Assembly and Targeting of Adaptin Chimeras in Transfected Cells

Margaret S. Robinson

Department of Clinical Biochemistry, University of Cambridge, Addenbrookes Hospital, Hills Road, Cambridge CB2 2QR, England

Abstract. Adaptors are the components of clathrin-coated pits and vesicles that attach the clathrin to the membrane. There are two types of adaptors in the cell: one associated with the plasma membrane and one associated with the TGN. Both adaptors are heterotetramers consisting of two adaptins (α and β for the plasma membrane; γ and β' for the TGN), plus two smaller proteins. The COOH-terminal domains of the adaptins form appendages that resemble ears, connected by flexible hinges. Unlike the other adaptor components, the COOH termini of the α- and γ-adaptins show no homology with each other, suggesting that they might provide the signal that directs the adaptors to the appropriate membrane. To test this possibility, the COOH-terminal ears were switched between α- and γ-adaptins and were also deleted. All of the constructs contained the bovine γ-adaptin hinge, enabling them to be detected with a species-specific antibody against this region when transfected into rat fibroblasts. Immunoprecipitation indicated that the engineered adaptins were still fully capable of assembling into adaptor complexes. Immunofluorescence revealed that in spite of their modified ears, the constructs were still able to be recruited onto the appropriate membrane; however, the ear-minus constructs gave increased cytoplasmic staining, and replacing the γ-adaptin ear with the α-adaptin ear caused a small amount of colocalization with endogenous α-adaptin in some cells. Thus, the major targeting determinant appears to reside in the adaptor "head," while the ears may stabilize the association of adaptors with the membrane.

ADAPTINS are proteins of ~100 kD which are associated with clathrin-coated vesicles. There are three classes of adaptins: α, β, and γ. In the cell, adaptins interact with other proteins to form heterotetrameric protein complexes known as adaptors, consisting of one β-adaptin subunit, one α- or γ-adaptin subunit, one medium chain of ~50 kD (AP50 or AP47), and one small chain of ~20 kD (AP17 or AP19) (see Pearse and Robinson, 1990; Robinson, 1992). Two types of adaptors have been identified, one associated with the plasma membrane and one associated with the TGN (Robinson, 1987; Ahle et al., 1988). The subunit composition of the plasma membrane adaptor is α-adaptin + β-adaptin +. AP50 + AP17, while the subunit composition of the TGN adaptor is γ-adaptin + β'-adaptin + AP47 + AP19 (Keen, 1987; Ahle et al., 1988). In vitro studies indicate that adaptors bind not only to clathrin, but also to the cytoplasmic domains of membrane proteins known to be concentrated in coated pits, such as the LDL receptor and the mannose-6-phosphate receptor (Pearse, 1988; Glickman et al., 1989). However, it seems unlikely that these interactions target the adaptors to the appropriate compartment, as the distribution of such membrane proteins is much more widespread than the disruption of the adaptors. Thus, the mechanism whereby adaptors are recruited onto the plasma membrane or the TGN is still unknown.

One approach that has been used to investigate the targeting of other molecules is the construction of chimeras, in which domains are switched between two proteins with different destinations. Because all the adaptor subunits have been cloned and sequenced, such an approach is now feasible as a means of locating the targeting signal on the adaptor complex. Sequencing reveals that the β-adaptin subunits of the plasma membrane and TGN adaptors are the most homologous, with 85% sequence identity (Kirchhausen et al., 1989; Ponnambalam et al., 1990). The medium chains are 40% identical (Nakayama et al., 1991), while the small chains are 45% identical (Kirchhausen et al., 1991). Least homology is seen between the α- and γ-adaptins, with only 25% identity overall and with COOH termini that appear to be completely unrelated (Robinson, 1989, 1990).

Construction of chimeras is facilitated by our knowledge of adaptor structure. The plasma membrane adaptor complex appears in the electron microscope as a brick-like core or "head" flanked by two smaller appendages or "ears," connected by stalks which are thought to act as flexible hinges (Heuser and Keen, 1988) (see the model in Fig. 8). Controlled proteolysis cuts at the hinges and releases the two ears. Sequencing of the ear fragments has shown that the ears correspond to the COOH-terminal domains of the proteins, while the hinges correspond to stretches of ~100 amino acids about two thirds of the way along the proteins with a high abundance of proline and glycine residues (Kirchhausen et al., 1989). Although the TGN adaptor complex has not yet been viewed by EM, its proteolytic cleavage pattern and

the amino acid content of its putative hinge region suggest that it has a structure similar to that of the plasma membrane adaptor (Schroder and Ungewickell, 1991; Robinson, 1990).

Here I report experiments in which the COOH-terminal ears were switched between the α- and γ-adaptins, and were also completely deleted. The COOH-terminal domains of α and γ were chosen for two reasons. First, because they do not appear to interact with the other subunits of the adaptor complex, it should be possible to modify them without affecting adaptor assembly; and second, because of their lack of homology with each other, it seems likely that they are required for functions specific to the plasma membrane and TGN adaptors. The constructs were all designed to contain the bovine γ-adaptin hinge, allowing them to be detected with a species-specific antibody, mab 100/3, when transfected into Rat 1 fibroblasts, since the antibody reacts with the γ-adaptin hinge from most mammals but does not recognize the rodent protein (Ahle et al., 1988; Robinson, 1990). The antibody proved useful both for localizing the constructs by immunofluorescence and for immunoprecipitating adaptor complexes containing the engineered adaptins.

Materials and Methods

Construction of Plasmids

Most of the DNA manipulations that were carried out followed protocols described by Sambrook et al. (1989). The strategy that was adopted for the construction of chimeras was to introduce new restriction sites on either side of the hinge-encoding sequences in α-adaptin (αC) and γ-adaptin (see Fig. 1). The hinge was defined as amino acids 620–700 in α and 594–703 in γ, based on the abundance of prolines and glycines in these regions and the optimum alignment of α- and γ-adaptins. In both proteins the hinge begins with the sequence GP and ends with the sequence PG, allowing ApaI and SmaI sites to be introduced into the DNA without changing the sequence of the protein products (see Robinson, 1989, 1990).

Both the α-adaptin and the γ-adaptin cDNAs had been subcloned into the EcoRI site of pBluescript SK-. The γ-adaptin that was used was a mouse-cow chimera, joined at a PstI site near the middle of the cDNA, which contains the epitope recognized by mAb 100/3 (Robinson, 1990). Because there are already endogenous ApaI and SmaI sites in both plasmids, it was first necessary to eliminate some of them. The cDNA encoding α-adaptin contains an ApaI site at precisely the right position (base 1857). There are two SmaI sites in the α-adaptin plasmid that needed to be eliminated, however: one in the 5' untranslated region and another in the polylinker of the vector upstream from the insert. These were removed by cutting with SmaI and BamHI (which only cuts in the polylinker, upstream from the SmaI site), blunting, and religating the plasmid. The same strategy was used to remove SmaI sites in the 5' untranslated region and polylinker of the γ-adaptin plasmid. In addition, it was necessary to remove an ApaI site in the 3' untranslated region of the γ-adaptin plasmid and another ApaI site in the polylinker downstream from the insert. This was achieved by cutting with ApaI, blunting, and religation.

New restriction sites were engineered into the constructs by oligonucleotide-directed mutagenesis. Three mutagenic primers were synthesized: Oligo 1, containing one mismatch for introducing the SmaI site into α-adaptin at base 2025; Oligo 2, containing one mismatch for introducing the ApaI site into γ-adaptin at base 1779; and Oligo 3, containing three mismatches for introducing the SmaI site into γ-adaptin at base 2104. The sequences of the three oligonucleotides are shown below:

Oligo 1: TTGCACCCGGGTCTGAAGACA
Oligo 2: AGTGACCACAAAGGGCCCTACTGAG
Oligo 3: AATGACATTGCTCCCGGGATCCCCTCCATC

Single-stranded DNA was prepared either by using the helper phage K07 (for introducing the SmaI site into γ-adaptin) or by subcloning into M13, using a SacI-EcoRI fragment for γ-adaptin and a KpnI fragment for α-adaptin, both of which contained the region to be mutagenized (see Fig. 1). The mutagenic primers were annealed to the single stranded DNA and second strands were synthesized, ligated, and used to transform TG2 host cells.

