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The Aftiphilin/p200/γ-Synergin Complex

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Aftiphilin is a protein that was recently identified in database searches for proteins with motifs that interact with AP-1 and clathrin, but its function is unknown. Here we demonstrate that aftiphilin has a second, atypical clathrin binding site, YQW, that colocalizes with AP-1 by immunofluorescence, and that is enriched in clathrin-coated vesicles (CCVs), confirming that it is a bona fide component of the CCV machinery. By gel filtration, aftiphilin coelutes with two other AP-1 binding partners, p200a and γ-synergin. Antibodies against any one of these three proteins immunoprecipitate the other two, and knocking down any of the three proteins by siRNA causes a reduction in the levels of the other two, indicating that they form a stable complex. Like AP-1–depleted cells, aftiphilin-depleted cells missort a CD8-furin chimera and the lysosomal enzyme cathepsin D. However, whereas AP-1–depleted cells recycle endocytosed transferrin more slowly than untreated cells, aftiphilin-depleted cells accumulate endocytosed transferrin in a peripheral compartment and recycle it more rapidly. These observations show that in general, the aftiphilin/p200/γ-synergin complex facilitates AP-1 function, but the complex may have additional functions as well, because of the opposing effects of the two knockdowns on transferrin recycling.

INTRODUCTION

The AP-1 adaptor complex functions in the formation of clathrin-coated vesicles (CCVs) from intracellular membranes. AP-1 localizes to both the TGN and endosomes, and there is currently some controversy as to whether it facilitates trafficking in the TGN to endosome direction, in the endosome to TGN direction, between different populations of endosomes (e.g., early and recycling), from endosomes to the plasma membrane, or in more than one of these pathways (Robinson, 2004). Like all of the AP complexes, AP-1 is a heterotetramer, consisting of two large subunits, γ and β1; a medium subunit, μ1; and a small subunit, σ1 (Robinson and Bonifacino, 2001). The COOH-terminal domains of the two large subunits project from the complex like ears, forming binding platforms for accessory proteins. So far most of our knowledge of accessory proteins comes from studies on AP-2, where >20 ear binding partners have now been identified (Praefcke *et al.*, 2004). Some of these proteins appear to be adaptors in their own right, in that they bind not only to AP-2, but also to clathrin, to PIP2, and to certain types of CCV cargo (Traub, 2003), facilitating clathrin-mediated endocytosis even in the absence of any detectable AP-2 (Motley *et al.*, 2003).

Considerably less is known about binding partners for AP-1. The first AP-1 binding partner to be identified was γ-synergin, a protein isolated in a yeast two-hybrid library screen for proteins that interact with the γ subunit (Page *et al.*, 1999). Unusual features of the γ-synergin sequence include the existence of several different splice variants, the presence of an EH (Eps15 homology) domain, and multiple repeats of the sequence DDFX[D/E]F, which we initially proposed might constitute a γ ear binding motif (Page *et al.*, 1999). Subsequent biochemical and structural studies have pinpointed the sequence [F/W/Y]G[D/E/P][F/W/I/L/M] as the consensus for binding to the γ ear, as well as to the ears of the GGAs, a family of monomeric adaptors with a COOH-terminal domain related to the γ ear (Collins *et al.*, 2003; Miller *et al.*, 2003; Mattera *et al.*, 2004). A number of other AP-1 binding partners have been identified by GST pulldown using the γ ear domain as bait, including Eps15 (Kent *et al.*, 2002), epsinR, Snx9, (Hirst *et al.*, 2003), and p200 (Lui *et al.*, 2003; a novel protein, not to be confused with other proteins that have sometimes been called p200). Another AP-1-interacting protein, aftiphilin, was identified by searching databases for sequences containing the γ ear binding motif (Mills *et al.*, 2003; Mattera *et al.*, 2004; and our own unpublished observations).

Although all of these proteins have been shown to bind to AP-1 in vitro, the physiological relevance of some of the interactions is still unclear, and in most cases the function of the protein is unknown. One notable exception is epsinR, which shows excellent colocalization with AP-1, indicating that it interacts with AP-1 in vivo as well as in vitro, and which appears to function as a cargo-selective adaptor for the SNARE protein vti1b (Hirst *et al.*, 2004). γ-Synergin also colocalizes with AP-1, and although its function is not yet known, its ability to bind to SCAMP1 has led to the suggestion that it might be a SCAMP-selective adaptor (Fernandez-Chacon *et al.*, 2000; Robinson, 2004). So far very little is known about aftiphilin, although a GFP-tagged version of the protein shows some colocalization with AP-1 in transiently transfected cells (Mattera *et al.*, 2004). p200 is even less well characterized, and—unlike most of the other AP-1 partners—it does not contain any obvious γ ear binding motifs, suggesting that its interaction with AP-1 may be indirect. In mammals there are two p200 isoforms, p200a (gi 55749742), which we identified in the pulldowns (Lui *et al.*, 2003), and p200b (gi 51471758), which is 68% identical to

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p200a. Attempts to localize p200 in mammalian cells have so far been unsuccessful, but there is a p200 homologue in yeast, Yjl207c, which colocalizes with clathrin when expressed as a GFP-tagged construct (Huh *et al.*, 2003), indicating that p200 is a bona fide component of the CCV machinery.

In the present study, we started out by trying to characterize aftiphilin. In the course of our experiments, we found that aftiphilin forms a stable complex with p200 and γ-synergin. Using siRNA knockdowns in HeLa cells, we have investigated the function of this complex in various trafficking pathways, and we have compared the phenotype of aftiphilin or p200 depletion with that of AP-1 depletion.

MATERIALS AND METHODS

Cells and Ligand Assays

Unless otherwise specified, reagents were purchased from Sigma (Poole, Dorset, United Kingdom). HeLaM cells (Tiwari *et al.*, 1987) were used throughout this study and will hereafter be referred to as HeLa cells. Stable line of these cells expressing CD8-furin and CD8-CIMPR were kind gifts from Matthew Seaman (University of Cambridge; Seaman, 2004). To monitor cell surface expression of the CD8 chimeras, cells were incubated with a 1:100 dilution of anti-CD8 (153–020); Ancell, Bayport, MN) in serum-free medium (SFM; DMEM containing 20 mM HEPES and 1% bovine serum albumin) for 45 min at 4°C. The cells were then washed, incubated for a further 45 min at 4°C with SFM containing ^{125}I-labeled protein A (1:1000; Amersham Biosciences, Piscataway, NJ), washed again, and then total cell-associated radioactivity was extracted with two washes of 0.8 ml 1 M NaOH and quantified using a gamma counter (Nuclear Enterprises, Edinburgh, Scotland). An aliquot of the cell extract was used for protein determination using a Bradford assay. The cathepsin D sorting assay was performed essentially as described by Davidson (1995), using 5 μl ^{35}S Promix (Amersham Biosciences) per ml of medium. The cells were serum-starved, pulse-labeled for 15 min, and chased for 2.5 h in the presence of 5 mM mannose 6-phosphate before immunoprecipitation using rabbit anti-human cathepsinD (DAKO, High Wycombe, United Kingdom). Quantifications were carried out using a Packard Cyclone phosphorimager (Meriden, CT).

To investigate the rate of transferrin recycling in the various knockdowns, cells in 35-mm dishes were serum-starved for 15 min at 37°C in SFM and then incubated for 1 h at 37°C in SFM containing ^{125}I-labeled transferrin (Perkin Elmer-Cetus, Norwalk, CT) at a concentration of 500 nCi/ml. Dishes were washed three times with ice-cold SFM, and surface-bound transferrin was removed by stripping in 1 ml 0.1 M glycine, 150 mM NaCl, pH 3, twice for 4 min at 4°C, followed by two washes with SFM. Dishes were then incubated with 1.5 ml prewarmed DMEM containing 5% fetal calf serum. Medium was harvested and replaced at regular intervals. At the end of the experiment, the cells were extracted twice with 1 M NaOH as above, and counts in the medium and the cell extracts were quantified using a gamma counter. For some experiments, total surface binding of transferrin was quantified; this was done by incubating the cells at 4°C with the ^{125}I-labeled transferrin for 1 h, and then washing and extracting the cells and quantifying the radioactivity as above, saving an aliquot for protein determination.

Plasmid Construction and GST Pulldowns

An EST encoding aftiphilin (IMAGE Clone I.D. 6014735; gi 21175557), sequenced by the IMAGE Consortium (Lennon *et al.*, 1996), was obtained from the Sanger Centre (Hinxton, United Kingdom). The cDNA, which encodes a protein of 937 amino acids, is similar to the larger cDNA described by Mattera *et al.* (2004) and entered into the database as aftiphilin isoform c (gi 50409939), except that the serine at position 888 is not deleted. For pulldown experiments, GST fusion proteins were made with fragments of aftiphilin amplified by PCR. The SQS mutation was made using a QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Pulldowns were carried out essentially as previously described (Hirst *et al.*, 2000), using either HeLa or Jurkat cell cytosol prepared in phosphate-buffered saline (PBS) containing 0.1% NP-40 and the protease inhibitor AEBSF (4-[2-aminoethyl]benzenesulfonylfluoride), at a protein concentration of either 1.5 mg/ml (HeLa cells) or 3.5 mg/ml (Jurkat cells). Bound proteins were eluted with sample buffer and subjected to SDS-PAGE.

