

Targeting Signals and Subunit Interactions in Coated Vesicle Adaptor Complexes

Lesley J. Page and Margaret S. Robinson

Department of Clinical Biochemistry, University of Cambridge, Addenbrooke's Hospital, Cambridge CB2 2QR, England

Abstract. There are two clathrin-coated vesicle adaptor complexes in the cell, one associated with the plasma membrane and one associated with the TGN. The subunit composition of the plasma membrane adaptor complex is α -adapting, β -adapting, AP50, and AP17; while that of the TGN adaptor complex is γ -adapting, β' -adapting, AP47, and AP19. To search for adaptor targeting signals, we have constructed chimeras between α -adapting and γ -adapting within their NH₂-terminal domains. We have identified stretches of sequence in the two proteins between amino acids ~130 and 330–350 that are essential for targeting. Immunoprecipitation reveals that this region determines whether a construct coassembles with AP50 and AP17,

or with AP47 and AP19. These observations suggest that these other subunits may play an important role in targeting. In contrast, β - and β' -adapting are clearly not involved in this event. Chimeras between the α - and γ -adapting COOH-terminal domains reveal the presence of a second targeting signal. We have further investigated the interactions between the adaptor subunits using the yeast two-hybrid system. Interactions can be detected between the β/β' -adapting and the α/γ -adapting, between the β/β' -adapting and the AP50/AP47 subunits, between α -adapting and AP17, and between γ -adapting and AP19. These results indicate that the adaptor subunits act in concert to target the complex to the appropriate membrane.

CLATHRIN-coated pits and vesicles bud from two membrane compartments of the cell: the plasma membrane and the TGN. Although the clathrin associated with these two membranes is the same, the adaptor complexes, which attach the clathrin to the membrane, are different (Robinson, 1987; Ahle et al., 1988). There is some evidence that the adaptors bind to the cytoplasmic domains of selected membrane proteins (e.g., the receptors for LDL, transferrin, and EGF at the plasma membrane; and the mannose-6-phosphate receptor at the TGN) (Pearse, 1988; Glickman et al., 1989; Sorkin and Carpenter, 1993; Sorkin et al., 1995; Sosa et al., 1993), causing such proteins to become concentrated in coated pits and vesicles for transport to another compartment. However, it seems unlikely that these interactions are responsible for targeting the adaptors to the plasma membrane or the TGN, since proteins such as the transferrin receptor are often found at higher concentrations in endosomal compartments where adaptors are absent. Thus, it has been proposed that adaptors are recruited onto the appropriate membrane by interacting with specific adaptor docking proteins (Robinson, 1993; Traub et al., 1993). Although these docking proteins have yet to be identified,

they presumably act by recognizing targeting signals on the two adaptor complexes.

Both adaptor complexes are heterotetramers, consisting of two 100-kD subunits or adaptins, a medium chain of ~50 kD, and a small chain of ~20 kD. The plasma membrane adaptor complex is composed of α -adapting, β -adapting, AP50, and AP17, while the TGN adaptor complex is composed of γ -adapting, β' -adapting, AP47, and AP19 (Keen, 1987; Ahle et al., 1988). Rotary shadowing of isolated adaptors shows that they consist of a brick-like core or "head" attached to two smaller appendages or "ears" by stalks that are thought to act as flexible hinges (Heuser and Keen, 1988). The ears correspond to the COOH-terminal domain of the two adaptins, while the head consists of the NH₂-terminal domains of the adaptins together with the medium and small chains (Kirchhausen et al., 1989) (see the model in Fig. 1). But although the general organization of the complex is known, there is little information on exactly how the adaptor subunits interact with each other, or on what the function of each domain or subunit is. It is not known, for example, which part of the complex is responsible for targeting the adaptor to the correct membrane compartment.

It seems unlikely that the β - and β' -adapting are involved in adaptor targeting for several reasons. They are the most homologous of the four subunits (Kirchhausen et al., 1989; Ponnambalam et al., 1990); they have already been assigned a role in clathrin binding (Ahle and Unger-

Address correspondence to Margaret S. Robinson, Dept. of Clinical Biochemistry, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QR, England. Tel.: 011 44 1223 336141. Fax: 011 44 1223 330598.

wickell, 1989; Gallusser and Kirchhausen, 1993); and Camidge and Pearse (1994) have recently shown that when *Drosophila* β -adaptin (which shows homology to both β - and β' -adaptins) is transfected into mammalian cells, it localizes to both the TGN and the plasma membrane and assembles with both α - and γ -adaptin. The α - and γ -adaptins are better candidates for proteins containing targeting signals because they are the least homologous of the four subunits, and their ear domains in particular are completely unrelated (Robinson, 1990). In an earlier study we investigated the possibility that the α - and γ -adaptin ears might be responsible for targeting by constructing chimeras. A chimera consisting of the α -adaptin NH₂-terminal domain, the γ -adaptin hinge, and the γ -adaptin ear ($\alpha\gamma\gamma$) was found to be localized to the plasma membrane (Robinson, 1993), suggesting that the γ -adaptin ear domain is not involved in targeting. However, a chimera consisting of the γ -adaptin NH₂-terminal domain, the γ -adaptin hinge, and the α -adaptin ear ($\gamma\gamma\alpha$) was found to be mainly associated with the TGN, but in some cells a fraction of the construct was localized to the plasma membrane. These results suggested that the ear may play a minor role in targeting, but that the major targeting signal is likely to be present in the adaptor head. Thus, any of the subunits with the possible exception of β/β' could potentially be involved in adaptor localization.

The aim of the present study was to determine which part of the complex is responsible for targeting by making chimeras between the NH₂-terminal domains of α - and γ -adaptin. Although there is no reason necessarily to assume that these domains contain the adaptor targeting signals, their localization within the complex suggests that they interact with the other adaptor subunits, and therefore the prediction was that some of the chimeras should assemble into complexes containing a mixture of plasma membrane and TGN adaptor subunits. Thus, this approach should allow us to test whether targeting can be correlated with the presence or absence of a particular subunit, while at the same time providing information about how the subunits interact with each other.

Materials and Methods

Construction of Plasmids

The adaptins have three domains: NH₂-terminal (head), hinge, and ear. The NH₂-terminal domain is defined as amino acids 1–619 in α -adaptin and 1–593 in γ -adaptin; the hinge is defined as amino acids 620–700 in α -adaptin and 594–703 in γ -adaptin; and the ear domain is defined as amino acids 701–938 in α -adaptin and 704–822 in γ -adaptin (see Robinson, 1989, 1993).