Mutant colonies or plaques were identified by hybridizing with the mutagenic oligonucleotides at different stringencies and were sequenced to check that the correct mutations had been engineered into the constructs. In addition, most of the rest of the DNA was re-sequenced to ensure that no errors had been introduced when the second strand was copied. The two fragments were then religated back into the original plasmids to create plasmids pαAS and pγAS (see Fig. 1).

To construct the chimera γγα, plasmids pγAS and pαAS were cut with EcoRI, which cuts near the 3' end of the insert in pγAS and in the polylinker downstream from the insert in pαAS, and SmaI, and the insert from pαAS was ligated into the pγAS vector. The chimera αγγ was made by cutting pγAS and pαAS with NotI, which cuts in the polylinker upstream from the insert in both plasmids, and ApaI, after which the insert from pαAS was ligated into the pγAS vector. The chimera αγα was constructed by cutting the plasmid containing αγγ and pαAS with SmaI and EcoRI and inserting the insert from pαAS into αγγ.

The chimeras were then subcloned into pHYKS3 (Robinson, 1990), an expression vector containing the SV-40 early promoter and a polylinker with unique cloning sites for HindIII, BglII, NotI, KpnI, and EcoRI. NotI and EcoRI were used to make the expression plasmids pγγα and pαγα. To construct the expression plasmid pαγγ, the insert needed to be moved in two pieces because of the presence of an endogenous EcoRI site in the coding region and the destruction of the EcoRI site in the polylinker. First a 5' NotI-KpnI fragment was subcloned, followed by the 3' KpnI fragment (see Fig. 1). The ear deletion constructs were prepared from pαγα and pγγα using Oligo 4, a double stranded oligonucleotide containing a stop codon and an EcoRI site:

Oligo 4: GGGTAGGAATTCCTACCC

The oligonucleotide was annealed to itself (not that it is a palindrome), kinased, and ligated into pαγα and pγγα, both of which had been cut with SmaI and phosphatased, to make pαγ- and pγγ-. Aliquots of the plasmids were cut with EcoRI to ensure that the double stranded oligonucleotide had been successfully ligated.

Transfection into Rat 1 Cells

Rat 1 fibroblasts in culture were transfected with all the above constructs by the calcium phosphate method, as previously described (Robinson, 1990). Transiently expressing cells were examined 2 d after transfection. To make stable transfectants, the cells were co-transfected with the adaptin chimeras and pRSVneo, a plasmid containing the G418 resistance gene downstream from the RSV promoter, constructed and generously provided by P. Goodell (MRC Laboratory of Molecular Biology, Cambridge, England). After 2 d in culture, the cells were trypsinized and plated into three 9-cm-diam tissue culture dishes for each 25-cm² flask. On day three, G418 was added at a concentration of 0.5 mg/ml. Medium containing 0.5 mg/ml G418 was changed every 3 d. After 2 wk, individual G418-resistant colonies were picked with sterile yellow tips and transferred to multiwell plates. Expression of adaptins was assayed by immunofluorescence (see below).

Detection of Constructs with mAb 100/3

Immunofluorescence. Cells on multiwell test slides were fixed in methanol and acetone, as previously described (Robinson, 1990). Immunofluorescence was carried out using the mouse anti-γ-adaptin antibody mAb 100/3 (Ahle et al., 1988), and some cells were double labeled with either a rabbit antiserum against TGN38 (Luzio et al., 1990) or antibody C8. Antibody C8 is a rabbit antiserum raised against a pGEX fusion protein containing part of the hinge region of αC (amino acids 619–656) and absorbed with a fusion protein containing the comparable part of αA before affinity purification to make it monospecific (Ball, C. L., and M. S. Robinson, manuscript in preparation). Antibody incubations, labeling with secondary antibodies, mounting, and examination of the slides were all carried out as previously described (Robinson, 1990).

Western Blotting. Stably transfected cells were grown in 9-cm-diam dishes until confluent, and similar cultures were prepared for Vero cells (positive control) and nontransfected Rat 1 cells (negative control and positive control for endogenous α-adaptin). Two dishes were pooled for each cell line. The cells were trypsinized, washed, and resuspended in 150 μl lysis buffer (Buffer A [0.1 M MES, pH 6.5, 0.2 mM EGTA, 0.5 mM MgCl₂, 0.02% NaN₃] containing 1% NP-40, 10 mg/ml BSA, 1 mM PMSF, 0.4 mg/ml BPTI, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). After a 5-min incubation at room temperature, the cells were spun at 50,000 RPM for 15 min in a Beckman micro-ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA). The pellets were resuspended in the same volume of lysis

buffer as the supernatants, and the samples were boiled with an equal volume of 2× SDS-PAGE and sonicated before being subjected to SDS-PAGE. Gels were blotted onto nitrocellulose and labeled either with mAb 100/3 followed by rabbit anti-mouse IgG and ^{125}I-protein A (for endogenous γ-adaptin and the constructs), or with mAb AC1-M11 followed by ^{125}I-protein A (for endogenous α-adaptin), as previously described (Robinson, 1987; Robinson and Kreis, 1992).

Immunoprecipitation. The immunoprecipitation protocol was based on a method developed by B. Pearse (personal communication), which involves extracting a high speed pellet of detergent-lysed cells with Na_2CO_3 and then precipitating adaptors from the extract. Three 9-cm-diam dishes of confluent cells were used for each stably transfected cell line and for the positive and negative controls (see above). The cells were grown overnight in 15 ml methionine-free medium per dish, containing 3% dialyzed FCS and 20 μCi/ml Tran^{35}S Label (Amersham International, Amersham, UK). The following morning, the cells were trypsinized, pooled, washed, and resuspended in 300 μl lysis buffer. After a 5-min incubation at room temperature, the cells were spun 5 min in a microfuge and the supernatants were then spun at 50,000 RPM for 15 min in a Beckman micro-ultracentrifuge. Pellets from this step were resuspended in 150 μl lysis buffer to which 7.5 μl 1 M Na_2CO_3 was then added. After a 5-min incubation on ice, the samples were spun at 50,000 RPM for 15 min in the micro-ultracentrifuge, after which the supernatants were incubated on ice with 7.5 μl 1 M KH_2PO_4 for 1 h. These samples were then spun at 70,000 RPM for 20 min in the micro-ultracentrifuge. Supernatants were mixed with an equal volume of lysis buffer, and 100 μl aliquots were incubated with 2 μl mAb 100/3 (the antibody concentration had been estimated to be 0.5 mg/ml). After a 1-h incubation at room temperature, the samples were spun for 5 min in a microfuge, and the supernatants were incubated for a further 10 min with 0.3 μl rabbit anti-mouse IgG (Sigma Immunochemicals, St. Louis, MO). Protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) (30 μl of a 50% slurry) was then added and the samples were incubated for another hour. All incubations involving antibodies were carried out on a rotator. The Sepharose was then pelleted by a 1-min spin in the microcentrifuge, and the pellets were extensively washed: first 3× in 0.15 M NaCl, 50 mM Tris, pH 8.2, 2 mM EDTA, and 0.2% NaN_3, containing the same concentrations of NP-40, BSA, and protease inhibitors as the lysis buffer; then 3× in the above solution but with 0.5 M NaCl; and finally 2× in the high NaCl buffer but without the extra additions. The final pellets were resuspended in 15 μl 2× sample buffer, boiled, and subjected to SDS-PAGE. Gels were fluorographed, dried down, and autoradiographed.

Immunoprecipitation was also carried out on the high speed supernatants. Although the samples were heavily contaminated with additional proteins (also seen in the negative control lane), for each cell line the ratios of the adaptin constructs to the other adaptor subunits in the immunoprecipitates from the high speed supernatants appeared to be similar to the ratios in the immunoprecipitates from the extracted high speed pellets.