Protein identities were obtained by the now-standard technique of peptide mass fingerprinting using in-gel trypsin digestion and MALDI-TOF mass spectrometry. Gel bands were excised and digested with trypsin according to the method of Shevchenko *et al.* (1996). Samples of tryptic peptide mixtures were mixed with alpha-cyano-4-hydroxy-transcinnamic acid matrix and analyzed with a Micromass (Manchester, United Kingdom) TofSpec2e MALDI mass spectrometer in reflectron mode. Callibration was performed from each spectrum using internal features, specifically the matrix-related ion peak at 1060.048 Da and the trypsin autolysis peaks at 2163.057 and 2273.160 Da. Spectra were interpreted by the Mascot search engine (http://www.matrixscience.com) configured with mass tolerance of 70 ppm and the gel-derived variable modifications "Propionamide Cysteine" and "Methionine Sulfoxide."

Antibodies, Immunoprecipitations, and Western Blotting

Rabbit polyclonal antibodies against γ-synergin, γ-adaptin, μ1, μ2, σ1, σ2, clathrin, epsinR, p200, and Snx9 were raised in house and have already been described (Seaman *et al.*, 1993; Page *et al.*, 1999; Hirst *et al.*, 2003; Lui *et al.*, 2003). Monoclonal antibodies against γ-adaptin (monoclonal antibody 100/3; Sigma) and CD8 (Ancell), and polyclonal antibodies against cathepsinD (DakoCytomation) and TGN46 (Serotec, Oxford, United Kingdom) were purchased from the manufacturers. The polyclonals antibodies against CI-MPR (Reaves *et al.*, 1996), SCAMP1 (Wu and Castle, 1997), eps15, and furin were kind gifts from Paul Luzio (University of Cambridge, United Kingdom), David Castle (University of Virginia), Phil Evans (MRC LMB, Cambridge, United Kingdom), and Liz Ledgerwood (University of Otago, New Zealand), respectively. Aftiphilin antibodies were raised against GST-afti(383–610) and affinity-purified, as described for other GST constructs (Hirst *et al.*, 1999). Immunoprecipitations were carried out on HeLa extracts under nondenaturing conditions, as previously described (Hirst *et al.*, 1999). For gel filtration, HeLa cytosol was prepared in PBS at 10 mg/ml and fractionated on a Superose 6 column. Fractions were concentrated by trichloroacetic acid precipitation followed by acetone washing, resuspended in sample buffer, and subjected to SDS-PAGE. Western blots were probed with various antibodies, followed by a rabbit anti-mouse linker where appropriate and then by ^{125}I-protein A, as previously described (Hirst *et al.*, 2000). Samples included GST pulldowns, whole-cell extracts, and clathrin-coated vesicles purified from control and siRNA-treated cells (see below). Quantifications were carried out using a Packard Cyclone phosphorimager.

Immunolocalization

For immunofluorescence, HeLa cells were fixed either with 3% paraformaldehyde followed by permeabilization with 0.1% TX-100, or with methanol followed by acetone at −20°C. Primary antibodies are described above; secondary antibodies were purchased from Molecular Probes (Eugene, OR). For transferrin uptake experiments, cells were serum-starved in SFM for 15 min at 37°C, incubated with AlexaFluor 594-transferrin (Molecular Probes) for 10 min at 37°C, washed with PBS, and fixed with 3% paraformaldehyde. The cells were viewed using a Zeiss Axiophot fluorescence microscope equipped with a CCD camera (Princeton Instruments, Monmouth Junction, NJ) and photographs were recorded using IP Labs software (Scanalytics, Billerica, MA).

RNA Interference

siRNA duplexes designed against sequences of the target cDNAs were purchased from Dharmacon (Boulder, CO). An epsinR sequence, which had previously been found to be ineffective for knockdown experiments, was used as a control. The sequences were as follows: AACCAUUGAUCUUGGAGCAGC (control); AAUACAGAUAUGGUCCAGAAA (epsinR); AAGGCAUCAAGUAUCGGAAGA (μ1a); AAGCAGUUGCUAGUGGCCAUU (aftiphilin); UAAUCCAAUUCGAAGACCAAU (clathrin heavy chain); siGENOME SMARTpool XM_042685 (p200a); siGENOME SMARTpool XM_113763.5 (p200b); CAGCAGCTCCTATTCCAACTT (γ-synergin). HeLa cells were transfected using Oligofectamine (Invitrogen, Inchinnan, United Kingdom) as specified by the manufacturer. The transfection mixture was left on the cells for 4 h, after which DMEM/20% fetal calf serum without antibiotics was added. For efficient knockdown two transfections were performed 2 d apart, and experiments were carried out 2 d after the second knockdown.

Clathrin-coated Vesicle Preparation

For preparation of CCVs, six confluent dishes of HeLa cells treated with control, clathrin, or aftiphilin siRNAs were used, essentially as described (Hirst *et al.*, 2004). Briefly, the cells were scraped in buffer A (0.1 M MES, pH 6.5, 0.2 mM EGTA, 0.5 mM $MgCl_2$, 0.02% NaN_3) containing AEBSF (0.2 mM) and homogenized, the postnuclear supernatant was treated with 50 μg/ml RNAse for 30 min, and the membranes were pelleted. The pellet was resuspended and mixed with an equal volume of 12.5% Ficoll/12.5% sucrose and spun to pellet most of the contaminants. The Ficoll/sucrose supernatant (containing the CCVs) was diluted with buffer A, and the CCVs (and other particles) were pelleted and resuspended in buffer A.

RESULTS

Binding of Aftiphilin to AP-1 and Clathrin

Aftiphilin was first identified as a sequence in the database containing multiple copies of the γ ear binding motif. There are two splice variants of aftiphilin in mammals, one of