Most of the constructs that were made contain the α -adaptin ear, and all have the bovine γ -adaptin hinge, which is required for recognition by the species-specific antibody mAb 100/3. The basic techniques used in making the constructs are described by Sambrook et al. (1989). The general strategy was first to identify stretches of identical or highly conserved protein sequence in the NH₂-terminal domain where α - and γ -adaptin could be joined together. A naturally occurring restriction site was then sought in one DNA sequence and PCR was used to introduce the same site, in frame, into the other sequence. For example, N γ -331 (the nomenclature refers to the NH₂-terminal domain having γ -adaptin sequence up to amino acid residue 331) was made by introducing an ApaLI site into γ -adaptin by PCR in the middle of a sequence that is conserved between the two adaptins. The α -adaptin naturally contains this restriction site so a three-way ligation of the following was performed: the γ -adaptin PCR product digested with NotI at the 5' end and ApaLI at the 3' end (to give

the first half of the chimera's NH₂-terminal sequence), α -adaptin digested with ApaLI and ApaI (to provide the rest of the NH₂-terminal sequence), and the expression vector encoding $\alpha\gamma\alpha$ (Robinson, 1993) cut with NotI and ApaI (which releases the NH₂-terminal domain of the construct, leaving the vector plus the bovine γ -adaptin hinge and α -adaptin ear). The digests were run on an agarose gel, the appropriate bands were isolated, the DNA was extracted using a GeneClean kit (BIO 101, Inc., Vista, CA), and the fragments were ligated together. In some cases no natural site could be exploited so restriction sites were introduced by PCR into both sequences. All of the joins between α - and γ -adaptin sequences and much of the PCR products were sequenced.

The following chimeras were constructed (see Fig. 2): N γ -36: introduced a SnaBI site into both sequences; N γ -132: made use of the BanII site in γ -adaptin; N γ -190: made use of the NcoI site in γ -adaptin; N γ -331: made use of the ApaLI site in α -adaptin; N γ -566: made use of the SacI site in γ -adaptin; N α -132: made use of the NheI site in γ -adaptin; N α -171: made use of the DraIII site in α -adaptin; N α -265: made use of the HindIII site in α -adaptin; N α -350: introduced an EcoRV site into both sequences; N α -430: made use of the BglI site in α -adaptin; N α -566: made use of the SacI site in γ -adaptin.

Since alignment of the two sequences results in several gaps, the nomenclature does not always reflect the relationship between chimeras of the different series. For example, γ -adaptin residue 330 aligns with α -adaptin residue 350 (for alignment see Robinson, 1990), so that constructs N γ -331 and N α -350 are actually joined within the same conserved stretch of sequence, but one consists of γ followed by α while the other consists of α followed by γ .

All of the chimeras were made using DNA from constructs $\alpha\gamma\alpha$ and $\gamma\gamma\alpha$ in the expression vector pHYKS3 (Robinson, 1993). However, because the NH₂-terminal domain of the original $\gamma\gamma\alpha$ construct was a chimera between mouse and cow, this construct was modified to obtain an NH₂-terminal γ -adaptin sequence entirely derived from mouse using a similar strategy to that already described, making use of NotI, SacI, and ApaI sites. This facilitated the construction of other chimeras as the bovine NH₂-terminal domain has not been entirely sequenced.

The ear swap constructs were made by digesting $\alpha\gamma\gamma$ with NotI and ApaI to obtain an expression vector containing the hinge and ear domains only. The appropriate plasmid was digested with the same enzymes but in this case the NH₂-terminal domain was isolated and ligated into the cut vector.

Transfection into Rat 1 Cells

Rat 1 fibroblasts maintained in DME containing 10% FCS (DME-FCS) were cotransfected with the various constructs and the G418-selectable plasmid pRSVneo (Robinson, 1993) using one of the following protocols: (a) the calcium phosphate method as previously described (Robinson, 1990). (b) The DEAE Dextran method. Cells were grown in 25-cm² flasks and washed in DME, and ~5 μ g of construct DNA plus 1 μ g of pRSVneo in 0.5 ml of DME was added, followed by 0.5 ml of 1 mg/ml DEAE Dextran in TBS, pH 7.4. The cells were then incubated for 30 minutes at 37°C, after which the medium was replaced with DME-FCS containing 0.1 mg/ml chloroquine, and the cells were incubated in this solution for 3 hours at 37°C. The cells were then washed in DME-FCS and incubated overnight. (c) Transfectam (Promega Corp., Madison WI). Cells grown in 25-cm² flasks were washed and incubated in 0.5 ml DME. A solution of 10 μ l Transfectam in 0.5 ml DME and a solution of DNA (5 μ g of construct plus 1 μ g of pRSVneo) in 0.5 ml DME were mixed together and added to the cells, which were then incubated overnight at 37°C.

In all procedures the cells were trypsinized the next day and split into 3 dishes. They were left for two more days before G418 was added to the medium at a concentration of 0.5 mg/ml. After 2 wk, individual G418-resistant colonies were picked with a sterile yellow tip and transferred to a multiwell plate. Expression of the chimeras was assayed by immunofluorescence. Even after two rounds of cloning, some of the cell lines showed heterogeneity in their expression levels.

Immunofluorescence

Cells grown on multiwell slides were fixed in methanol and acetone as previously described (Robinson, 1990). Immunofluorescence was carried out using the mouse antibody mAb 100/3 (Ahle et al., 1988) which recognizes the bovine but not the rat γ -adaptin hinge. For some experiments the cells were double labeled either with the rabbit antibody C619-656, raised to the α C-adaptin hinge (amino acids 619–656) (Ball et al., 1995) or with an antibody raised against a β -adaptin fusion protein (see below).

Antibody incubations, labeling with secondary antibodies, and mounting and examination of the slides were carried out as previously described (Robinson, 1990).