Results

Localization of adaptin Constructs

To investigate the possible role of the α- and γ-adaptin ears in adaptor targeting, new restriction sites were engineered into both adaptin cDNAs on either side of the hinge, allowing the hinges and/or ears to be either exchanged between the two proteins or deleted (Fig. 1). The nomenclature for the constructs refers to their three domains: NH_2 terminus, hinge, and ear. All of the constructs contain the bovine γ-adaptin hinge to enable them to be detected by mAb 100/3, a species-specific antibody that recognizes the hinge region of the protein but does not react with endogenous γ-adaptin in rodent cells (Ahle et al., 1988; Robinson, 1990).

Rat 1 fibroblasts were transfected both with normal γ-adaptin and with the five new constructs, and were examined by immunofluorescence. The distribution of the constructs was first studied in transiently expressing cells, but because the transfection efficiency was low, stably transfected cells were generated by co-transfecting with a plasmid containing the G418 resistance gene. Although some of the cell lines showed heterogeneous patterns of expression, it was possible to obtain lines for all of the constructs in which the majority of the cells were expressing at comparable levels. Fig. 2 shows immunofluorescence staining of Rat 1 fibroblasts stably transfected with the normal γ-adaptin construct (Fig. 2 *a*) and the constructs γγα (Fig. 2 *b*) and γγ- (Fig. 2 *c*), double labeled with a rabbit antiserum against TGN38 (Fig. 2, *d–f*), an integral membrane protein of the TGN (Luzio et al., 1990). Normal γ-adaptin (Fig. 2 *a*) has a perinuclear distribution in the cell, similar to that of TGN38 (Fig. 2 *d*), but more punctate and extending to more peripheral regions of the cell. This is the same sort of labeling that is seen in cells stained for endogenous γ-adaptin (see Ahle et al., 1988; Robinson, 1990).

Fig. 2 *b* shows the distribution of the construct γγα, containing the γ-adaptin NH_2 terminus and hinge, but with the α-adaptin ear. The distribution of the construct is very simi-
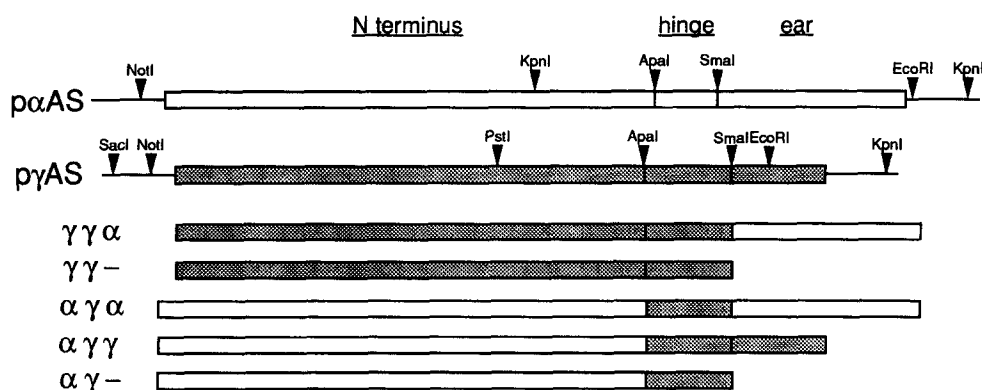

Figure 1. Construction of adaptin chimeras. Oligonucleotide-directed mutagenesis was used to introduce ApaI and SmaI sites on either side of the hinge in the α- and γ-adaptin cDNAs. The resultant plasmids, pαAS and pγAS, were cut and re-ligated to produce the five new constructs: γγα, γγ-, αγα, αγγ, and αγ-. The nomenclature refers to the three domains, NH_2 terminus, hinge, and ear; α sequences are white and γ sequences are stippled. All the restriction sites shown in the figure were used for subcloning and are mentioned in the text.
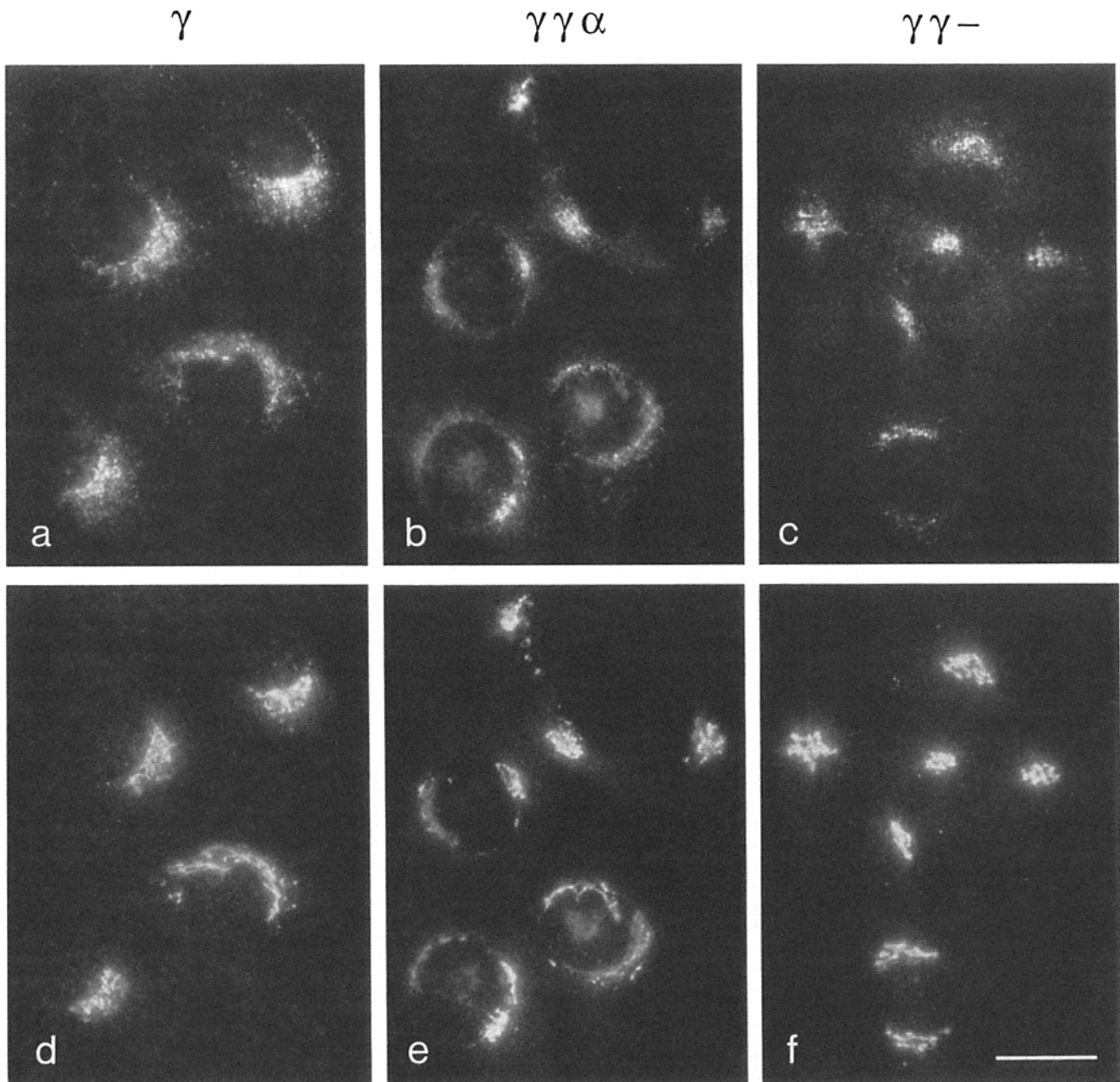
$$\gamma \qquad \gamma\gamma\alpha \qquad \gamma\gamma-$$

Figure 2. Immunofluorescence localization of constructs containing the γ-adaptin NH$_2$ terminus, double labeled for TGN38. Stably transfected Rat 1 cells were double labeled with mAb 100/3 (*a–c*) and an antibody against TGN38 (*d–f*). The cells in a and d were transfected with normal γ-adaptin containing the epitope recognized by mAb 100/3, *b* and *e* with $\gamma\gamma\alpha$, and *c* and *f* with $\gamma\gamma-$. All of the constructs are targeted to the TGN. Bar, 20 μm.

lar to that of normal γ-adaptin, although careful examination of the cells suggests that there may be a slight increase in the relative amount of cytoplasmic and/or peripheral staining.