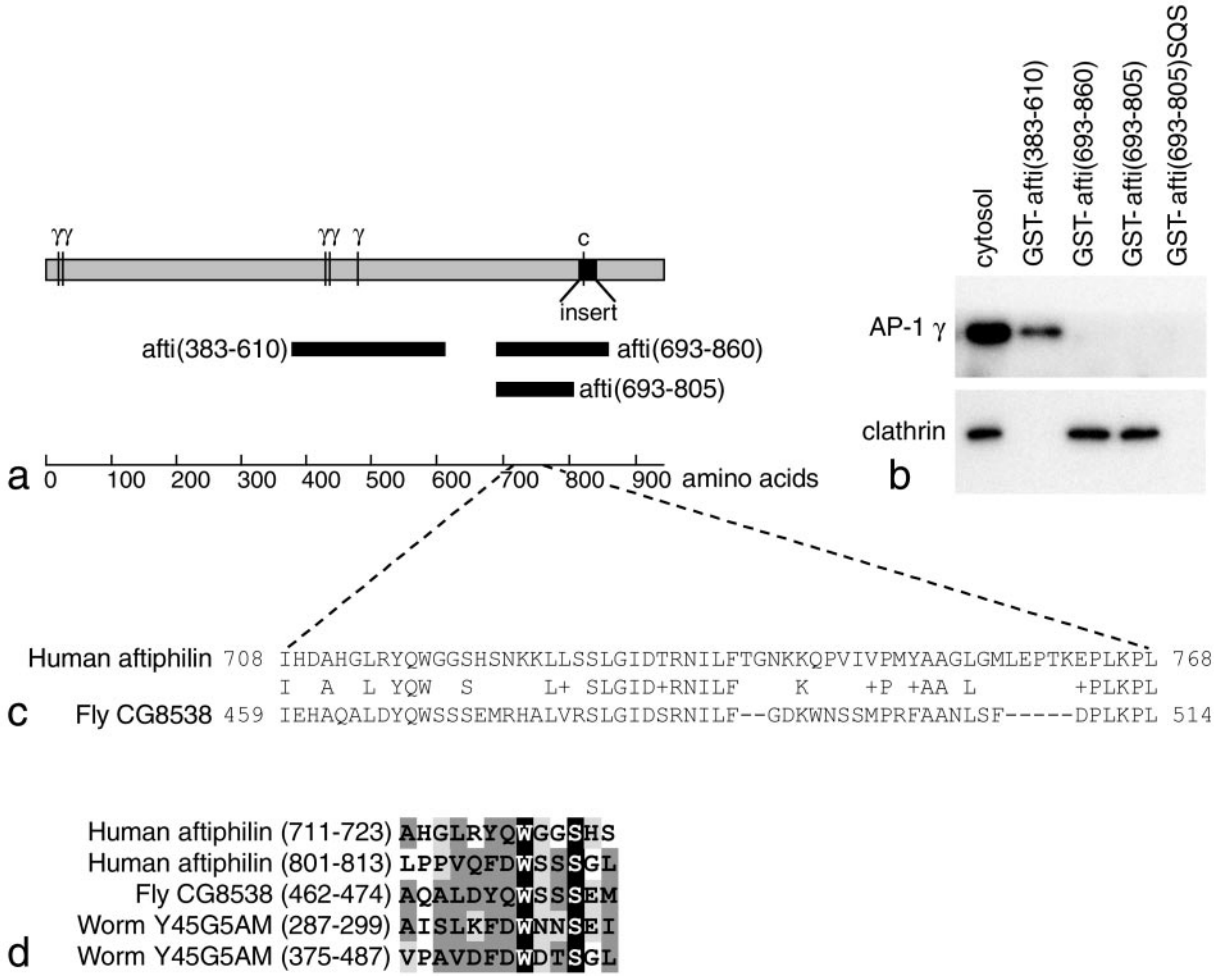

Figure 1. Binding domains on aftiphilin. (a) Schematic diagram of aftiphilin. The larger version of the protein is 937 amino acids long and contains a 28-amino acid insert with a clathrin box (marked c). Upstream of the clathrin box are five γ ear binding motifs (marked γ). The regions of aftiphilin used in the construction of GST fusion proteins are indicated. Bovine serum albumin, GST pulldowns of HeLa cell cytosol using constructs indicated diagramatically in a. GST-afti(383–610) brings down AP-1 but not clathrin, whereas GST-afti(693–860) brings down clathrin but not AP-1. A shorter construct, GST-afti(693–805), also brings down clathrin, and this interaction appears to depend on the YQW motif, because when it is mutated to SQS, the construct no longer brings down clathrin. (c) Comparison of aftiphilin with its closest homologue in *Drosophila*, CG8538. (d) "Pile-up" alignments of sequences from human aftiphilin, fly CG8538, and Y45g5aM, a homologue of aftiphilin in *C. elegans*.

which contains a clathrin box, LLNLD, within a 28-amino acid insert (marked "c" in Figure 1a). To investigate the binding of aftiphilin to AP-1 and clathrin, we constructed several GST chimeras for pulldown assays. Figure 1b shows that GST-afti(383–610), which contains three of the γ ear binding motifs, is able to pull down AP-1 from HeLa cell cytosol but not clathrin. In contrast, GST-afti(693–860), which contains the clathrin box but none of the γ ear binding motifs, brings down clathrin but not AP-1. Surprisingly, however, GST-afti(693–805), which terminates before the clathrin box, also brings down clathrin, indicating that there is a second clathrin-binding site upstream of the clathrin box.

A possible clue as to the identity of this site came from a BLAST search for aftiphilin homologues in *Drosophila*. The closest match was with a protein of unknown function, CG8538 (Figure 1c). Although the homology between aftiphilin and CG8538 is weak, being mainly confined to a stretch of ~60 amino acids, they both have similarly low secondary structure predictions, and CG8538 contains at least two γ ear binding motifs, suggesting that it may be the fly orthologue of aftiphilin. Like the shorter isoform of mammalian aftiphilin, CG8538 contains no clathrin box, but both proteins contain the sequence YQW, which is reminiscent of the WDW clathrin binding site that has been identified in auxilin (Scheele *et al.*, 2003). Mutating this sequence to SQS in GST-afti(693–805) resulted in a construct that was no longer able to bring down clathrin (Figure 1b), indicating that the YQW motif is the second clathrin-binding site in our construct and that both splice variants of aftiphilin are able to interact with clathrin.

We also found an aftiphilin homologue in *Caenorhabditis elegans*, Y45g5aM, which has three copies of the γ ear binding motif. Although this protein does not contain the YQW tripeptide, it contains two copies of a related sequence, FDW. Interestingly, human aftiphilin also contains an FDW

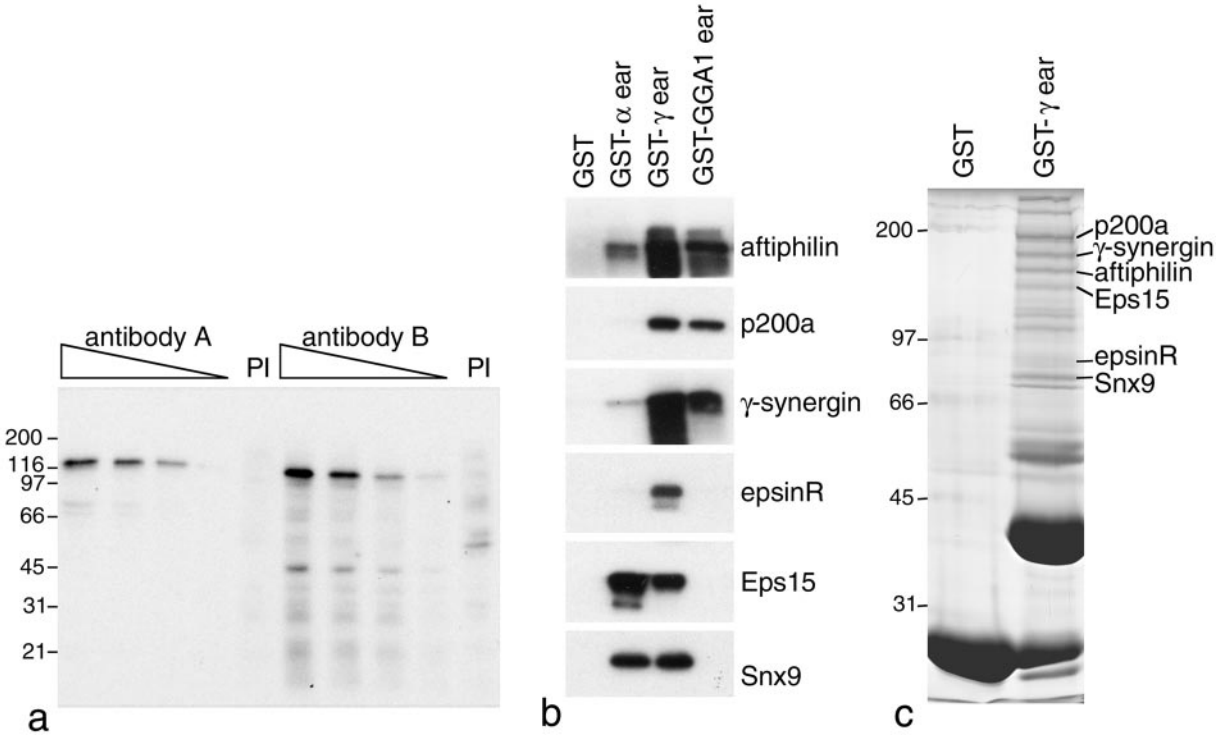

Figure 2. Binding of aftiphilin to adaptor ears. (a) Western blots of HeLa cell extracts probed with serial dilutions of two aftiphilin antibodies, A and B, as well as with the two preimmune sera (PI). Both antibodies label a band with an apparent molecular weight of ~126 kDa, which is somewhat larger than aftiphilin's predicted size (99 and 102 kDa for the two isoforms), but this sort of aberrant mobility is typical of many of the CCV accessory proteins, which have long regions with little or no secondary structure. Bovine serum albumin, GST pulldowns of HeLa cell cytosol using α ear, γ ear, and GGA ear constructs. Aftiphilin, p200a, and γ-synergin all show the same preferences for the γ ear. (c) Coomassie blue–stained gel of a GST-γ ear pulldown of Jurkat cells. The indicated bands were identified by mass spectrometry.

sequence. Alignment of these sequences from humans, flies, and worms indicates that there is a conserved consensus sequence, [L/V]X[F/Y][D/Q]WXXS, which may constitute a novel clathrin-binding motif.

To investigate whether aftiphilin binds to other adaptor ear domains, we carried out pulldowns on HeLa cell cytosol using GST alone, GST-α ear, GST-γ ear, or GST-GGA1 ear. Blots were then probed with new antibodies against aftiphilin (characterized in Figure 2a) as well as with antibodies against other γ ear binding partners. Figure 2b shows that aftiphilin is brought down most efficiently by the GST-γ ear construct, followed by GST-GGA1 ear, and only weakly by GST-α ear. Similar binding preferences are shown by p200a and γ-synergin. In contrast, three other ear binding partners, epsinR, Eps15, and Snx9, show different preferences from aftiphilin and from each other. These results were the first indication that aftiphilin, p200a, and γ-synergin might be binding to adaptor ears in a similar manner, at least in vitro.

We were also able to detect aftiphilin in γ ear pulldowns by mass spectrometry. Figure 2c shows a Coomassie blue-stained gel of a pulldown using Jurkat cell cytosol, with aftiphilin and other bands indicated. This is the first time that we have been able to see aftiphilin as a major band in a pulldown, even though we have carried out many γ ear pulldowns using different sources of cytosol (e.g., see Hirst *et al.*, 2000, 2003). We suspect that aftiphilin may be particularly susceptible to proteolysis, and tends to get degraded during the preparation of cytosol for pulldowns.