Production of Monospecific Antibodies

To identify the adaptor subunits that coassemble with the various constructs, monospecific antibodies were raised. First, DNA encoding the subunits was amplified from rat liver cDNA (Clontech) by PCR and ligated into pBluescript, and the identity of the clones was confirmed by sequencing. A second PCR reaction was then used to amplify specific sequences and to introduce restriction sites compatible with pGEX3X. The sequences that were amplified encoded: amino acids 583–619 for β -adap-
tin; amino acids 583–619 for β' -adap-
tin; amino acids 16–78 and 278–384 for AP50 (AP50-1 and AP50-2); amino acids 17–80 and 268–374 for AP47 (AP47-1 and AP47-2); amino acids 20–61 and 107–142 for AP17 (AP17-1 and AP17-2); and amino acids 20–61 and 107–158 for AP19 (AP19-1 and AP19-2). These sequences were chosen because they are where the two homologues show the most divergence (Kirchhausen et al., 1989, 1991; Ponnambalam et al., 1990; Nakayama et al., 1991). The PCR products were ligated into pGEX3X and transformed into MC1060 host cells for sequencing and expression. Of the ten fusion proteins, only the β and β' constructs and AP19-2 were found to be soluble, and these were purified on GSH-Sepharose (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) according to the manufacturer's instructions. To purify the other seven fusion proteins, inclusion bodies were prepared (Bohmann and Tijian, 1989) and the proteins solubilized in sample buffer and subjected to preparative SDS PAGE. Up to 5 mg purified protein was obtained from each 100-ml culture. Rabbits were injected with 0.5 mg fusion protein in complete Freund's adjuvant, followed by boosts of 0.5 mg fusion protein in incomplete Freund's adjuvant at 2 and 8 wk after the primary injection. 10 d after the final injection, the rabbits were bled out and their sera tested on blots of purified adaptors. 7 of the 10 fusion proteins elicited a response against the appropriate adaptor subunit (the ones that did not were AP50-1, AP17-1, and AP19-1, and these were discarded). To ensure that the antibodies would be monospecific, and to improve the signal relative to background, the seven positive antisera were cross-absorbed and affinity purified. Each fusion protein was coupled to CNBr-activated Sepharose (Pharmacia), and 20 ml of each serum were absorbed over two nights at 4°C with resin containing 0.67 mg of the "wrong" fusion protein (e.g., anti- β was absorbed with β' and vice versa). The resin was then removed by centrifugation, and the sera were absorbed overnight at 4°C with resin containing 0.33 mg of the "right" fusion protein. Bound antibodies were eluted with 0.2 M gly, pH 2.3, as previously described (Robinson and Pearce, 1986).

Immunoprecipitation and Western Blotting

The immunoprecipitation protocol was based on a method developed by B. Pearce that has previously been described (Robinson, 1993). Cells were lysed with 1% NP-40 and centrifuged at high speed to obtain a membrane pellet enriched in assembled adaptor complexes. The pellet was then extracted with 0.05M Na₂CO₃ to solubilize the membrane-associated adaptors, which were then immunoprecipitated using mAb100/3, followed by rabbit anti-mouse IgG (Sigma Immunochemicals, St. Louis, MO) and protein A-Sepharose 4B Fast (Sigma). After extensive washing (see Robinson, 1993), the pellets were resuspended in 2× sample buffer, boiled, and subjected to SDS PAGE. Gels were blotted onto nitrocellulose and labeled with the antibodies raised against the various adaptor subunits, followed by ¹²⁵I-protein A (Amersham Corp., Arlington Heights, IL). For maximum resolution, the x-ray film was exposed at room temperature without an intensifying screen.

Yeast Two-Hybrid System

The "Matchmaker" two-hybrid system was obtained from Clontech Labs. (Palo Alto, CA) and all procedures were carried out as described in the manufacturer's instructions. PCR was used to generate appropriate restriction sites in the adaptor subunit cDNAs (some of which were generously provided by T. Kirchhausen) for expression in the yeast vectors pGBT9 and pGAD424. The yeast strain SFY526 was cotransformed with the plasmids using a PEG/lithium acetate protocol and grown on plates lacking in leucine and tryptophan to select for colonies containing both plasmids. The colonies were transferred onto filter paper and permeabilized by freezing in liquid nitrogen and thawing at room temperature. The filters were then placed on another piece of filter paper that had been pre-

soaked in a solution containing 0.33 mg/ml X-gal and were incubated at 30°C until the color developed.

β -Galactosidase activity was also measured using a liquid culture assay that makes use of the substrate ONPG and measures the release of *o*-nitrophenol. Cells were grown at 30°C in media lacking in tryptophan and leucine to an OD of ~1.0, and aliquots were permeabilized by the addition of chloroform and SDS 0.16 ml of *o*-nitrophenylgalactoside (4 mg/ml) was added, and the tubes were incubated at 30°C until the color developed. The reactions were quenched by the addition of 0.4 ml 1 M Na₂CO₃ and their absorbance at 420 nm was measured. One unit of activity was defined as OD₄₂₀ divided by (OD₆₀₀ × ml × min) × 1,000.

Results

Localization of the N γ Constructs

To investigate the role of the α - and γ -adap-
tin NH₂-terminal domains in adaptor targeting, chimeras were constructed within this region, joining the two adaptins together at points where they show the most homology (Fig. 2). Constructs in the N γ series (*a*) all have NH₂-terminal domains consisting of γ -adap-
tin sequence followed by α -adap-
tin sequence, while constructs in the N α series (*b*) all have NH₂-terminal domains consisting of α -adap-
tin sequence followed by γ -adap-
tin sequence. This is then followed by the bovine γ -adap-
tin hinge, which allows the constructs to be detected by the species-specific antibody, mAb 100/3. Most of the constructs end with the α -adap-
tin ear. The constructs were stably transfected into Rat 1 cells and localized by immunofluorescence. Fig. 3 shows the localization of the constructs in the N γ series.

N γ -36 (Fig. 3 *a*) and N γ -132 (*b*) both show a punctate pattern characteristic of plasma membrane coated pits. Double labeling with an antibody specific for the α -adap-
tin hinge (Ball et al., 1995) reveals that the constructs colocalize with endogenous α -adap-
tin (*d* and *e*). There is however some cytoplasmic staining as well, and this is observed for most of the NH₂-terminal domain chimeras. The next construct in the series, N γ -190 (*c*), is not recruited to either the plasma membrane or the TGN but has an exclusively cytoplasmic distribution, suggesting that the targeting signal may have been disrupted. N γ -331 (*g*) is localized mainly to a perinuclear compartment, shown by double labeling with anti-TGN38 (Luzio et al., 1990) to be the TGN (not shown). However, some plasma membrane recruitment can also be seen (*g* and *i*, arrowheads). This varied in intensity from cell to cell but generally appeared to be

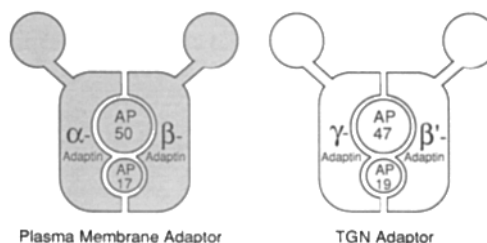


Figure 1. Schematic diagram of the two adaptor complexes. The heads consist of the NH₂-terminal domains of the adaptins (α and β in the plasma membrane adaptor complex; γ and β' in the TGN adaptor complex), plus the medium chains (AP50 or AP47) and the small chains (AP17 or AP19). The COOH-terminal domains of the adaptins form the ears, which are connected to the head by flexible hinges.