In Fig. 2 *c*, the localization of the ear-minus γ-adaptin construct, $\gamma\gamma-$, is shown. There is a marked increase in the relative amount of cytoplasmic staining when compared with the other two constructs. This is not related to the level of expression of the construct: in both high-expressing and low-expressing cell lines, the amount of cytoplasmic staining relative to perinuclear staining is consistently much higher than in cells expressing either γ or $\gamma\gamma\alpha$. Nevertheless, the labeling pattern indicates that at least some of the truncated pro-

tein is associated with the TGN compartment. Thus, all of the constructs containing the γ-adaptin NH$_2$ terminus and hinge are able to be correctly targeted to the TGN.

The localization of the construct $\alpha\gamma\alpha$, which contains the α-adaptin NH$_2$ terminus and ear, but with the γ-adaptin hinge, is shown in Fig. 3 *a*. Numerous dots are seen extending out to the cell periphery, often aligned in rows. There is no apparent concentration of the label in the Golgi region (see Fig. 3 *d*), and focusing up and down on the cells indicates that the dots are in the plane of the plasma membrane. Thus, $\alpha\gamma\alpha$ has a similar distribution to that of endogenously expressed wild-type α-adaptin (see Robinson, 1987), indi-

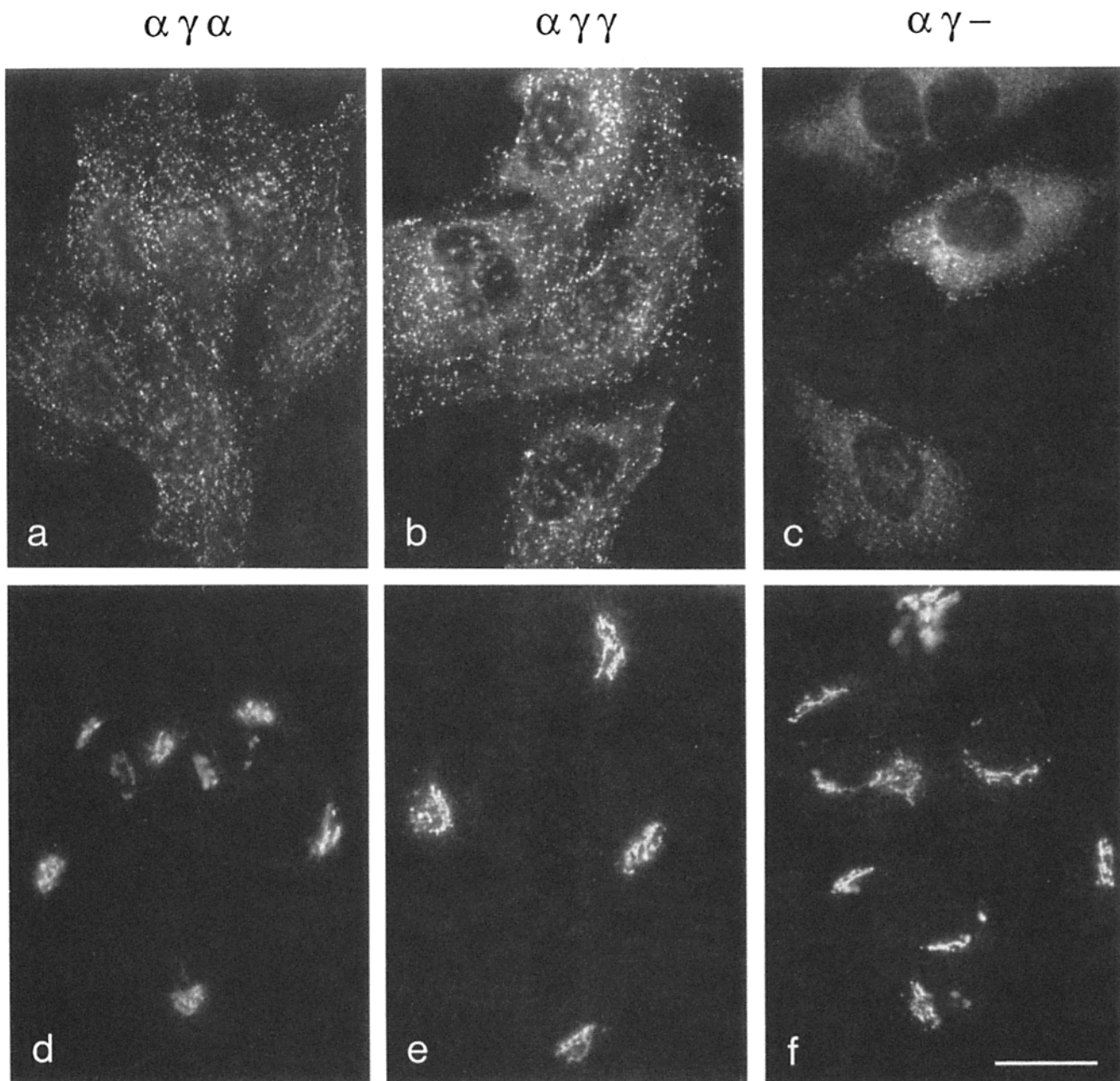α γ α   α γ γ   α γ −

Figure 3. Immunofluorescence localization of constructs containing the α-adaptin NH₂ terminus, double labeled for TGN38. Stably transfected Rat 1 cells were double labeled with mAb 100/3 (*a–c*) and an antibody against TGN38 (*d–f*). The cells in *a* and *d* were transfected with αγα, *b* and *e* with αγγ, and *c* and *f* with αγ–. All of the constructs show punctate labeling extending out to the cell periphery without any obvious colocalization with TGN38, indicating that they are able to be targeted to the plasma membrane, although αγγ shows increased cytoplasmic staining and αγ– appears to be predominantly cytoplasmic (especially in the upper two cells). Some non-expressing cells are seen in *c*. Bar, 20 μm.

cating that replacing the α-adaptin hinge with the γ-adaptin hinge does not affect the localization of the protein.

When the γ-adaptin ear is transplanted onto α-adaptin (Fig. 3 *b*), the staining pattern looks somewhat different from that of the endogenous protein or of αγα. Increased cytoplasmic staining is seen, particularly around the nucleus. However, when the cells are double labeled for TGN38 (Fig. 3 *e*), the construct does not appear to be especially concentrated in the Golgi region: the TGN38 labeling tends to be on one side of the nucleus, while the αγγ labeling is more

uniformly distributed and probably reflects an increase in the cytoplasmic pool of the protein which accumulates where the cell is thickest.

