenates of control and $\mu 2$-depleted cells were centrifuged at high speed, and supernatants and pellets were probed with anti-α. Knocking down $\mu 2$ 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 $\mu 2$ siRNA (e), and cells treated with clathrin heavy chain siRNA (f). The $\mu 2$ 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 $\mu 2$ (i) siRNAs, and cells treated twice with α (j) and $\mu 2$ (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.
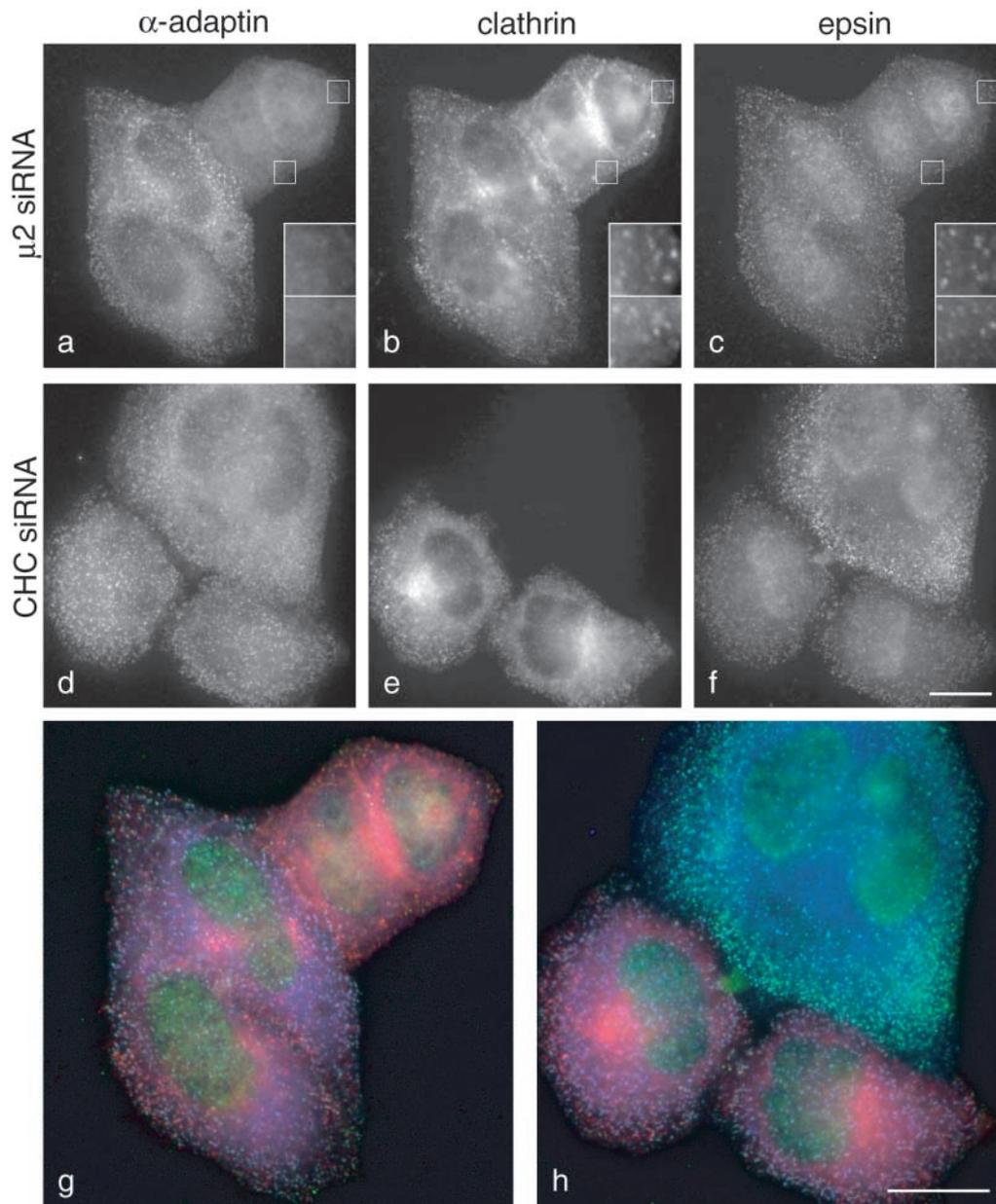
Figure 2. **Immunofluorescence triple labeling of control and siRNA-treated cells plated together.** (a–c and g). Cells treated with μ2-2 and control cells were labeled with mouse anti-α-adaptin (a; blue in g), rabbit anti-clathrin (b; red in g), and goat anti-epsin 1 (c; green in g). α-Adaptin is cytosolic in the μ2-depleted cells. Clathrin is still membrane-associated; however, many of these membranes are intracellular. Epsin spots are reduced in number, but are still present in the μ2-depleted cells. Many of these spots are also positive for clathrin. The two boxed-in regions are shown at higher magnification in the bottom right corner of all the panels in a–c. (d–f and h) Cells treated with chc-2 and control cells were labeled with mouse anti-α-adaptin (d; blue in h), rabbit anti-clathrin (e; red in h), and goat anti-epsin (f; green in h). Clathrin is undetectable in the siRNA-treated cells. α-Adaptin labeling is not markedly different; however, epsin labeling is brighter in the clathrin-depleted cells. 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. 3 f 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 (un-published data). However, in clathrin-depleted cells, there is a striking amount of swelling of post-Golgi compartments (Fig. 3 g), 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 up-take 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 μ2 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 μ2 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 μ2, also strongly inhibits uptake of the transferrin receptor (Nesterov et al., 1999).