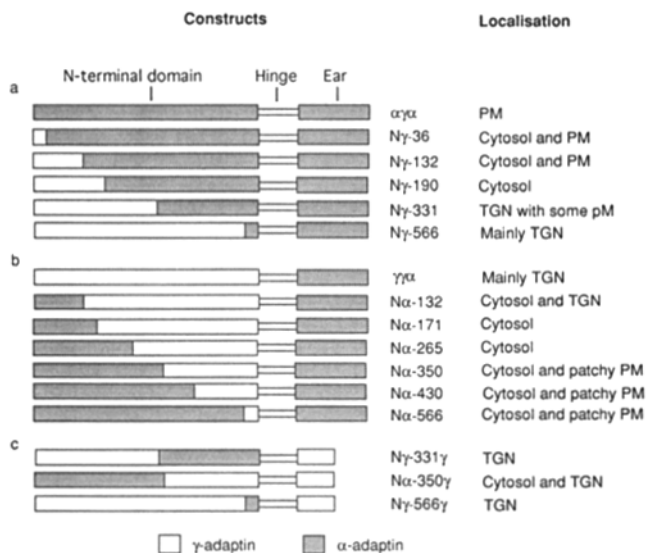


Figure 2. Diagram of the NH₂-terminal domain chimeras. Constructs in the N γ series (a) contain γ -adapitin sequence at the NH₂-terminal end followed by α -adapitin sequence. The nomenclature refers to the protein at the NH₂-terminal end and to the amino acid where the join was made. Constructs in the N α series (b) contain α -adapitin sequence at the NH₂-terminal end followed by γ -adapitin sequence. Two previously described constructs, $\alpha\gamma\alpha$ and $\gamma\gamma\alpha$ (Robinson, 1993), are also shown. All of the constructs contain the bovine γ -adapitin hinge. The constructs in (a) and (b) all have the α -adapitin ear, while those in (c) were made with the γ -adapitin ear.

stronger than that observed for the chimera $\gamma\gamma\alpha$ (see Robinson, 1993). The last construct in the series, N γ -566, is localized mainly to the TGN (h) and looks similar in its distribution to $\gamma\gamma\alpha$.

Assembly of the N γ Constructs

The immunofluorescence results suggest that a targeting signal may exist in the α - and γ -adapitins somewhere between amino acids 132 and 331. However, the α - and γ -adapitins are part of a complex, and an alternative explanation is that this stretch of sequence may be indirectly involved in targeting by binding to one or more of the other adaptor subunits. Thus, we carried out studies to determine whether there was any correlation between targeting and the presence of a particular subunit. For these experiments it was essential to be able to identify the other subunits unambiguously. To date, no antibodies against the medium and small chains have been described, and the two antibodies available that recognize the β subunits cross-react with both β -adapitin and β' -adapitin (Robinson, 1987; Ahle et al., 1988). In order to raise specific antibodies against each of the subunits, GST fusion proteins were constructed containing unique regions of β -adapitin, AP50, AP17, β' -adapitin, AP47, and AP19, and were used to immunize rabbits. The resulting antisera were tested on blots of purified plasma membrane and TGN adaptors. Fig. 4 shows that all six of the antibodies only recognize the subunit against which they were raised and do not cross-react with the homologous protein in the other complex.

To examine the composition of the adaptor complexes assembled from the chimeric adaptins, mAb 100/3 was used to immunoprecipitate each construct plus associated proteins from a carbonate-extracted high speed pellet of detergent-lysed transfected cells. Similar immunoprecipitates were prepared from cells expressing the constructs $\alpha\gamma\alpha$ and $\gamma\gamma\alpha$, and from nontransfected Rat 1 cells. The samples were subjected to SDS PAGE, blotted, and probed with antibodies against the plasma membrane and TGN adaptor subunits (Fig. 5). The antibodies against the adaptor medium chains produced some background labeling of the immunoglobulin heavy chain band, which can be seen in the control immunoprecipitates from the nontransfected cells as well as in the immunoprecipitates of the constructs. However, the specifically labeled adaptor medium chains can be clearly resolved from the immunoglobulin band: they run as much sharper bands just below it.

The $\alpha\gamma\alpha$ construct has been shown to localize to the plasma membrane (Robinson, 1993), and Fig. 5 demonstrates that this construct associates with the plasma membrane adaptor subunits β , AP50, and AP17, although there is also a small amount of β' -adapitin present. Complexes containing the chimeras N γ -36 and N γ -132, which are also targeted to the plasma membrane, were also found to contain β , AP50, and AP17. They, too, therefore associate with the subunits normally found in the plasma membrane adaptor complex. No other subunits could be detected in immunoprecipitates of N γ -190; however, because this construct was found to be present in the high speed pellet in very small amounts, it is possible that the antibodies were not sensitive enough to detect them. The next two chimeras in the series, N γ -331 and N γ -566, were found to be associated with β , AP47, and AP19. A small amount of β' could also be detected, suggesting that both β - and β' -adapitin can associate with these two chimeras. However, they appear to assemble preferentially with β -adapitin even though they are recruited to the TGN. A similar pattern was seen with the construct N γ -331 γ (see below). The $\gamma\gamma\alpha$ construct, which is targeted to the TGN (Robinson, 1993), associates with the normal subunits for the TGN adaptor complex, β' -adapitin, AP47, and AP19, although again some heterogeneity is observed for the β/β' subunits as there is a small amount of β -adapitin also present.

These results show a correlation between the presence of a particular medium and small chain and the targeting of the complex to a particular membrane. However, no correlation is seen for β - and β' -adapitin, indicating that the β subunits are not involved in this event. The results also show that unlike the medium and small chains, β - and β' -adapitins behave somewhat promiscuously: although all of the constructs appear to assemble preferentially with one of the two β subunits, small amounts of the other β subunit can also be detected.

Localization of the N α Constructs

An N α series of constructs was also made (see Fig. 2), in which α -adapitin sequence is followed by γ -adapitin sequence in the NH₂-terminal domain, which is in turn followed by the bovine γ -adapitin hinge and the α -adapitin ear. This series was more difficult to study by immunoprecipitation because only small amounts of protein were