A larger increase in the relative amount of cytoplasmic staining is seen in cells expressing the ear-minus construct αγ– (Fig. 3 *c*). These cells tended to be somewhat heterogeneous in their labeling patterns, but in most of them a characteristic α-adaptin staining pattern can be seen (e.g., in the lower cell in Fig. 3 *c*), although it is often obscured by the cytoplasmic staining (e.g., in the upper cells in Fig. 3 *c*).
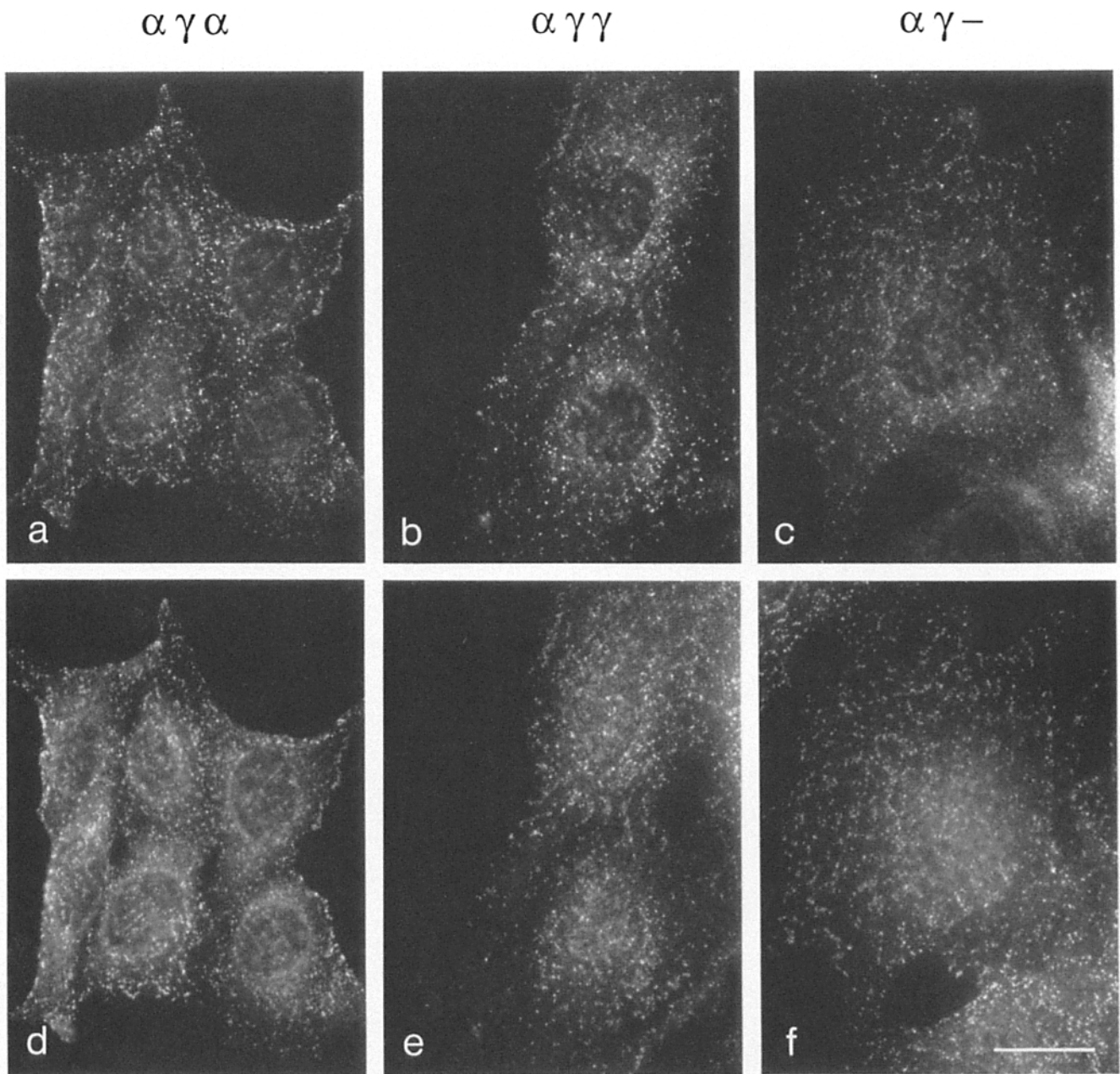
$\alpha\gamma\alpha$ $\alpha\gamma\gamma$ $\alpha\gamma-$

Figure 4. Immunoflourescence localization of constructs containing the α-adaptin NH_2 terminus, double labeled for endogenous α-adaptin. Stably transfected Rat 1 cells were double labeled with mAb 100/3 (*a-c*) and an antibody that recognizes only endogenous α-adaptin (*d-f*). The cells in *a* and *d* were transfected with $\alpha\gamma\alpha$, *b* and *e* with $\alpha\gamma\gamma$, and *c* and *f* with $\alpha\gamma-$. The fluorescent dots labeled with the two antibodies coincide (a particularly well spread cell is shown in *c*, so the characteristic cytoplasmic distribution of $\alpha\gamma-$ is less apparent). Thus, all the constructs with the α-adaptin NH_2 terminus are able to be incorporated into plasma membrane coated pits. Some non-expressing cells are seen in *b* and *c*. Bar, 20 μm.

Thus, although much of the labeling is cytoplasmic, a fraction of the construct appears to be capable of associating with the plasma membrane.

Colocalization with Endogenous α-Adaptin

To confirm that the constructs $\alpha\gamma\alpha$, $\alpha\gamma\gamma$, and $\alpha\gamma-$ are associated with plasma membrane coated pits, cells were double labeled for endogenous α-adaptin. This was achieved by using a rabbit antibody raised against a fusion protein containing the α-adaptin hinge, which does not cross react with any of the constructs since they all contain the γ-adaptin

hinge (see Fig. 1). Fig. 4 shows the three cell lines expressing constructs containing the α-adaptin NH_2 terminus (Fig. 4, *a-c*), double labeled with the antibody against endogenous α-adaptin (Fig. 4, *d-f*). In all cases, the fluorescent dots coincide, confirming that these constructs are correctly targeted to the plasma membrane and incorporated into coated pits.

Because the cells expressing γ-adaptin NH_2-terminal constructs always show a certain amount of punctate labeling which does not coincide with TGN 38, these cells were also double labeled for endogenous α-adaptin to see whether there was any colocalization. As shown in Fig. 5, there is no

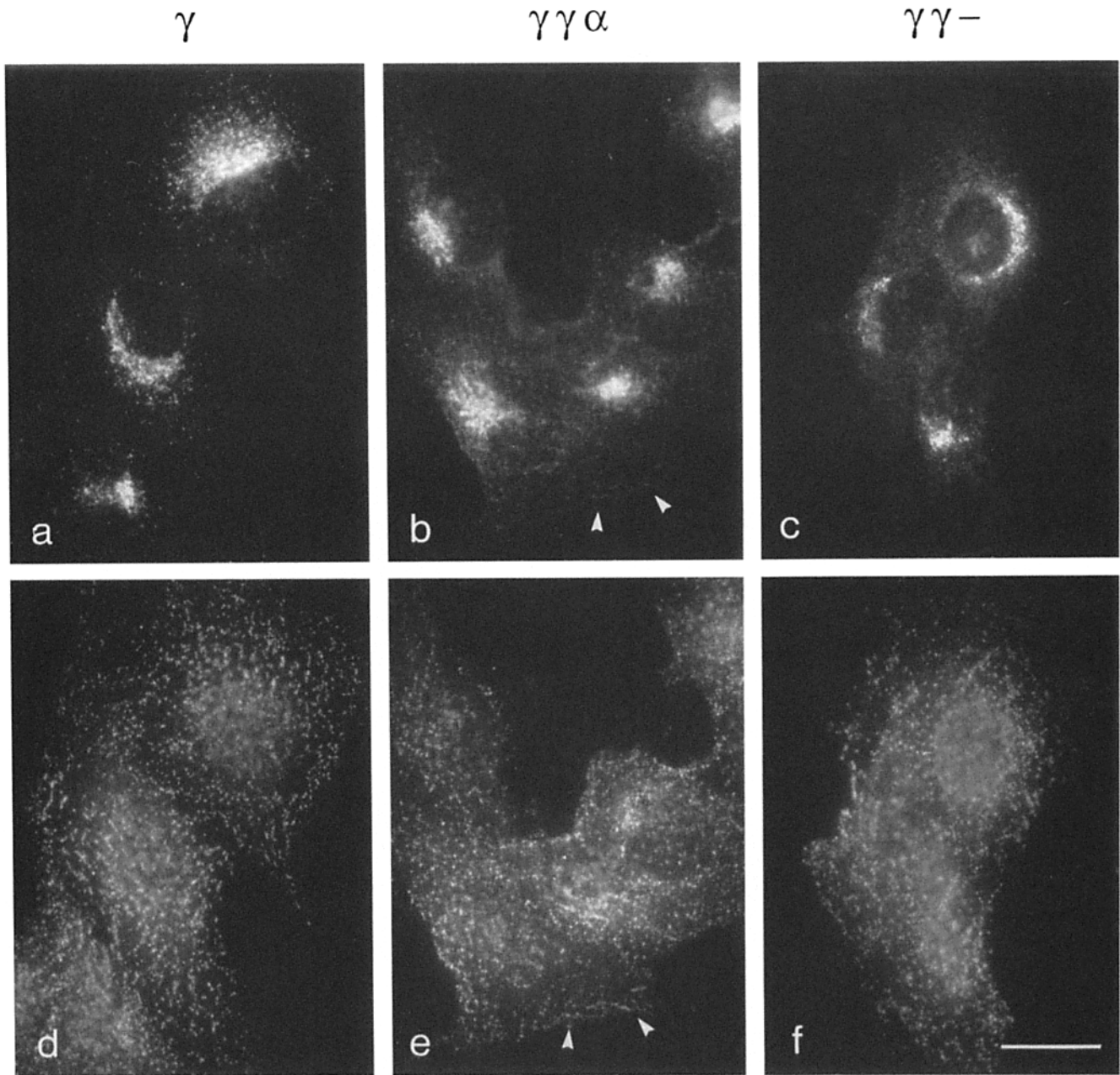$$\gamma \qquad \gamma\gamma\alpha \qquad \gamma\gamma-$$