Fig. 5 a 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
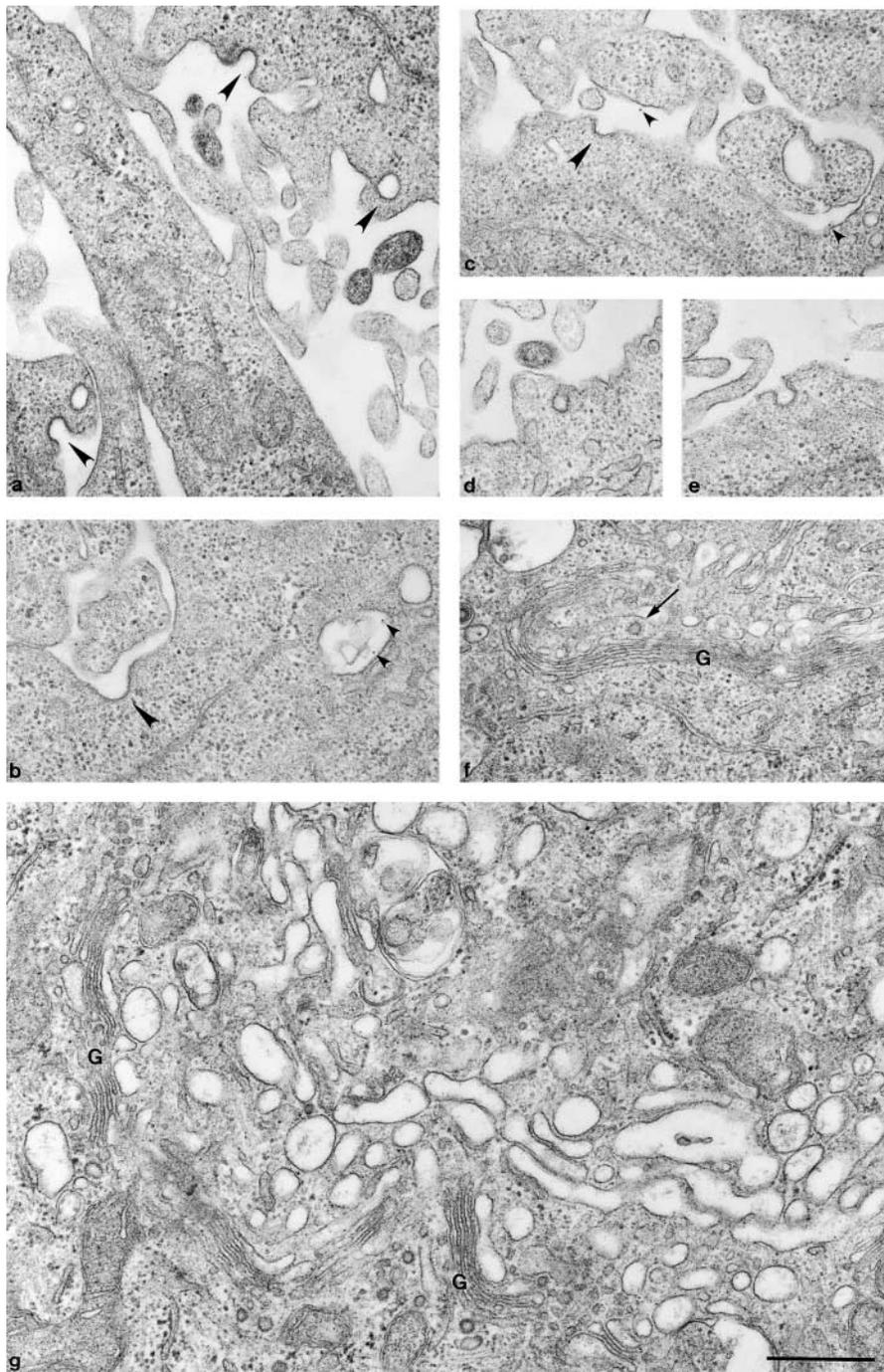
Figure 3. **Electron micrographs of control and siRNA-treated cells.** The cells in these experiments were incubated with an antibody against the transferrin receptor coupled to 8-nm gold before fixation to monitor the efficiency of transferrin receptor endocytosis. (a and b) Control cells have abundant 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 μ2-2 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.