Localization of Aftiphilin

Although the two aftiphilin antibodies were better for Western blotting than for immunofluorescence, they both gave the same localization pattern, showing significant colocalization with AP-1 (Figure 3, a and b). Both aftiphilin and AP-1 become cytosolic in brefeldin A–treated cells (Figure 3, c and d), indicating that the membrane association of aftiphilin, like that of AP-1, is dependent on ARF. Using two different siRNAs to knock down the expression of aftiphilin (i.e., in separate experiments), we are able deplete the protein by ~95% as assayed by Western blotting (see Figure 5d), resulting in a complete loss of aftiphilin labeling by immunofluorescence (Figure 3e), although AP-1 still localizes to the perinuclear region in the aftiphilin-depleted cells (Figure 3f). In contrast, in AP-1–depleted cells the perinuclear localization of aftiphilin is lost (Figure 3, g and h), indicating that AP-1 recruits aftiphilin onto membranes rather than vice versa.

We also investigated the effect of epsinR depletion on the localization of aftiphilin. We have previously shown that knocking down epsinR leads to a more pronounced perinuclear distribution of AP-1, possibly because there is a block in the formation of free CCVs (Hirst *et al.*, 2004). Figure 4, a–d, shows that there is also a striking increase in the amount of aftiphilin labeling on perinuclear membranes. In addition, perinuclear staining with anti-γ-synergin is enhanced after epsinR knockdown (Figure 4, e–h), and our antibody against p200a, which only gives background labeling of the nucleus in untreated cells (Figure 4, i and j), now

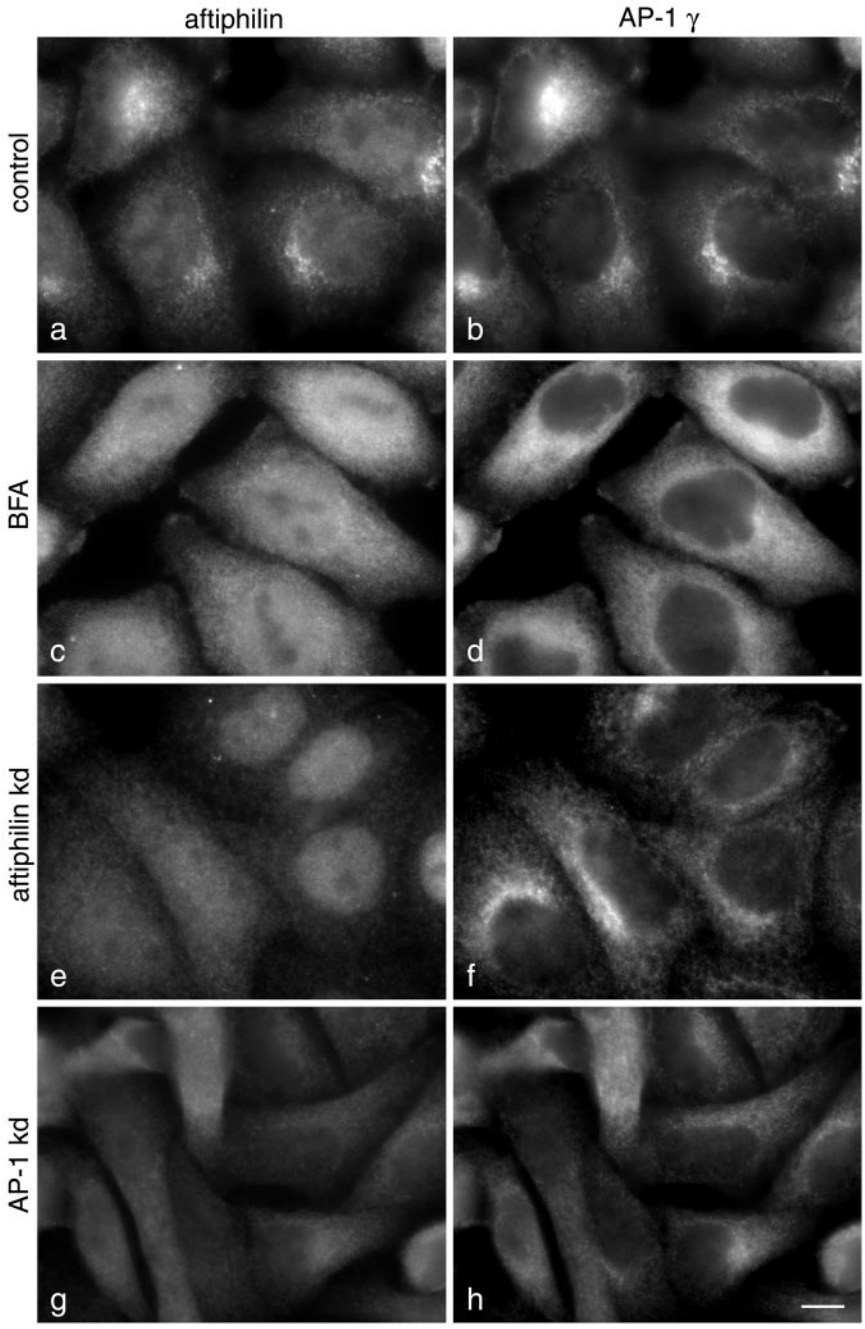

Figure 3. Immunofluorescence double labeling for aftiphilin and the AP-1 γ subunit. Both proteins are sensitive to BFA, but whereas knocking down aftiphilin does not affect the membrane localization of AP-1, knocking down AP-1 causes aftiphilin to redistribute to the cytoplasm. Scale bar, 10 μm.

produces a faint but distinct perinuclear pattern that colocalizes with AP-1 (Figure 4, k and l, arrows). These observations, together with the similar ear binding profiles of aftiphilin, p200a, and γ-synergin, suggested to us that the three proteins might be functioning together as a complex.

Evidence for an Aftiphilin/p200a/γ-Synergin Complex

To determine whether aftiphilin, p200a, and γ-synergin form a complex, we first investigated whether they coelute by gel filtration. HeLa cell cytosol was fractionated on a Superose 6 column, and Western blots were probed with antibodies against various proteins. Figure 5, a and b, shows that clathrin, which has a native molecular weight of ~630 kDa, peaks in fraction 13, whereas AP-1, which has a native molecular weight of ~270 kDa, peaks in fraction 27. Aftiphilin, p200a, and γ-synergin all comigrate up to fraction 19, peaking in fraction 17. There are then additional peaks for p200a and for γ-synergin, which runs as multiple bands by SDS PAGE, most likely due to proteolysis. Nevertheless, the identical high molecular weight peaks of the three proteins is consistent with their forming a stable complex in cytosol.

We next investigated whether aftiphilin, γ-synergin, and p200a are able to be coimmunoprecipitated. Figure 5c shows Western blots of HeLa cells extracted with 0.5% NP40, to try to preserve protein-protein interactions that take place on membranes, and then immunoprecipitated using antibodies against the AP-2 α subunit, the AP-1 γ subunit, aftiphilin, p200a, and γ-synergin. The antibody against the AP-2 α

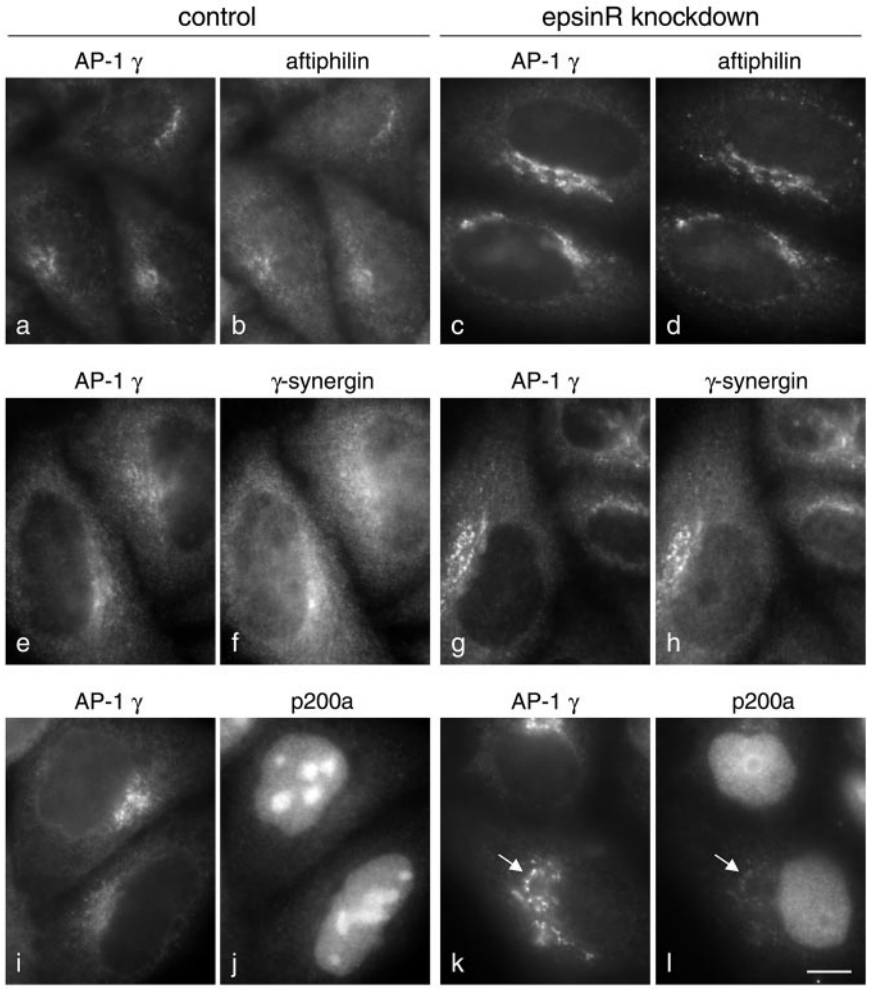

Figure 4. Localization of aftiphilin, γ-synergin, and p200a in epsinR-depleted cells. EpsinR depletion increases the perinuclear distribution of all three proteins, as well as that of AP-1. The arrows mark regions of overlap between AP-1 and p200. Scale bar, 10 μm.