Figure 5. Immunofluorescence localization of constructs containing the γ-adaptin NH_2 terminus, double labeled for endogenous α-adaptin. Stably transfected Rat 1 cells were double labeled with mAb 100/3 (*a–c*) and an antibody against endogenous α-adaptin (*d–f*). The cells in *a* and *d* were transfected with normal γ-adaptin, *b* and *e* with γγα, and *c* and *f* with γγ-. Although neither normal γ-adaptin nor γγ- coincides with α-adaptin, a small amount of γγα shows colocalization, indicated by the arrowheads. Bar, 20 μm.

obvious colocalization of either normal γ-adaptin or the construct γγ- with α-adaptin (Fig. 5, compare *a* with *d*, and *c* with *f*). However, in some of the cells expressing the construct with the α-adaptin ear, γγα, there is significant colocalization with endogenous α-adaptin at the cell periphery, marked with arrowheads. Thus, although this construct is predominantly perinuclear, in some cells a small fraction of the protein appears to be associated with plasma membrane coated pits.

Solubility of the Constructs

To test whether the increased cytoplasmic localization of some of the constructs could be correlated with an increase in their solubility, Western blots were carried out on cells that had been extracted with 1% NP-40, then pelleted at high speed. Fig. 6 shows blots of the supernatants and pellets (made up to the same volume) of all 6 cell lines probed with mAb 100/3, together with positive control lanes containing Vero cells (which express endogenous γ-adaptin that reacts with the antibody), negative control lanes containing non-transfected Rat 1 cells, and lanes containing supernatants and pellets from non-transfected Rat 1 cells labeled for endogenous α-adaptin (α). All of the constructs migrate with approximately their expected mobilities, since the γ-adaptin ear is nearly twice the size of the γ-adaptin ear (see Fig. 1). However, when constructs with the same ear are compared, those with the γ-adaptin NH_2 terminus consistently migrate
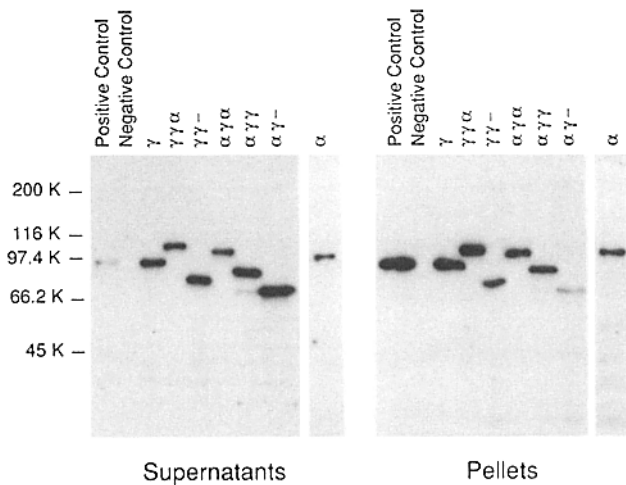
Figure 6. Western blot of high speed supernatants and pellets of transfected cell extracts. Cells were extracted with 1% NP-40 and centrifuged at high speed, and both the supernatants and the pellets (made up to equal volumes) were subjected to SDS-PAGE and Western blotting. All the lanes except the ones marked α were probed with mAb 100/3; the α lanes containing nontransfected cells probed with the anti–α-adaptin mAb AC1-M11. The positive control lane contains Vero cells, the negative control lane contains non-transfected Rat 1 cells, and the other six lanes contain the cell lines shown in Figs. 2–5. The constructs that show increased cytoplasmic staining by immunofluorescence also appear to be more soluble.

slower than those with the α-adaptin NH_2 terminus (compare γ with αγγ, γγα with αγα, and γγ- with αγ-), in spite of the fact that the α-adaptin NH_2-terminal domain is somewhat larger than the γ-adaptin NH_2-terminal domain (see Fig. 1). Since endogenous γ-adaptin migrates with an apparent molecular mass of 95–100 kD, while its actual molecular weight (determined from its amino acid sequence) is 91,352 (Robinson, 1990), these observations indicate that the anomalous migration of γ-adaptin is a function of its NH_2 terminus.

A comparison of the relative intensity of labeling in the supernatant and pellet lanes for endogenous γ- and α-adaptins indicates that very little of the γ-adaptin is soluble, while nearly half of the α-adaptin is soluble. Quantification of the autoradiographs by densitometry revealed that in this experiment, 8% of the γ-adaptin signal was found in the supernatant and 36% of the α-adaptin signal. The normal γ-adaptin construct and the construct γγα are both found mainly in the pellet, although both are more soluble than the endogenous protein (23 and 33% of the signal, respectively), possibly because they are expressed at somewhat higher levels. In contrast, the ear-minus construct, γγ-, is more concentrated in the supernatant (66%), consistent with its increased cytoplasmic staining by immunofluorescence (see Figs. 2 and 5). Of the constructs with the α-adaptin NH_2-terminus, the solubility of αγα appears to be similar to that of endogenous α-adaptin (32%), while αγγ is somewhat more soluble (56%) and αγ- is much more soluble (92%). These results are also consistent with the immunofluorescence data. Thus, the tendency of some of the constructs to accumulate in the cytosol can be demonstrated by Western blotting as well as immunofluorescence.
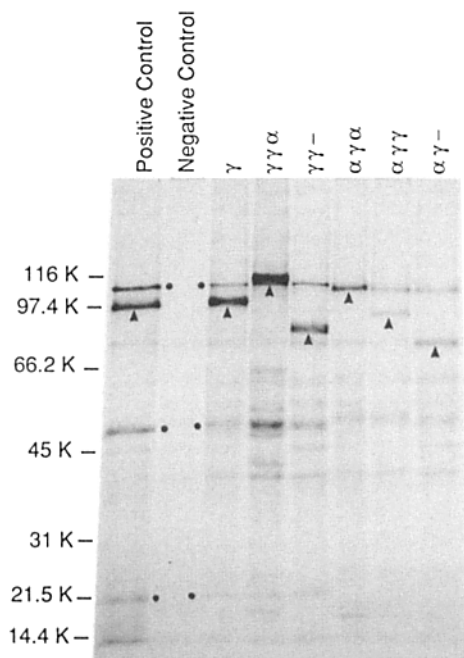

Figure 7. Immunoprecipitation of adaptor complexes containing adaptin chimeras. Extracts from the cell lines shown in Fig. 6 were immunoprecipitated with mAb 100/3 under nondenaturing conditions. The band that is recognized by the antibody (either endogenous γ-adaptin or a construct) is indicated in each of the lanes by an arrowhead. The bands marked with dots are the other subunits of the adaptor complex which co-precipitate: either β′, AP47, and AP19 in the positive control, γ, γγα, and γγ- lanes; or β, AP50, and AP17 in the αγα, αγγ, and αγ- lanes. Because the αγα construct and β-adaptin have virtually identical mobilities in this gel system, the two proteins cannot be resolved in the αγα lane. Other bands that are present in the lanes appear to be contaminants: relative amounts of them varied from one sample to another, even from the same cell line; and they could also be detected in nontransfected cells and in controls from which the first antibody had been omitted.

Assembly of Adaptor Complexes

To investigate whether the constructs are capable of assembling into adaptor complexes, immunoprecipitation of extracts from ^{35}S-labeled cells was carried out under nondenaturing conditions (Fig. 7). In the positive control lane, a Vero cell extract precipitated with mAb 100/3 is shown. It is clear that the antibody has brought down not only γ-adaptin (arrowhead), but also β′-adaptin, AP47, and AP19 (indicated with dots). None of these bands are seen in the negative control lane containing non-transfected Rat 1 cells immunoprecipitated with mAb 100/3, while all of them are seen in the lane containing the precipitate from cells transfected with normal γ-adaptin. In contrast to the positive control lane, however, in this lane the other adaptor subunits appear to be substoichiometric with γ-adaptin, presumably because the γ-adaptin DNA is coupled to a strong constitutive promoter and thus is somewhat overexpressed. The same three bands can be seen in the lanes containing immunoprecipitates of extracts from cells expressing γγα and γγ-.