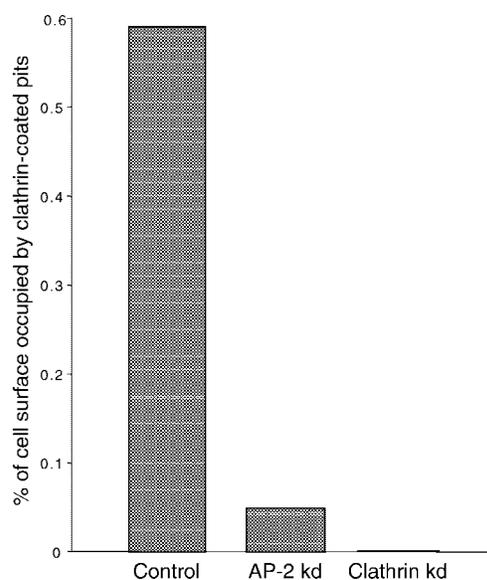
Figure 4. **Morphometric analysis of the EM data, using 128 images of each condition.** For the control cells, 7,630 plasma membrane intersections were scored, of which 45 were coated pit intersections (0.6%). For the μ2-2–treated cells, 11,361 plasma membrane intersections were scored, of which six were coated pit intersections (0.05%). For the chc-2–treated cells, 7,585 plasma membrane intersections were scored, of which none were coated pit intersections.

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 ^{125}I-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.
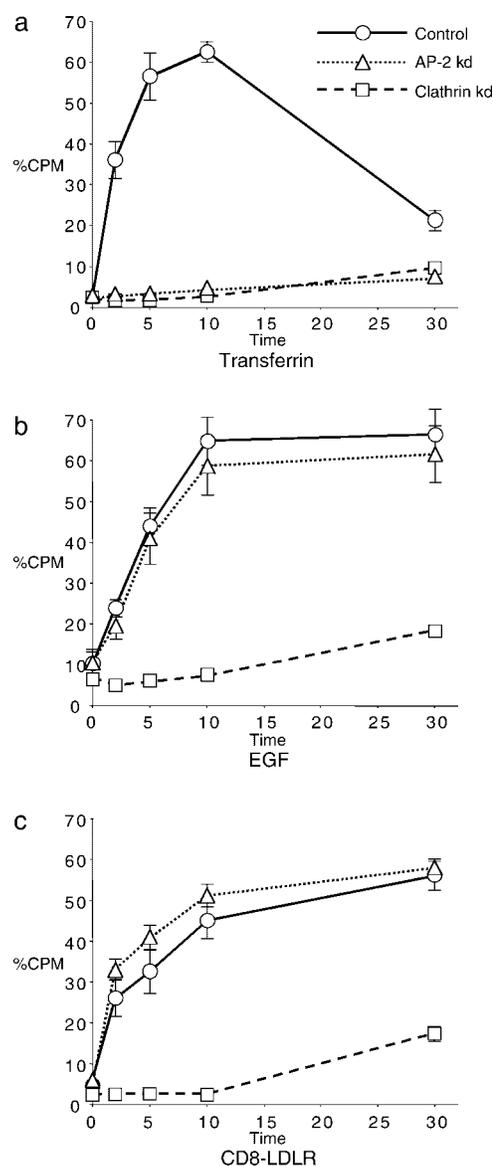
Figure 5. **Kinetics of ligand uptake in control and siRNA-treated cells.** (a) Cells were allowed to bind ^{125}I-labeled transferrin at 4°C, and were then shifted to 37°C for the indicated length of time, after which the medium was harvested, ligand remaining on the cell surface was released with an acid wash, the cells were solubilized with NaOH, and all three fractions were quantified and expressed as a percentage of total counts/m. The graph shows percentage of total counts recovered in the NaOH extract (i.e., internalized transferrin). Each point is derived from at least three separate experiments; the error bars show the SEM. Both μ2- and clathrin-depleted cells are strongly impaired in their ability to internalize transferrin. (b) Cells were treated exactly as in a, but using ^{125}I-labeled EGF. Uptake is strongly inhibited in the clathrin-depleted cells, but it is not significantly different from control in the μ2-depleted cells. (c) Cells expressing a chimera consisting of the CD8 extracellular and lumenal domains fused to the LDL receptor cytoplasmic tail were incubated at 4°C with an mAb against CD8 followed by ^{125}I-labeled protein A, and were then shifted to 37°C and treated as in a and b. Again, uptake is strongly inhibited in the clathrin-depleted cells, but similar to controls in the μ2-depleted cells. Virtually identical results were obtained using α-depleted cells, except that the effect on transferrin uptake was not quite so profound, presumably because the knockdown was less complete (not depicted).