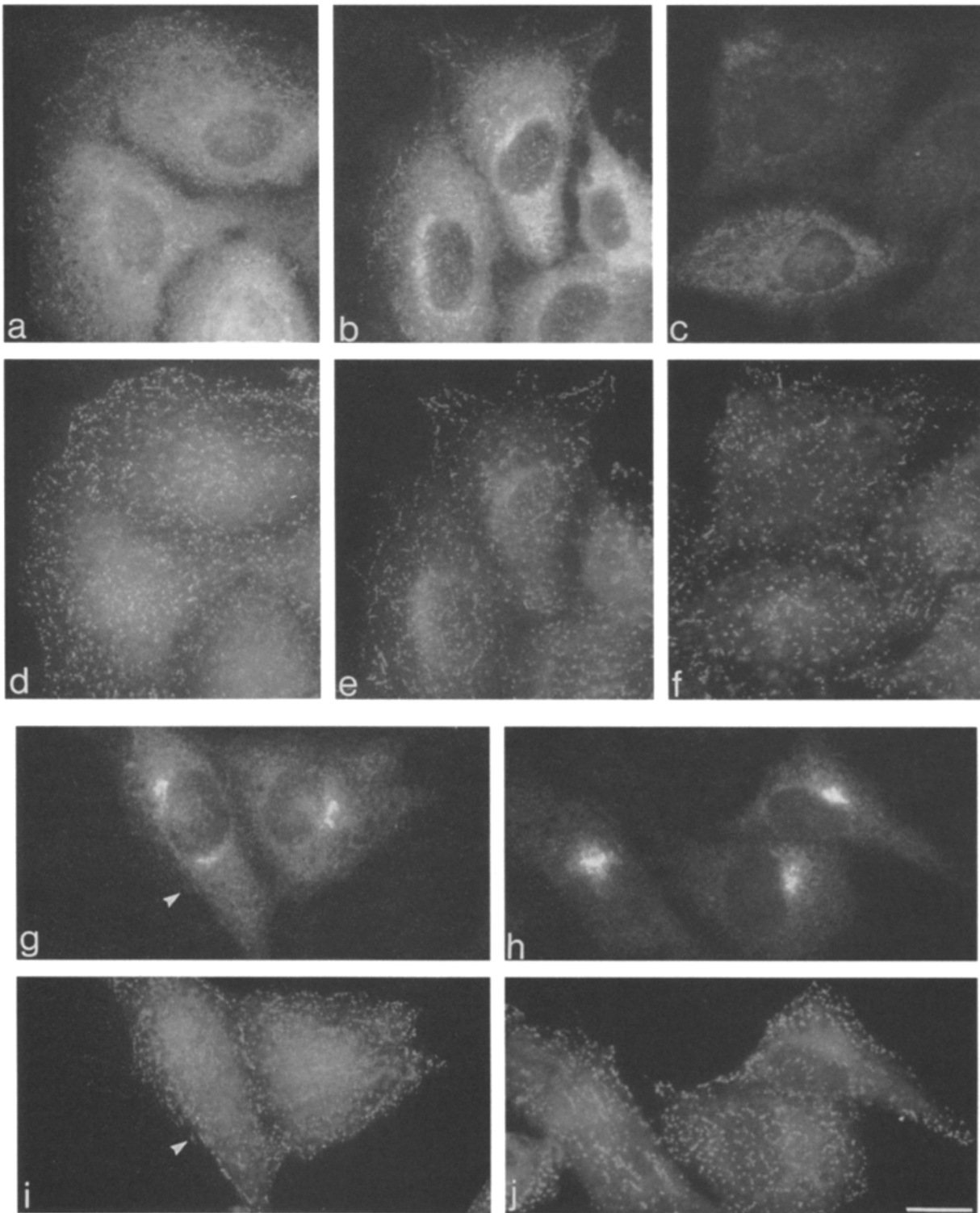


Figure 3. Immunofluorescence localization of the $N\gamma$ series of constructs. Stably transfected cells were labeled with mAb 100/3, which recognizes the bovine γ hinge and thus only labels the chimeric protein and not endogenous α -adaptin (*a-c*, *g*, and *h*). The cells were double labeled with a rabbit antibody against the α -adaptin hinge, which only recognizes endogenous α -adaptin (*d-f*, *i*, and *j*). $N\gamma$ -36 (*a*) and $N\gamma$ -132 (*b*) are targeted to the plasma membrane, $N\gamma$ -190 (*c*) is cytoplasmic, and $N\gamma$ -331 (*g*) and $N\gamma$ -566 (*h*) are targeted primarily to the TGN although some plasma membrane labeling can also be seen, particularly in the cells expressing $N\gamma$ -331 (*arrowheads*). Bar, 15 μ m.

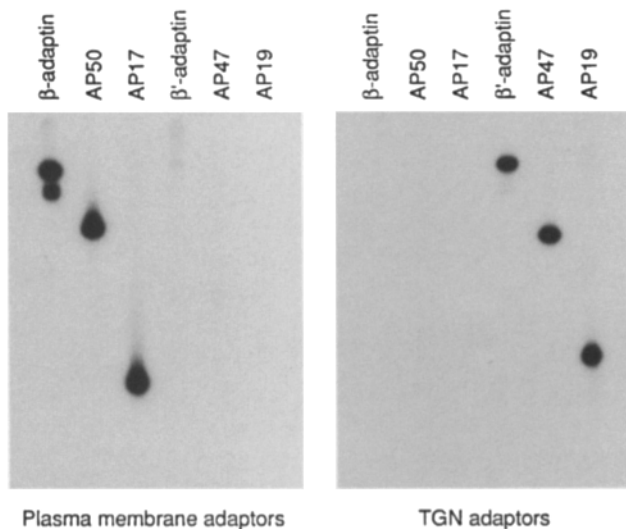


Figure 4. Western blots of purified adaptors labeled with antibodies against the various subunits. Strips were cut from blots of either plasma membrane adaptors (*left*) or TGN adaptors (*right*) and probed with antibodies raised against nonhomologous stretches of sequence expressed as GST fusion proteins. The labeling shows that the antibodies are specific for the subunit against which they were raised. The lower molecular mass band labeled with the antibody against β -adaptin is presumably a breakdown product, since β -adaptin is known to be susceptible to proteolysis (Kirchhausen et al., 1989).

brought down, which appeared to be below the limit of detection for most of the antibodies. However, the immunofluorescence results shown in Fig. 6 reveal a very similar pattern to that seen for the $N\gamma$ series.

$N\alpha$ -132 (Fig. 6, *a*) is localized to the TGN, with considerable cytoplasmic staining but no detectable plasma membrane staining (*d*). The next two chimeras in this series, $N\alpha$ -171 (*b*) and $N\alpha$ -265 (*c*), appear to be completely cytoplasmic with no recruitment to either the plasma membrane (*e* and *f*) or the TGN. The last three chimeras, $N\alpha$ -350 (*g*), $N\alpha$ -430 (*h*) and $N\alpha$ -566 (*i*), are recruited to the plasma membrane as shown by their colocalization with endogenous α -adaptin (*j-l*). Thus, there is again a stretch of ~ 200 amino acids, between 132 and 350, which appears to be responsible for targeting the adaptors to the appropriate membrane, and chimeras made within this region do not associate with any membrane.