In the other three lanes, which contain immunoprecipitates from cells expressing constructs with the α-adaptin
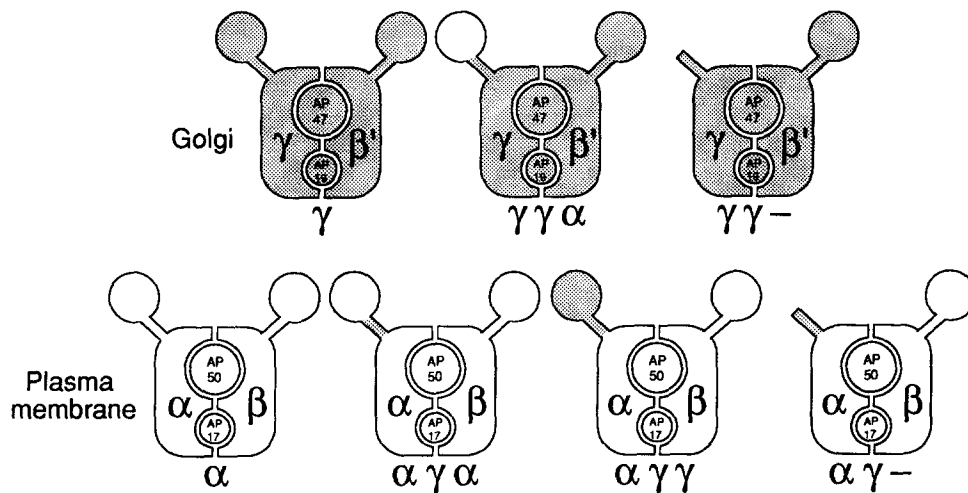
Figure 8. Models of the adaptor complexes assembled from wild-type or engineered adaptins. Proteins normally found in the Golgi adaptor complex are stippled; those normally found in the plasma membrane adaptor complex are white; and chimeras are indicated accordingly. The membranes with which the various adaptor complexes associate are indicated.

NH₂ terminus, the three co-precipitating bands are different. In place of the β' band, there is a slightly faster migrating band, presumably β-adaptin which runs somewhat ahead of β' (see Ahle et al., 1988). The AP47 band has disappeared, and there is a new, somewhat slower migrating band, presumably AP50. At the bottom of the gel, the AP19 band can no longer be seen and a new band is apparent, which has the expected mobility for AP17.

Thus, in spite of the changes that have been engineered into the COOH-terminal ear domains, all of the constructs with the γ-adaptin NH₂ terminus appear to be capable of assembling into Golgi adaptor complexes, while all of the constructs with the α-adaptin NH₂ terminus appear to be capable of assembling into plasma membrane adaptor complexes; and these complexes are able to be targeted to the appropriate membrane. These results are summarized in Fig. 8.

Discussion

The results presented in this study indicate that the α- and γ-adaptin ears are not required for adaptor targeting. This raises two questions: first, what is the function of the ears if not targeting; and second, where is the targeting information if not in the ears?

A potential insight into the function of the ears comes from the observation that the ear-minus α- and γ-adaptins show relatively more cytoplasmic staining than either wild-type or ear-swapped constructs. Similarly, Western blots of high speed supernatants and pellets from extracts of transfected cells reveal that the ear-minus constructs are more soluble. Thus, without their ears, the proteins appear to be impaired in their ability to attach to membranes and/or to be incorporated into coated pits. One possible explanation for this finding might be that the ear-minus constructs are simply misfolded; however, the immunoprecipitation experiments indicate that the truncated adaptins are fully capable of assembling into adaptor complexes. Alternatively, the ears could stabilize the association of adaptor complexes with the membrane: for instance, they could participate in clathrin binding, in the binding of adaptors to the cytoplasmic tails of selected membrane proteins, or in adaptor–adaptor interactions.

A number of experiments have been carried out using proteolytically cleaved adaptors to assess the potential role of the adaptor ears in clathrin binding and assembly. Results of these studies show that earless adaptors are unable to promote clathrin assembly in solution (Schroder and Ungewickell, 1991). However, there is some evidence that the ears do not interact directly with clathrin: plasma membrane adaptor heads alone are able to bind to preformed clathrin cages (Keen and Beck, 1989); proteolysis of coats assembled from clathrin plus adaptors results in the release of the ears while the heads stay behind with the clathrin (Matsui and Kirchhausen, 1990); and clathrin can co-assemble with proteolyzed adaptors on exposed plasma membranes (Peller et al., 1993). Certainly the lack of homology between the α- and γ-adaptin ears tends to argue against a role in clathrin binding.

However, this lack of homology is an argument in favor of the idea that the ears may bind to the cytoplasmic tails of membrane proteins, since the signals that direct membrane proteins into coated pits on the plasma membrane are thought to be different from the signals that direct them into coated pits in the TGN (Lobel et al., 1989). Although so far the only published experiment addressing this question failed to show any interaction between adaptor ears and membrane protein tails, the study was done using proteolyzed adaptors immobilized on a Western blot (Beltzer and Spiess, 1991), and it is possible that the ears need to be correctly folded for binding to occur. One attraction of the ear-tail interaction model is that the flexible hinges would allow the ears to make contact with internalization or sorting signals which can be either very close to the membrane or a considerable distance away (Trowbridge, 1991). Although it seems unlikely that this interaction is what directs the adaptors to the appropriate membrane, it is possible that once the adaptors are membrane bound, they make contact with the cytoplasmic tails of other proteins in the same compartment and that this interaction may help to anchor them onto the membrane. Thus, deleting the ears might result in a less stable association of adaptors with the membrane, while swapping the ears between α- and γ-adaptins might cause the adaptors to bind to a different set of membrane proteins.

Another possibility is that the ears might participate in interactions between adaptor complexes of the same type.

Plasma membrane adaptors have a tendency to self-associate in vitro and although this process seems mainly to occur via the heads (Beck and Keen, 1991), it is possible that the ears may also be involved. If so, this might explain both the increased cytoplasmic distribution of the ear-minus constructs and the observation that a small amount of the construct γγα is associated with plasma membrane coated pits: Golgi adaptors with one α-adaptin ear might interact to some extent with plasma membrane adaptors and thus be incorporated into coated pits assembling on the plasma membrane. One way to find out more about the function of the ears might be to overproduce the ear-minus and ear-swapped constructs to such high levels that virtually all of the adaptor complexes would contain mutant rather than endogenous adaptins, and then to study the phenotype of the transfected cells further (e.g., whether they are defective in receptor-mediated endocytosis or sorting at the TGN).

So far only one other study has been carried out to address the question of whether the adaptin ears are involved in the association of adaptors with the membrane. Purified plasma membrane adaptors had previously been shown to be able to bind to the exposed cytoplasmic surface of the plasma membrane in sonicated cells (Mahaffey et al., 1990), and this interaction is still able to occur after removal of the α- and β-adaptin ears by proteolysis (Peeler et al., 1993). These results are largely in agreement with the results described here, except that the authors did not find that the earless adaptors were in any way impaired in their ability to associate with the plasma membrane. It is possible that adaptors assembled de novo from ear-minus α-adaptin may behave somewhat differently from pre-assembled adaptors from which the ears are then removed. Moreover, the in vitro system used by Peeler et al. (1993) is designed to study the ability of adaptors to bind to sites previously occupied by endogenous adaptors, while the in vivo results reflect the distribution of adaptors at steady state, where other interactions may be involved.