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 ^{125}I-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 ^{125}I-labeled protein A as the ligand. Fig. 5 c 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 α 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; Aguilar 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 10^6 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 d 0 and d 2. 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-CACGCUGGCCA and the μ2-2 target sequence was AAGUGGAUGCCU-UUCGGGUCA (other sequences, designated α-1 and μ2–1, were ineffective). The clathrin heavy chain target sequences were AAG-CUGGGAAAACUCUUCAGA (chc-1) and UAAUCCAAUUCGAAGAC-CAAU (chc-2). The control siRNA was a nonfunctional oligo, μ2–1, originally designed to knock down the μ2 subunit, target sequence AACACAG-CAACCUCUACUUGG. 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 ^{125}I-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 cacody-late, pH 7.2, containing 6 mM CaCl$_2$) to an equal volume of tissue culture
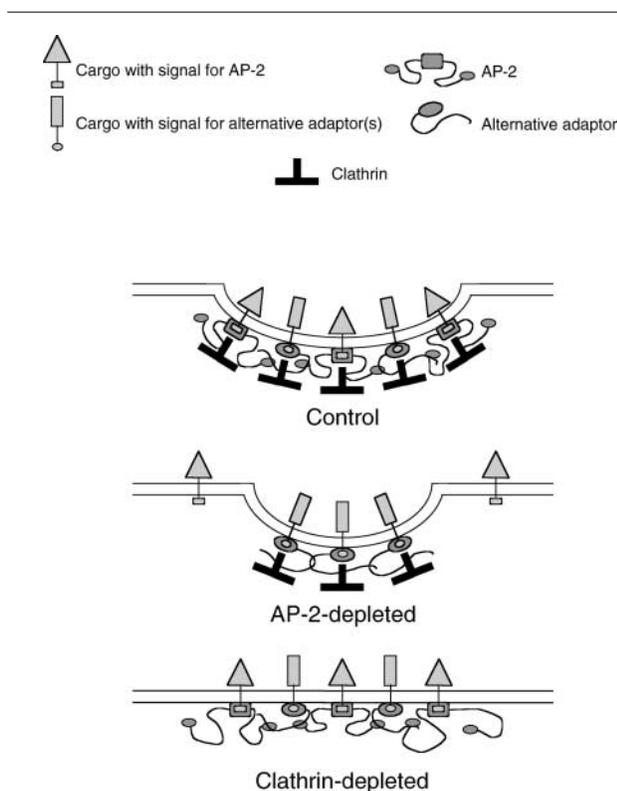
Figure 6. **Model showing interactions at the plasma membrane in control and siRNA-treated cells.** In control cells, AP-2 and alternative adaptors are both recruited onto the plasma membrane, where they interact with each other and recruit clathrin through interactions mainly involving their flexible linker and appendage domains, whereas their more highly structured domains interact with phosphoinositides and with cargo. In AP-2–depleted cells, only alternative adaptors are recruited onto the plasma membrane, but these can still associate with each other (e.g., through EH domain–NPF interactions and SH3 domain–proline-rich domain interactions), and can still recruit clathrin and bring a subset of cargo proteins into the coated pit. However, without AP-2 there is less clathrin on the plasma membrane and fewer coated pits per cell, and cargo proteins that only have signals for AP-2 do not get internalized into coated vesicles. In clathrin-depleted cells, AP-2 and alternative adaptors are both recruited onto the plasma membrane, where they interact with each other and with cargo, but the membrane does not invaginate and there is no selective uptake of cargo.

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 CaCl$_2$. 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 Ultracut S; 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 13,500, 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 ^{125}I-labeled EGF or ^{125}I-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 ^{125}I-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 Gudrun Ihrke (University of Cambridge, Cambridge, UK; Ihrke 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′ end. The resulting fragment was ligated to the AflII site at the end of the transmembrane domain coding sequence of CD8, and the chimera was cloned into pIRES2Neo (CLONTECH Laboratories, Inc.). The construct was sequenced to confirm that a correct in-frame fusion had been achieved, and was then transfected into HeLaM cells. Stably transfected cells were selected and maintained in the presence of 500 μg/ml G418 (GIBCO BRL).

To monitor uptake of the chimera, the cells were treated as above, but incubated with SFM containing 1:100 diluted anti-CD8 (153–020; Ancell Corp.) instead of with a radiolabeled ligand, and the incubation was for 45 min at 4°C instead of for 30 min. The cells were then washed and incubated for a further 45 min at 4°C with SFM containing ^{125}I-labeled protein A (1:1,000 diluted; Amersham Biosciences). The rest of the experiment was performed exactly as above.

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