Although the chimeras $N\alpha$ -350, $N\alpha$ -430, and $N\alpha$ -566 are recruited to the plasma membrane, they have a rather patchy distribution. Not every coated pit and vesicle detected with the antibody against endogenous α -adaptin contains the chimeric constructs, and the regions of colocalization generally occur at areas where linear arrays are observed rather than discrete dots. Furthermore, endogenous α -adaptin tends to be depleted from the patches containing the chimeras (Fig. 6, *arrowheads*). In particularly

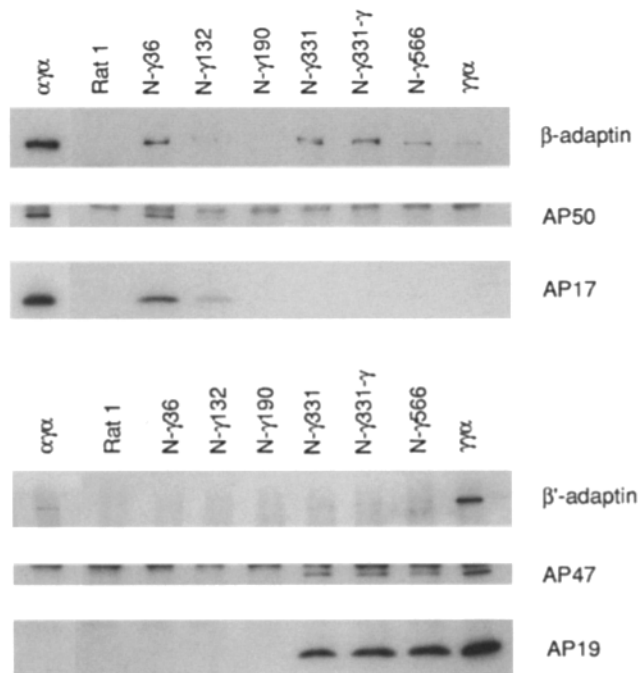


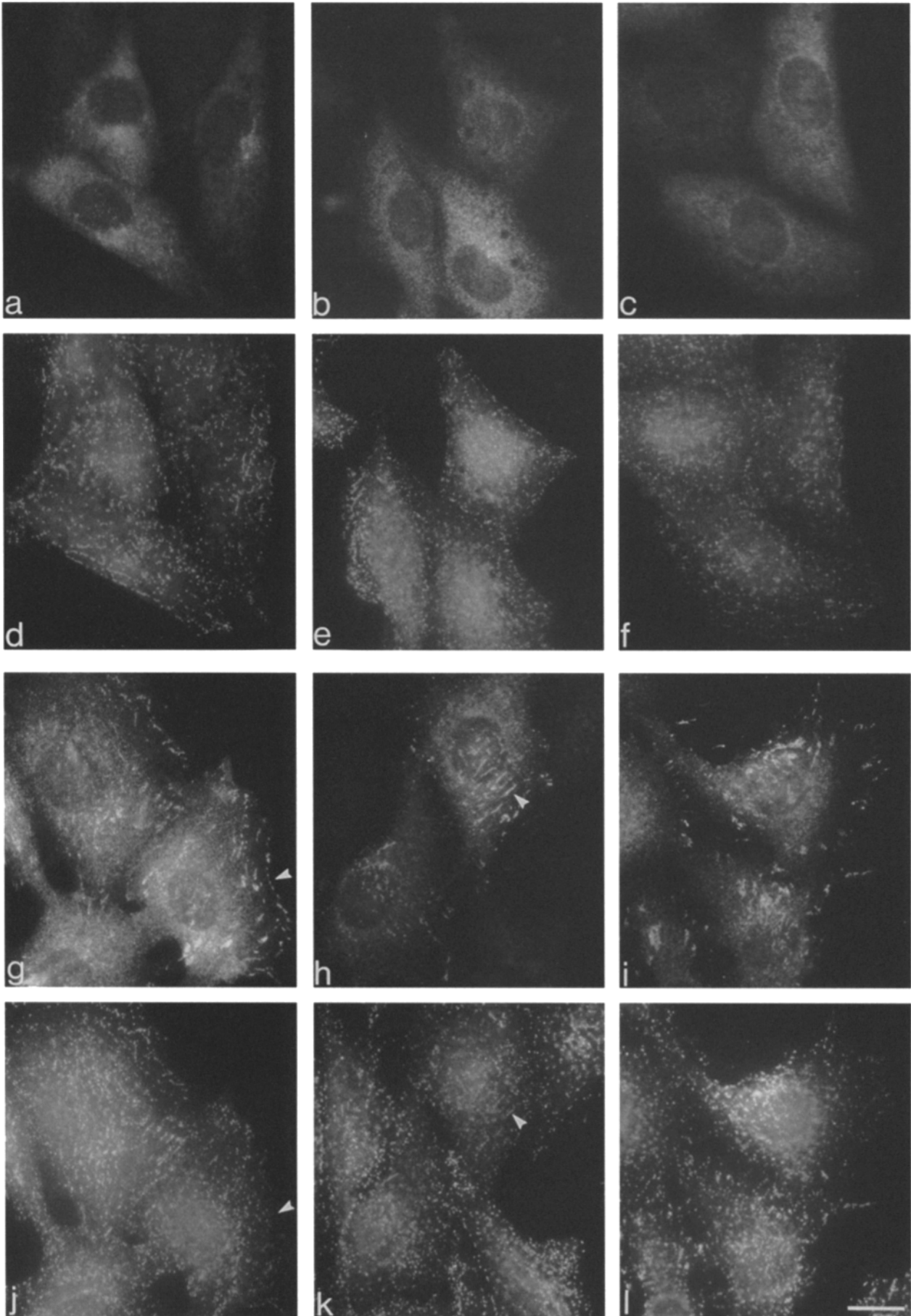
Figure 5. Immunoprecipitation of adaptor complexes containing chimeric adaptins. Extracts from stably transfected cell lines expressing constructs in the $N\gamma$ series, nontransfected Rat 1 cells, and cell lines expressing $\alpha\gamma\alpha$ and $\gamma\gamma\alpha$ were immunoprecipitated with mAb100/3 under nonreducing conditions. The samples were subjected to SDS PAGE, Western blotted, and probed with antibodies against the plasma membrane adaptor subunits β -adaptin, AP50, and AP17 (*top*); and against the TGN adaptor subunits β' -adaptin, AP47, and AP19 (*bottom*). In the blots probed for the medium chains AP50 and AP47, a contaminating band of ~ 50 kD can be seen that probably is the immunoglobulin heavy chain. The medium chains appear as sharper bands just below the contaminant. The higher intensity of labeling of the subunits that coprecipitate with $\alpha\gamma\alpha$ and $\gamma\gamma\alpha$ appears to be a reflection of the total amount of adaptor complex immunoprecipitated, since Western blotting with an antibody against the γ -adaptin hinge also gave a stronger signal (not shown).

highly expressing cells, such as those shown in Fig. 7, *a* and *c* (*small arrowheads*), there often appears to be less endogenous α -adaptin in general than nonexpressing cells (*large arrowheads*). These observations suggest that the chimeras may be competing with endogenous α -adaptin for the other adaptor subunits and/or for docking sites on the membrane. The linear arrays containing the chimeras tend to be concentrated at the cell periphery (Fig. 7, *b* and *d*), suggesting that they might be associated with adhesion plaques. However, double labeling with anti-talin revealed only limited colocalization (not shown).