The second question raised by this study concerns the nature of the targeting signal on the adaptor complex. Although the partial colocalization of the construct γγα with endogenous α-adaptin suggests that there may be multiple targeting signals, it is clear that the contribution of the α- and γ-adaptin ears to adaptor targeting is at most a very minor one, and possibly a passive one. The hinges are unlikely to play any role in adaptor targeting since the γ-adaptin hinge does not affect the localization of α-adaptin; indeed, the hinge appears to be the least conserved domain when adaptin isoforms of the same class are compared (Robinson, 1989; Kirchhausen et al., 1989), and it may simply need to be present as a flexible connection between the head and ear.

Thus, the NH₂-terminal domains of the α- and γ-adaptins appear to determine whether the proteins associate with the plasma membrane or the TGN. However, this does not necessarily imply that there is actually a targeting signal in the NH₂-terminal domain: because all the constructs assemble into adaptor complexes, the signal could be present in one of the other subunits. The β-adaptins are perhaps the least likely candidates for proteins containing the targeting signal since they are highly homologous in the two adaptor complexes and have already been assigned a role in clathrin binding (Ahle and Ungewickell, 1989). However, the functions of the medium and small chains, which are <50% iden-

tical in the two complexes, are still completely unknown. By constructing chimeras between AP50 and AP47 and between AP17 and AP19, as well as between the NH₂-terminal domains of α- and γ-adaptins, it should be possible to investigate the potential role of these other proteins in adaptor targeting.

There is much that is not understood about how adaptors are recruited from the cytoplasm onto the appropriate membrane. Presumably there are special adaptor receptors on the plasma membrane and the TGN, which may correspond to the protease-sensitive factors shown to be required for the membrane association of plasma membrane adaptors in vitro (Mahaffey et al., 1990; Peeler et al., 1993). However, such receptors do not have to be stoichiometric with the adaptors nor do they need to be incorporated into coated vesicles: binding could be transient, and the association of adaptors with the membrane could then be stabilized by binding to the cytoplasmic tails of membrane proteins or possibly by interacting with lipids. Recent experiments making use of in vitro systems have shown that the recruitment of coat proteins onto the membrane is a complex process, and one that is likely to be an important regulatory step in the control of vesicular traffic, both clathrin mediated and non-clathrin mediated (Donaldson et al., 1991; Robinson and Kreis, 1992; Wong and Brodsky, 1992; Seaman et al., 1993). By extending the in vivo approach described here to investigate the localization of other chimeric adaptor subunits, and by using in vitro approaches to find components of the cellular machinery involved in adaptor recruitment, it should be possible not only to identify the targeting signal on the adaptor complex, but also to learn how that signal functions.

References

Ahle, S., and E. Ungewickell. 1989. Identification of a clathrin binding site in the HA2 adaptor protein complex. *EMBO (Eur. Mol. Biol. Organ.) J.* 264:20089–20093.

Ahle, S., A. Mann, U. Eichelsbacher, and E. Ungewickell. 1988. Structural relationships between clathrin assembly proteins from the Golgi and the plasma membrane. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:919–929.

Beck, K. A., and J. H. Keen. 1991. Self-association of the plasma membrane-associated clathrin assembly protein AP-2. *J. Biol. Chem.* 266:4437–4441.

Beltzer, J. P., and M. Spiess. 1991. In vitro binding of the asialoglycoprotein receptor to the β adaptin of plasma membrane coated vesicles. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3735–3742.

Donaldson, J. G., R. A. Kahn, J. Lippincott-Schwartz, and R. D. Klausner. 1991. Binding of ARF and β-COP to Golgi membranes: possible regulation by a trimeric G protein. *Science (Wash. DC).* 254:1197–1199.

Glickman, J. N., E. Conibear, and B. M. F. Pearse. 1989. Specificity of binding of clathrin adaptors to signals on the mannose-6-phosphate/insulin-like growth factor II receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1041–1047.

Heuser, J. E., and J. H. Keen. 1988. Deep-etch visualization of proteins involved in clathrin assembly. *J. Cell Biol.* 107:877–886.

Keen, J. H., and K. A. Beck. 1989. Identification of the clathrin binding domain of assembly protein AP-2. *Biochem. Biophys. Res. Commun.* 158:17–23.

Kirchhausen, T., K. L. Nathanson, W. Matsui, A. Vaisberg, E. P. Chow, C. Burne, J. H. Keen, and A. E. Davis. 1989. Structural and functional division into two domains of the large (100- to 115-kDa) chains of the clathrin-associated protein complex AP-2. *Proc. Natl. Acad. Sci. USA.* 86:2612–2616.

Kirchhausen, T., A. C. Davis, S. Frucht, B. O. Greco, G. Payne, and B. Tubb. 1991. AP17 and AP19, the mammalian small chains of the Clathrin Associated Protein complexes, show homology to Yap17p, their putative homologue in yeast. *J. Biol. Chem.* 266:11153–11157.

Lobel, P., K. Fujimoto, R. D. Ye, G. Griffiths, and S. Kornfeld. 1989. Mutants in the cytoplasmic domain of the 275kd mannose-6-phosphate receptor differentially alter lysosomal enzyme sorting and endocytosis. *Cell.* 57:787–796.

Luzio, J. P., B. Brake, G. Banting, K. E. Howell, P. Braghetta, and K. K. Stanley. 1990. Identification, sequencing and expression of an integral membrane protein of the trans-Golgi network, TGN38. *Biochem. J.* 270:97–102.

Mahaffey, D. T., J. S. Peeler, F. M. Brodsky, and R. G. W. Anderson. 1990. Clathrin-coated pits contain an integral membrane protein that binds the AP-2 subunit with high affinity. *J. Biol. Chem.* 265:16514–16520.

Matsui, W., and T. Kirchhausen. 1990. Stabilization of clathrin coats by the core of the clathrin-associated protein complex AP-2. *Biochemistry.* 29:10791–10798.

Nakayama, Y., M. Goebl, B. O'Brine Greco, S. Lemmon, E. Pingchang Chow, and T. Kirchhausen. 1991. The medium chains of mammalian clathrin associated proteins have an homolog in yeast. *Eur. J. Biochem.* 202:569–574.

Pearse, B. M. F. 1988. Receptors compete for adaptors found in plasma membrane coated pits. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3331–3336.

Pearse, B. M. F., and M. S. Robinson. 1990. Clathrin, adaptors, and sorting. *Annu. Rev. Cell Biol.* 6:151–171.

Peeler, J. S., W. C. Donzell, and R. G. W. Anderson. 1993. The appendage domain of the AP-2 subunit is not required for assembly or invagination of clathrin-coated pits. *J. Cell Biol.* 120:47–54.

Ponnambalam, S., M. S. Robinson, A. P. Jackson, L. Peiperl, and P. Parham. 1990. Conservation and diversity in families of coated vesicle adaptins. *J. Biol. Chem.* 265:4814–4820.

Robinson, M. S. 1987. 100-kD coated vesicle proteins: molecular heterogeneity and intracellular distribution studied with monoclonal antibodies. *J. Cell Biol.* 104:887–895.

Robinson, M. S. 1989. Cloning of cDNAs encoding two related 100-kD coated vesicle proteins (α-adaptins). *J. Cell Biol.* 108:833–842.

Robinson, M. S. 1990. Cloning and expression of γ-adaptin, a component of clathrin-coated vesicles associated with the Golgi apparatus. *J. Cell Biol.* 111:2319–2326.

Robinson, M. S. 1992. Adaptins. *Trends Cell Biol.* 2:293–297.

Robinson, M. S., and T. E. Kreis. 1992. Recruitment of coat proteins onto Golgi membranes in intact and permeabilized cells: effects of brefeldin A and G protein activators. *Cell.* 69:129–138.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 545 pp.

Schroder, S., and E. Ungewickell. 1991. Subunit interaction and function of clathrin-coated vesicle adaptors from the Golgi and plasma membrane. *J. Biol. Chem.* 266:7910–7918.

Seaman, M. N. J., C. L. Ball, and M. S. Robinson. 1993. Targeting and mistargeting of plasma membrane adaptors in vitro. In press.

Trowbridge, I. S. 1991. Endocytosis and signals for internalization. *Curr. Opin. Cell Biol.* 3:634–641.

Wong, D. H., and F. M. Brodsky. 1992. 100-kD proteins of Golgi and trans-Golgi network-associated coated vesicles have related but distinct membrane binding properties. *J. Cell Biol.* 117:1171–1179.