subunit brings down the AP-2 σ2 subunit, but not the AP-1 σ1 subunit or any of the other proteins. Similarly, the antibody against the AP-1 γ subunit brings down the AP-1 σ1 subunit, but not the AP-2 σ2 subunit. Aftiphilin, p200a, and γ-synergin also coimmunoprecipitate with AP-1, confirming that they interact with AP-1 on membranes, even though they do not comigrate with AP-1 on gel filtration columns of cytosol. However, much more aftiphilin, p200a, and γ-synergin come down when they are immunoprecipitated with antibodies against themselves or each other. These data confirm that aftiphilin, p200a, and γ-synergin form a complex with each other and are not simply brought together by the ability of all three proteins to interact with AP-1.

Subunits of stable protein complexes are often degraded when one of their partners is missing. For instance, the AP-3 μ3 subunit is degraded in β3-deficient mutants, and all of the subunits are degraded in mutants lacking the AP-3 δ subunit (Peden *et al.*, 2002). To investigate whether aftiphilin, p200a, and γ-synergin depend on each other for stability, we knocked down each one in turn, then probed Western blots with antibodies against the other proteins. Three separate knockdown experiments were performed for each protein and the results were averaged; Figure 5d shows a representative experiment. None of the proteins are destabilized by knocking down AP-1. However, knocking down aftiphilin results in a loss not only of itself, but also of p200a (down to 21.7 ± 1.3% of control levels), and, to a lesser extent, γ-synergin (down to 36.7 ± 5.9% of control levels). Similarly, knocking down γ-synergin depletes aftiphilin to 64.3 ± 2.3% and p200a to 49.6 ± 0.9% of control levels, and knocking down p200a depletes aftiphilin to 18.8 ± 3.9% and γ-synergin to 60.7 ± 6.3% of control levels.

Because of the possibility that p200a might be functionally redundant with p200b, we also investigated the effect of knocking down p200b. We do not have an antibody against p200b, but we used an siGENOME SMARTpool siRNA (Dharmacon), guaranteed to silence by at least 75% at the mRNA level. We found that knocking down p200b alone did not affect the stability of any of the other proteins, and knocking down both p200 isoforms together did not enhance the effects of p200a knockdown. These observations suggest either that p200b is functionally distinct from p200a, or alternatively that it is expressed at much lower levels. Nevertheless, our results confirm a relationship between aftiphilin, p200a, and γ-synergin, where the loss of any one affects the stability of the other two.

Enrichment of the Complex in CCVs

We have previously shown that γ-synergin is enriched in CCV preparations from rat liver by comparing Western blots of our purified fraction with an equal protein loading of crude rat liver membranes (Page *et al.*, 1999). However, there are two problems with this approach as a means of determining whether or not a protein is associated with CCVs

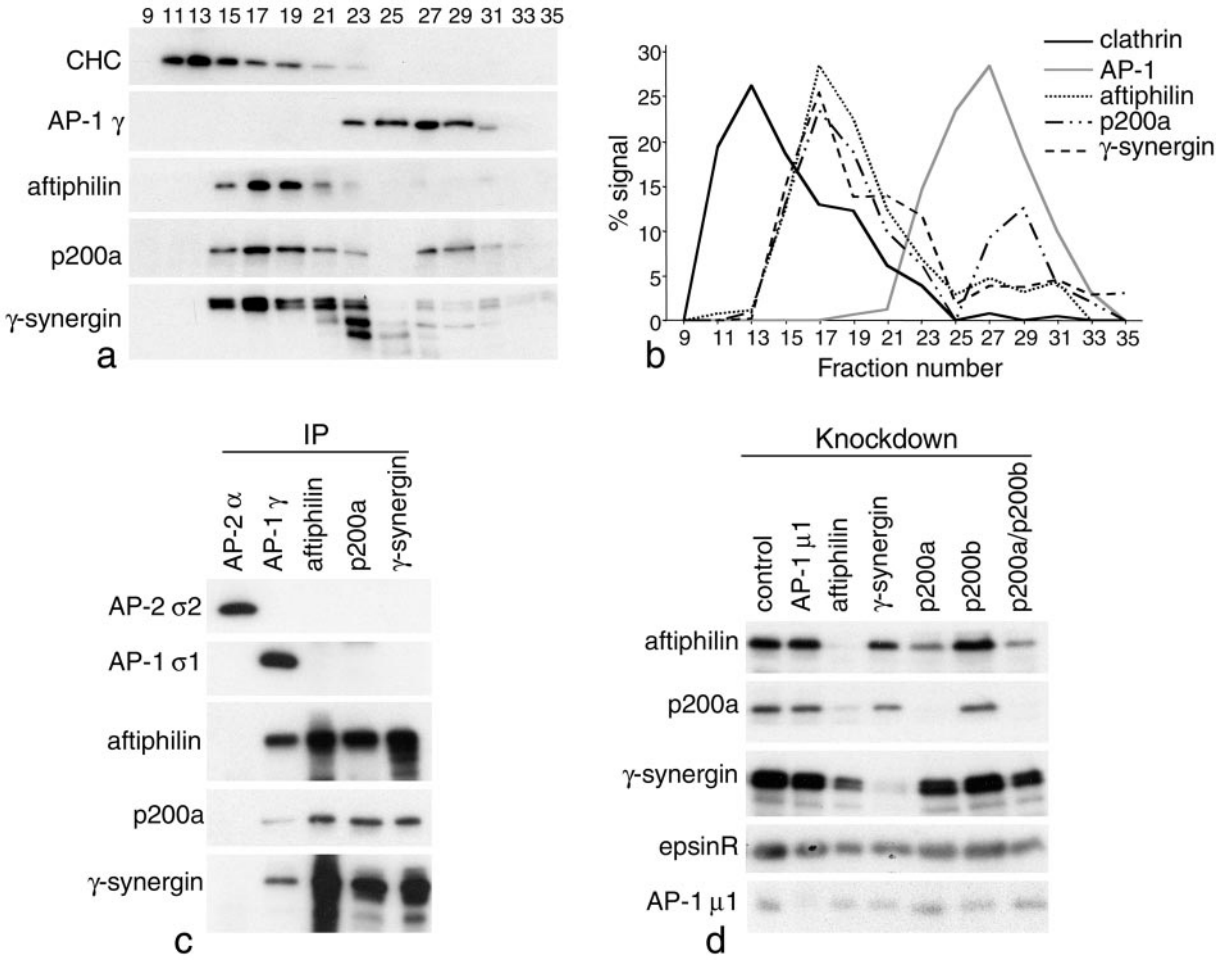

Figure 5. Evidence for a complex between aftiphilin, p200a, and γ-synergin. (a) HeLa cell cytosol was fractionated on a Superose 6 column, and fractions were probed with the indicated antibodies (CHC is clathrin heavy chain). Aftiphilin, p200a, and γ-synergin all show a strong peak in fraction 17, indicating that they form a complex that is intermediate in size between the clathrin heavy chain-light chain complex (~630 kDa) and the AP-1 complex (~270 kDa). Bovine serum albumin, Plot of the data shown in a. For γ-synergin, only the two upper bands were quantified. (c) Immunoprecipitation of NP-40 extracts of HeLa cells using the indicated antibodies. Antibodies against aftiphilin, p200a, and γ-synergin all bring down each other. (c) Effect of knocking down individual components of the complex. When any one component of the complex is knocked down, Western blots show that the remaining components are less stable.

First, proteins that have similar fractionation properties to CCVs but are not actually associated with them will also be enriched; and second, genuine CCV components might be missed if they are equally abundant in crude membranes. We have recently developed an alternative method: to isolate CCVs from HeLa cells either with or without first using siRNAs to knock down clathrin (Hirst *et al.*, 2004). In this way we can identify bona fide CCV components as proteins that are reduced or absent in the preparation from the clathrin-depleted cells. Figure 6a shows that aftiphilin, p200a, and γ-synergin are all reduced to undetectable levels in the preparation after clathrin knockdown, even though equal amounts of protein were loaded in the two lanes.

This same method can be used to investigate the function of other coat components. Figure 6b shows that depleting aftiphilin results in a concomitant loss of both p200a and γ-synergin from CCVs, while the levels of μ1 and epsinR remain unchanged. This is not simply a result of their decreased stability in aftiphilin-depleted cells; γ-synergin goes down to 14% of control levels in the CCVs, as opposed to ~40% of control levels in the whole cell homogenate.