Effect of the Ear Domain on Targeting

The constructs in both the $N\gamma$ series and the $N\alpha$ series

Figure 6. Immunofluorescence localization of the $N\alpha$ series of constructs. Stably transfected cells were double labeled with mAb 100/3 (*a, b, c, g, h, and i*) and the rabbit antibody against the α -adaptin hinge (*d, e, f, j, k, and l*). $N\alpha$ -132 (*a* and *d*) is targeted to the TGN, $N\alpha$ -171 (*b* and *e*) and $N\alpha$ -265 (*c* and *f*) are cytoplasmic, and $N\alpha$ -350 (*g* and *j*), $N\alpha$ -430 (*h* and *k*), and $N\alpha$ -566 (*i* and *l*) are targeted to the plasma membrane although the labeling is somewhat patchy. The arrowheads indicate regions of the cells where the construct is concentrated while endogenous α -adaptin is depleted. Bar, 15 μ m.



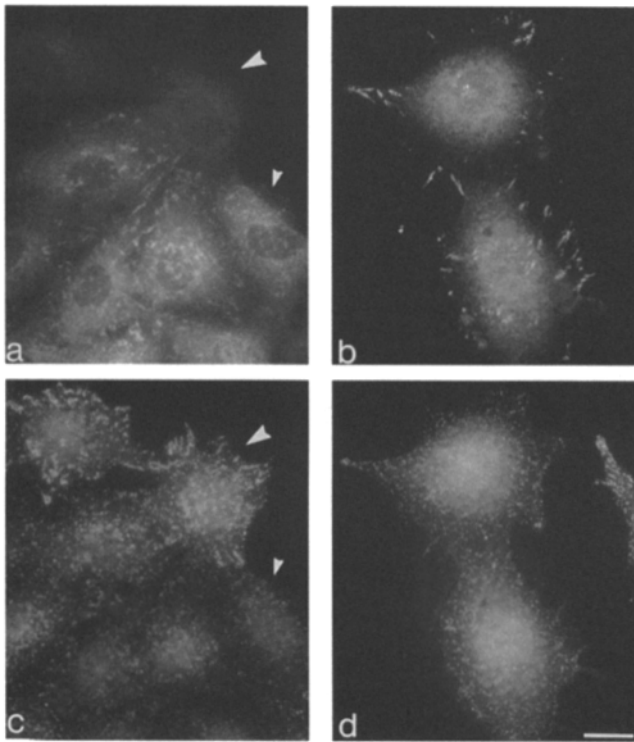


Figure 7. Immunofluorescence localization of the constructs N α -350 and N α -566 from the N α series, double labeled with mAb100/3 (*a* and *b*) and the rabbit antibody against the α -adaptin hinge (*c* and *d*). The constructs are generally found in linear arrays and tend to be concentrated at the cell periphery. In some of the highly expressing cells (*small arrowheads*), endogenous α -adaptin labeling appears to be less intense than in the nonexpressing cells (*large arrowheads*). Bar, 15 μ m.

generally support the view that the adaptor head contains the major targeting signal. However, one of the constructs, N γ -331, although primarily targeted to the TGN, also shows a substantial amount of labeling at the plasma membrane. To find out whether this plasma membrane localization is a result of the minor targeting signal in the α -adaptin ear, or whether there is another targeting signal between amino acid 331 and the hinge, an additional construct was made, N γ -331 γ , in which the α ear on N γ -331 was replaced with the γ -adaptin ear. An equivalent construct was also made from the N α series, N α -350 γ (see Fig. 2).

Unlike N γ -331 with the α ear (Fig. 8, *a* and *c*; see also Fig. 3), N γ -331 γ is localized exclusively to the TGN (Fig. 8, *b* and *d*). This indicates that the presence of the α -adaptin ear is solely responsible for the fraction of N γ -331 localized to the plasma membrane. The localization of the reverse construct, N α -350, is also influenced by its ear domain. This construct has a patchy plasma membrane distribution when the α -adaptin ear is present (Fig. 8, *e* and *g*; see also Figs. 6 and 7). However, with the γ -adaptin ear, although much of the construct is cytoplasmic, some of it is recruited to the TGN and no plasma membrane localization can be seen (Fig. 8, *f* and *h*). Thus, the ear has a much stronger effect on targeting when placed on constructs containing a chimeric NH₂-terminal domain.