To find out whether the aftiphilin complex helps to package particular cargo proteins into CCVs, we probed Western blots of CCVs from control and aftiphilin-depleted cells with antibodies against various membrane proteins known to be enriched in CCVs We were particularly interested in the possibility that the sorting of SCAMPs might be impaired because of the reported interaction between γ-synergin and SCAMP1 (Fernandez-Chacon *et al.*, 2000). However, neither SCAMP1 nor four other cargo proteins, the cation-independent mannose 6-phosphate receptor (CI-MPR), furin, TGN46, and the transferrin receptor (TfR), were significantly reduced from CCVs after aftiphilin depletion (Figure 6c), indicating that the complex is not an essential adaptor for any of these proteins.

Sorting of Furin and Cathepsin D

To try to establish a function for the aftiphilin/p200/γ-synergin complex, we looked at several different trafficking pathways that are known to depend upon AP-1. We have previously shown that knocking down AP-1 leads to missorting of a CD8-furin chimera (Hirst *et al.*, 2004). Normally

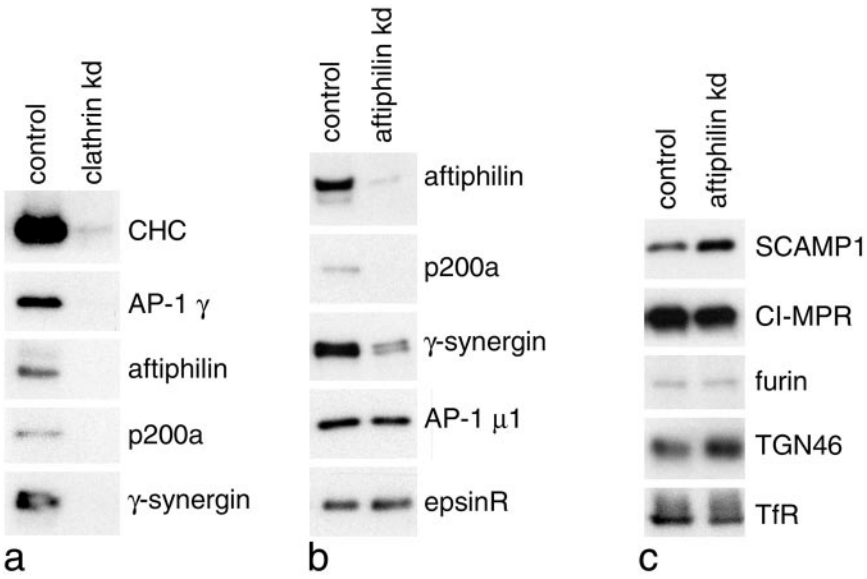

Figure 6. Enrichment of the complex in CCVs. (a) A CCV preparation was carried out using control and clathrin-depleted cells. The loss of aftiphilin, p200a, and γ-synergin from the preparation when clathrin heavy chain (CHC) is depleted indicates that they are all bona fide CCV components. Bovine serum albumin, knocking down aftiphilin causes a strong reduction in the amounts of p200a and γ-synergin in the CCV preparation. (c) Knocking down aftiphilin does not affect the enrichment of five cargo proteins in CCVs: SCAMP1, the cation-independent mannose 6-phosphate receptor, furin, TGN46, and the transferrin receptor.

this construct, like endogenous furin, cycles between the TGN and endosomes, with a steady state distribution mainly in the TGN (Figure 7a). However, knocking down AP-1 causes the construct to become much more peripheral (Figure 7b). We find that knocking down aftiphilin also causes a change in the localization of CD8-furin, causing it to become both more peripheral and more tubular (Figure 7c). These effects do not appear to be due to gross changes in TGN morphology, because the localization of TGN46 and the morphology of the TGN region by EM both look normal (unpublished observations). More subtle effects are seen when either p200a (Figure 7d) or p200b (Figure 7e) are knocked down, which become exacerbated when both p200 isoforms are knocked down together (Figure 7f).

Missorting of membrane proteins often causes them to accumulate on the cell surface, which can be quantified by an antibody binding assay. Figure 7g shows that the low levels of CD8-furin expressed on the surface of control cells go up ~11-fold when μ1 is depleted and ~4-fold when aftiphilin is depleted. Knocking down either p200a or p200b leads to a ~2-fold increase in the amount of construct on the cell surface, which goes up to ~3-fold when p200a and p200b are knocked down together. γ-Synergin knockdowns have a more modest effect, leading to a slight (<2-fold) increase, as do epsinR knockdowns, even though there is no obvious change in the distribution of the CD8-furin chimera after epsinR depletion by immunofluorescence (Hirst *et al.*, 2004, and unpublished observations).

Probably the best characterized AP-1–dependent pathway is the sorting of lysosomal enzymes via mannose 6-phosphate receptors, even though there is some controversy about precisely where AP-1 acts (Robinson, 2004). We have previously shown that knocking down AP-1 causes increased secretion of immature cathepsin D, whereas knocking down epsinR has no effect (Hirst *et al.*, 2003 and 2004). Here we carried out a similar experiment to investigate the potential role of aftiphilin and p200 in cathepsin D sorting. Cells were pulse-labeled for 15 min and chased for 2.5 h, after which cathepsin D was immunoprecipitated from both the media and the cells. Each immunoprecipitation was performed in duplicate and a representative image is shown in Figure 8a. The percentage of secreted cathepsin D relative to intracellular mature plus secreted cathepsin D was found to be 66% in the control cells, 85% in the μ1-depleted cells, 82% in the aftiphilin-depleted cells, and 78% in the p200a/p200b-depleted cells. Thus, knocking down either aftiphilin or p200 causes missorting of cathepsin D, similar to the phenotype of knocking down AP-1. However, AP-1 knockdowns have a more severe effect on mannose 6-phosphate receptor-mediated sorting, and this was also observed when we investigated the surface expression of a mannose 6-phosphate receptor chimera (see below).

Transferrin Recycling

The most striking phenotype that we have observed so far in aftiphilin-depleted cells is a change in the kinetics of transferrin recycling. Figure 9, a–f, shows cells incubated for 10 min at 37°C with transferrin coupled to AlexaFluor 594. In control cells (Figure 9a), the transferrin is localized to peripheral and perinuclear spots, presumably corresponding to early and recycling endosomes, respectively. A similar pattern is seen in μ1-depleted cells (Figure 9b). However, knocking down aftiphilin (Figure 9c), p200a (Figure 9d), p200b (Figure 9e), or both p200 isoforms together (Figure 9f) leads to a dramatic accumulation of the internalized transferrin at the cell periphery. These observations suggest that knocking down the aftiphilin/p200/γ-synergin complex, but not AP-1, causes the transferrin to accumulate in early endosomes and/or prevents it from moving to recycling endosomes.

To find out whether the knockdowns alter the rate of transferrin recycling, we incubated cells with iodinated transferrin for 1 h at 37°C to reach steady state. Surface-bound transferrin was then removed from the cells by acid stripping at 4°C, and fresh prewarmed medium was added to the cells, harvested, and replaced at regular intervals. Figure 9g shows that depleting aftiphilin causes the transferrin to be recycled more rapidly. p200 depletion has a similar but more modest effect at early time points, whereas AP-1 depletion decreases the rate of recycling. After 1 h, ~40% of the internalized transferrin is still intracellular in control and p200-depleted cells, 20% of the transferrin is still intracellular in aftiphilin-depleted cells, and 50% of the transferrin is still intracellular in μ1-depleted cells.

Because the effects of aftiphilin and p200 depletion are most pronounced at early time points, we carried out addi-

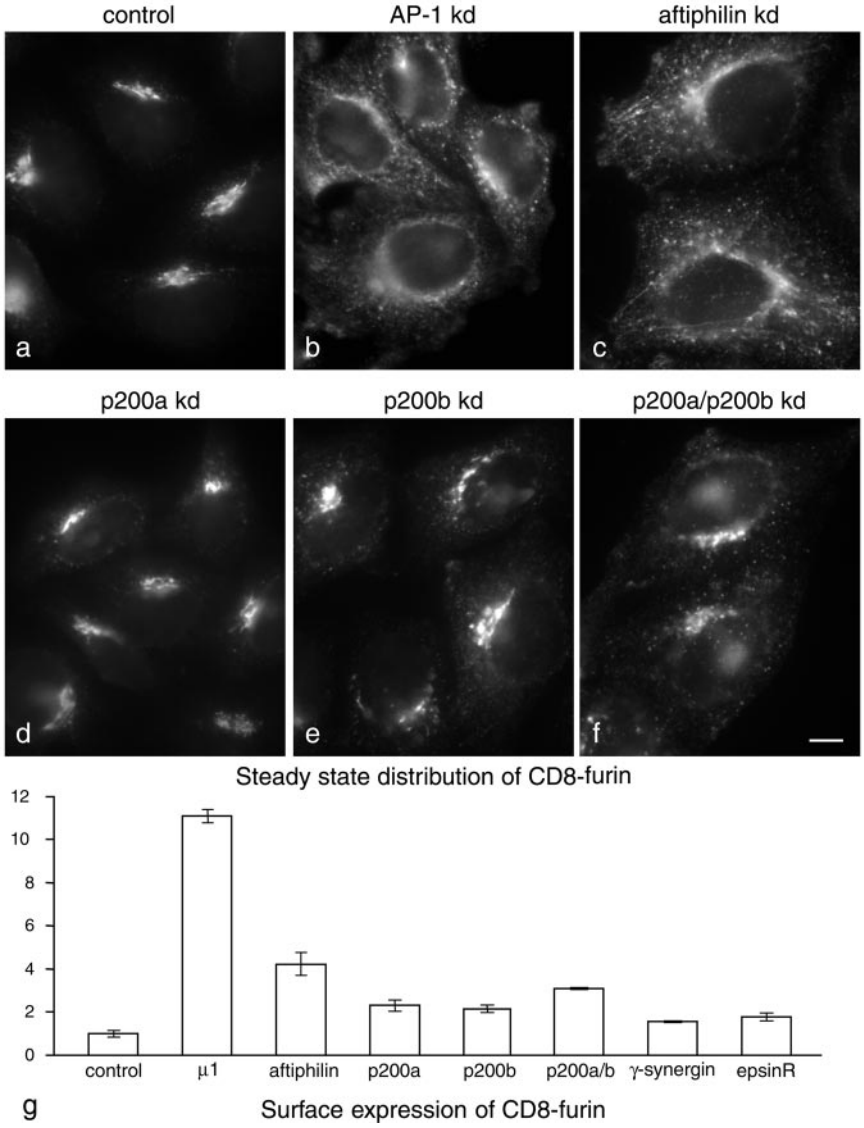

Figure 7. Steady state distribution of a CD8-furin chimera. (a–f) Localization of the chimera in control and siRNA-treated cells. The chimera normally localizes mainly to the TGN, but knocking down AP-1, aftiphilin, or p200 causes it to become more peripheral. The aftiphilin knockdown also causes the CD8-furin compartment to tubulate. Scale bar, 10 μm. (g) Surface expression of the chimera assayed using an antibody binding assay. All of the knockdowns cause an increase in the amount of chimera at the plasma membrane, with the AP-1 (μ1) knockdown having the strongest effect, followed by the aftiphilin knockdown.

tional experiments where we harvested the medium every 2 min. Figure 9h shows that although the effect of μ1 depletion is not apparent at very early time points, by 6–10 min transferrin recycling is down to ~60% of control levels. In contrast, there is a 3–4-fold increase in the amount of transferrin that gets recycled after 2–4 min in the aftiphilin-depleted cells, and a ~2-fold increase in the p200-depleted cells. These data provide further evidence that aftiphilin or p200 knockdowns may speed up the recycling of transferrin by preventing it from moving from early to recycling endosomes.

Are these changes reflected in the steady state distribution of the transferrin receptor? To quantify the amount of receptor on the cell surface, we incubated control and siRNA-treated cells at 4°C with radioiodinated transferrin. We also investigated whether depleting AP-1 or aftiphilin causes a change in the surface expression of a chimera consisting of the CD8 extracellular/lumenal domain and the cation-independent mannose 6-phosphate receptor tail (CD8-CIMPR). Figure 10 shows that knocking down AP-1 causes a significant decrease in the surface expression of transferrin receptor, and a significant increase in the surface expression of CD8-CIMPR. However, the surface expression of the two proteins in aftiphilin-depleted cells is not significantly different from controls. Thus, even though aftiphilin knockdowns cause increased secretion of both transferrin and cathepsin D, the receptors for the two ligands do not accumulate on the cell surface. This is further evidence that the AP-1 knockdown phenotype and the aftiphilin knockdown phenotype, although similar, are not identical.

DISCUSSION

As the most abundant components of purified CCVs, clathrin and the AP-1/AP-2 complexes were originally thought to be the only proteins needed for CCV formation. With the identification of >20 binding partners for AP-2, this idea has been modified, and the AP complexes are now considered to be just part of an extensive network of protein-protein interactions contributing to CCV formation. Among the binding partners for AP-2 are proteins that appear to function as cargo-selective adaptors (e.g., epsin, Dab2, ARH, β-arrestin), proteins that may facilitate membrane curvature (e.g., epsin, amphiphysin, endophilin), proteins required for vesicle scis-

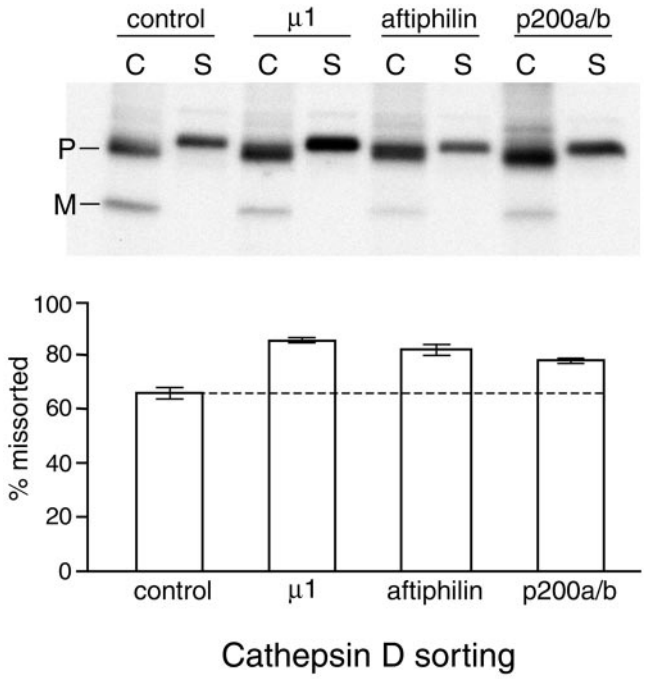

Figure 8. Cathepsin D sorting assay. Cells were pulse-labeled with ^{35}S for 15 min and chased for 2.5 h, and both cell-associated (C) and secreted (S) cathepsin D was immunoprecipitated. To calculate the % missorted, the amount of secreted cathepsin D was divided by the amount of intracellular mature plus secreted cathepsin D. Control HeLa cells already missort a significant amount of their cathepsin D, but this is increased by all three knockdowns.

sion and uncoating (e.g., dynamin, synaptojanin, auxilin), and proteins that provide links with the actin cytoskeleton and with signaling pathways (e.g., Dab2, Hip1/Hip1R, intersectin, syndapin; Praefcke *et al.*, 2004; Traub, 2003). The list of AP-1 binding partners is also starting to grow, but so far very little is known about their functions. Indeed, for many of these proteins, it is not even clear whether they have anything to do with clathrin-mediated trafficking or whether they just happen to interact with AP-1 in vitro.

Here we have investigated three such proteins: aftiphilin, p200, and γ-synergin. We find that all three proteins colocalize with AP-1 and are enriched in CCVs, indicating that they are all bona fide components of the CCV machinery. We also find that aftiphilin, p200, and γ-synergin behave as a complex: they cofractionate by gel filtration, they can be coimmunoprecipitated, and depleting any one of the three proteins using siRNAs reduces the stability of the other two. Whether the three proteins only exist as a complex, or whether they also have separate identities, is not yet clear. Both p200 and γ-synergin run as two peaks on a Superose 6 column: one high-molecular-weight peak containing all three proteins, and additional lower-molecular-weight peaks. Although this observation seems to support the idea that p200 and γ-synergin can exist outside of the aftiphilin complex, this is difficult to reconcile with the finding that both proteins show decreased stability when aftiphilin is depleted. One possibility is that the extra peaks represent complex that has been degraded. Both aftiphilin and γ-synergin are predicted to have largely disordered structures, and we have found that both are very sensitive to proteolysis, often appearing as multiple bands or streaks on Western blots even though we routinely add protease inhibitor to our buffers. Another puzzling feature of the complex is that p200 is very highly conserved, with clear orthologues in flies, worms, plants, and yeast; aftiphilin is less well conserved, although we have found likely orthologues in flies and worms, and γ-synergin appears to be expressed only in vertebrates. Thus, if p200 needs to be part of a complex for its stability, as suggested by our siRNA experiments, it would have to be able to interact with different partners in different organisms. We are currently trying to purify the aftiphilin/p200/γ-synergin complex, to get a better idea of its stoichiometry and to find out whether it contains any additional subunits.

To investigate the function of the complex, we used siRNA knockdowns to deplete the various subunits. We found that aftiphilin knockdowns have the most severe phenotype and that knocking down both p200 isoforms together appears to be more effective than knocking them down singly. Most organisms have only a single p200 gene, suggesting that gene duplication into the a and b isoforms occurred relatively recently. A search of the human EST database with the 3′ end of the two coding sequences pulled out 18 hits for p200a and only 7 hits for p200b, which suggests that p200a is expressed at higher levels. Thus, the two p200 isoforms may be functionally redundant, and the reason we only see effects on the stability of the other subunits when we knock down p200a may be because it is the more abundant isoform. γ-Synergin knockdowns have the weakest phenotype, indicating that complexes consisting only of aftiphilin and p200 are at least partially functional.

For the most part, the phenotype of depleting aftiphilin or p200 was similar to the phenotype of depleting AP-1, although less severe. Interestingly, however, knocking down AP-1 and the aftiphilin complex had opposing effects on transferrin recycling. The role of AP-1 in recycling from endosomes has only recently been appreciated; initially AP-1 was assumed to act only at the TGN. However, EM localization studies have shown that AP-1 is found not only on TGN membranes, but also on early and recycling endosomes (Futter *et al.*, 1998; Peden *et al.*, 2004). In addition, AP-1 has recently been shown to be required for the formation of recycling vesicles from endosomes in vitro (Pagano *et al.*, 2004). The present study extends these observations by showing that internalized transferrin is recycled more slowly, and the amount of transferrin receptor on the plasma membrane is decreased, when we deplete AP-1, even though the distribution of internalized transferrin looks normal. In contrast, internalized transferrin in aftiphilin- and p200-depleted cells is found mainly in peripheral endosomes, and it recycles more quickly than in controls. These observations suggest in the absence of AP-1, internalized transferrin may be unable to get into CCVs budding from both early and recycling endosomes, whereas in the absence of the aftiphilin complex, movement of internalized transferrin from early to recycling endosomes may be impeded, causing the transferrin to be recycled by the faster, more direct pathway from early endosomes back to the plasma membrane.

Why would the two knockdowns have different phenotypes, if the aftiphilin complex acts together with AP-1? So far we can only speculate, but perhaps the simplest explanation is that aftiphilin may only facilitate AP-1 function on certain membranes. Our aftiphilin antibodies are not good enough to determine whether or not the protein colocalizes completely or only partly with AP-1; however, we do see colocalization not only on perinuclear membranes but also on more peripheral membranes. In addition, we have previously shown that γ-synergin has near-perfect colocaliza-

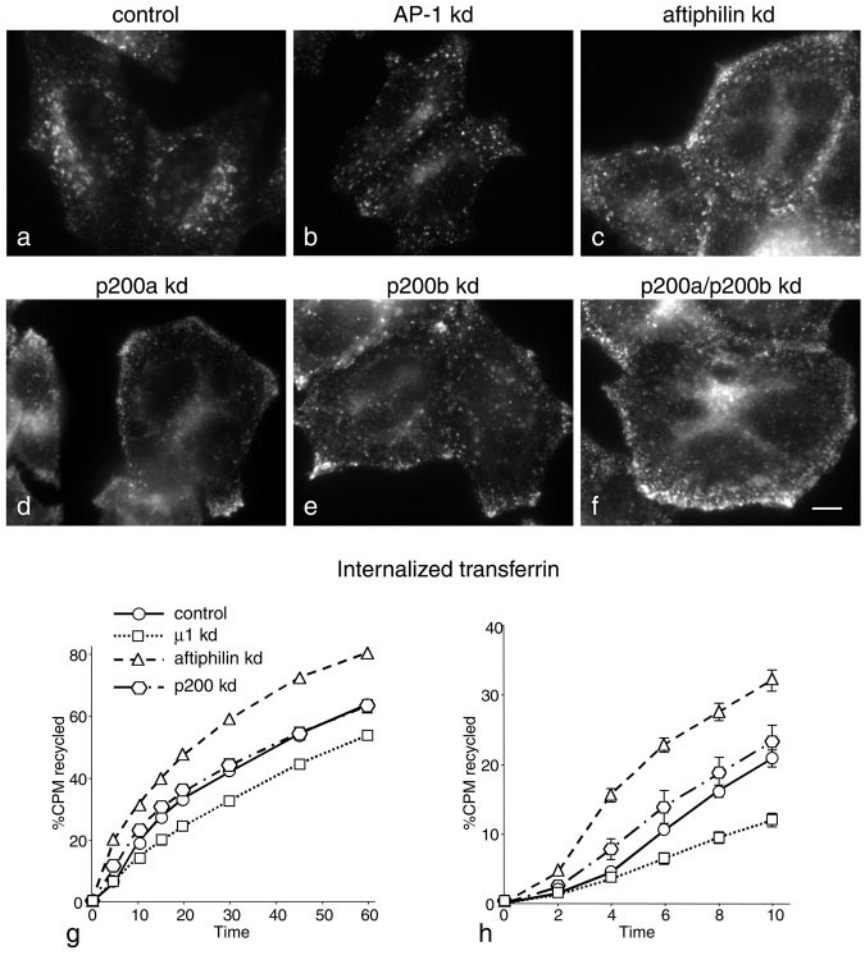

Figure 9. Effect of knockdowns on transferrin receptor recycling. (a–f) Localization of AlexaFluor 594–conjugated transferrin internalized for 10 min. In control and AP-1–depleted cells, the transferrin is present in both peripheral and perinuclear endosomes, but aftiphilin and p200 depletion cause the transferrin to accumulate mainly in peripheral endosomes. Scale bar, 10 μm. (g) Kinetics of transferrin recycling. Cells were allowed to endocytose ^{125}I-labeled transferrin for 1 h and then surface-bound transferrin was removed and recycled transferrin was assayed by collecting the medium at regular intervals. Aftiphilin and (to a lesser extent) p200 knockdowns increase the rate of recycling, whereas AP-1 knockdowns decrease the rate of recycling. (h) Early time points in a recycling assay similar to the one shown in g. The effects of the aftiphilin and p200 knockdowns are most pronounced in the first few minutes.

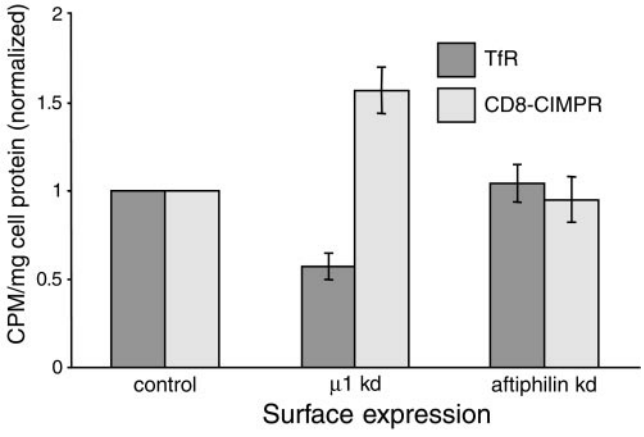

Figure 10. Surface expression of the transferrin receptor and of a CD8-CIMPR chimera assayed by binding either transferrin or anti-CD8 to the cells at 4°C. Results are pooled from two separate experiments for each condition, carried out in triplicate on different days. The AP-1 (μ1) knockdown causes a decrease in the amount of transferrin receptor at the cell surface, and an increase in the amount of CD8-CIMPR chimera at the cell surface. However, the aftiphilin knockdown does not have a significant effect on the surface expression of either protein.

tion with AP-1 (Page *et al.*, 1999; Lui *et al.*, 2003), and because γ-synergin and aftiphilin are part of the same complex, this suggests that aftiphilin is also found on all AP-1-positive membranes. A second—and perhaps more likely—possibility is that AP-1 and the aftiphilin complex may select different types of cargo. If the cargo includes proteins that actually contribute to the trafficking process, such as SNAREs, there may be secondary effects depending on which SNAREs get mislocalized. A third possibility is that the aftiphilin complex may act via GGAs as well as AP-1. Although aftiphilin, p200, and γ-synergin all show a preference for AP-1 over GGAs in pulldowns and although we have previously shown that γ-synergin colocalizes with AP-1 and not with GGAs in nocodazole-treated cells (Lui *et al.*, 2003), knocking down AP-1 might force the aftiphilin complex to associate with GGAs, and this could contribute to the difference between the two knockdown phenotypes.

What might be the function of the complex? One possibility is that, like several of the other AP binding partners, it may be a cargo-selective adaptor. The complex does not appear to be able to act independently of AP-1, because it becomes cytosolic in AP-1–depleted cells, but it does have its own binding sites for clathrin, including an atypical motif that is present in both splice variants of aftiphilin. Candidate cargo proteins for the aftiphilin complex include the SCAMPs, polytopic membrane proteins with multiple NPF motifs that bind to the EH domain of γ-synergin (Fernandez-Chacon *et al.*, 2000). Although SCAMP1 is still packaged

normally into CCVs in aftiphilin-depleted cells, it may contain other sorting signals in addition to the NPF motifs (e.g., it has five YXXΦ sequences in its cytoplasmic domains). Another possibility is that the aftiphilin complex might provide some sort of link with the cytoskeleton. This idea is supported by the phenotype of the aftiphilin-depleted cells, which have tubulated CD8-furin–containing membranes and peripherally localized transferrin, suggesting that there may be aberrant connections with microtubules and cortical actin, respectively. These two possibilities need not be mutually exclusive: Dab2 is an example of a protein that is part of the AP-2 interaction network, which acts both as a cargo adaptor for members of the LDL receptor family and as a link to the cytoskeleton by binding to myosin VI (Morris *et al*., 2002). Thus, the present study establishes that aftiphilin, together with its partners p200 and γ-synergin, plays a role in AP-1-mediated trafficking. However, further studies will be needed to define the precise function of the aftiphilin complex.

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