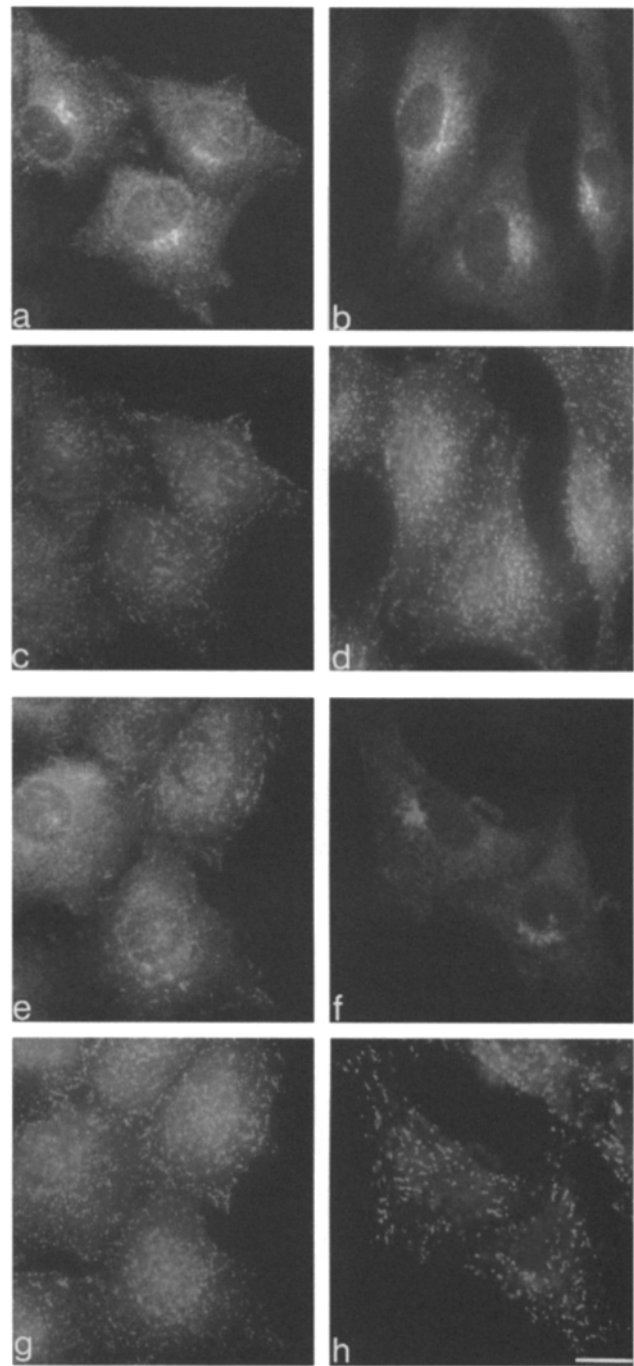


Figure 8. Effect of the ear domain on targeting. Cells expressing the constructs N γ -331 (*a*) and N α -350 (*e*) with the α -adaptin ear, and cells expressing the same constructs but with the γ -adaptin ear, N γ -331 γ (*b*) and N α -350 γ (*f*), were double labeled with mAb 100/3 (*a*, *b*, *e*, and *f*) and the rabbit antibody against the α -adaptin hinge (*c*, *d*, *g*, and *h*). The presence of the γ -adaptin ear results in more TGN labeling relative to plasma membrane labeling. Bar, 15 μ m.

Localization of β -Adaptin

Are the β subunits redistributed in cells expressing some of the chimeras? Because the antibody raised against β -adaptin was able to be used for immunofluorescence, it was possible to address this question by double labeling.

Some of the constructs in the N γ series, including N γ -566, were found to associate preferentially with β -adap-
tin although they are localized to the TGN, which suggests that
some of the β -adap-
tin in these cells should also be detect-
able at the TGN. Fig. 9 shows a mixture of transfected and
nontransfected cells double labeled for the construct (a)
and for β -adap-
tin (b), and reveals that in the transfected
cells, there is indeed a significant redistribution of β -adap-
tin to the TGN.

Another prediction is that by analogy with the N γ se-
ries, constructs such as N α -350 in the N α series should
preferentially coassemble with β' -adap-
tin rather than
 β -adap-
tin. Thus, since endogenous α -adap-
tin is depleted
from patches of coated pits containing the construct, β -adap-
tin should also be depleted. Fig. 9, c and d, shows that this
is in fact the case. However, because as yet we have no evi-
dence that the construct associates with β' -adap-
tin, we
cannot completely rule out the possibility that adaptor
complexes assembled from this construct contain no β
subunit of either type.

Interactions between the Adaptor Subunits

The results of the chimera experiments suggest that the

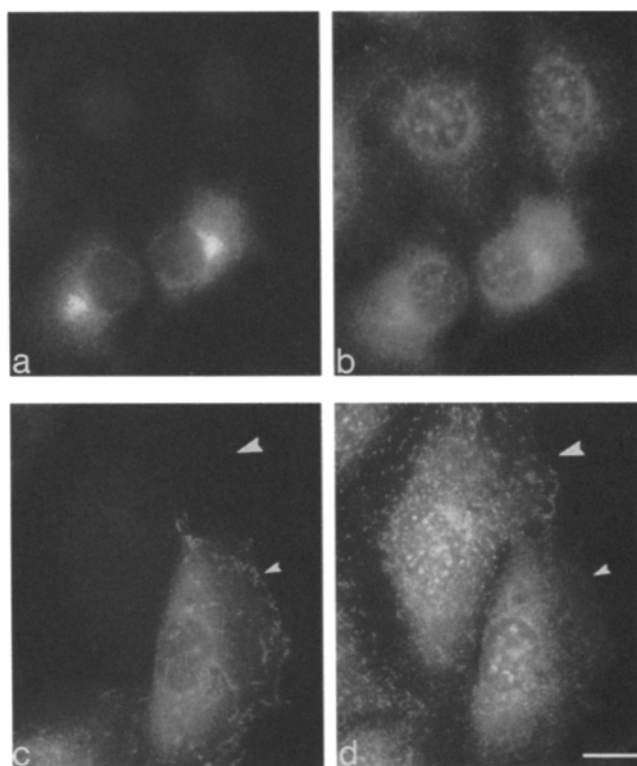


Figure 9. Immunofluorescence localization of β -adap-
tin in cells expressing the N α -350 (c and d) and N γ -566 (a and b) con-
structs. A mixture of transfected and nontransfected cells were
double labeled with mAb100/3 (a and c) and an antibody against
 β -adap-
tin (b and d). Expression of the constructs causes a redistri-
bution of β -adap-
tin. In the case of the N α -350-expressing
cells, β -adap-
tin is partially localized to the TGN region. In the
case of the N γ -566-expressing cells (small arrowheads), β -adap-
tin labeling is less intense, especially in regions containing the
construct, than in nonexpressing cells (large arrowheads). Bar,
15 μ m.

α - and γ -adap-
tins interact both with the β/β' -adap-
tins and
with the medium and/or small chains. However, it is not
possible using this method to show conclusively that two
subunits bind to each other, because all of the adaptor sub-
units are coexpressed within the same cell and interactions
could be indirect. To investigate interactions between defi-
ned pairs of subunits, we made use of the yeast two-
hybrid system. cDNAs encoding the various subunits were
inserted into the yeast vectors pGAD424 and pGBT9.
pGAD424 directs the expression of a transcriptional activator
fusion protein, while pGBT9 directs the expression
of a GAL 4 DNA binding domain fusion protein. If the
two fusion proteins interact, the transcriptional activator
domain is brought to the correct location to allow produc-
tion of β -galactosidase, which can be detected either by a
plate assay or by a liquid culture assay.

Table I shows the results of coexpressing different adap-
tor subunits using this system. As predicted by the immu-
noprecipitation experiments, β - and β' -adap-
tins were found
to behave promiscuously, binding to subunits in both adap-
tor complexes. Thus, β -adap-
tin is able to interact not only
with $\alpha\gamma\alpha$ and with the plasma membrane adaptor medium
chain AP50, but also with γ -adap-
tin ($\gamma\gamma\alpha$ and $\gamma\gamma\gamma$) and
with AP47. Similarly, β' -adap-
tin associates with both
plasma membrane and TGN adaptor α/γ subunits and me-
dium chains. No interactions could be detected between
the β subunits and the adaptor small chains.

Constructs containing the α - and γ -adap-
tin NH₂-termi-
nal domains, $\alpha\gamma\alpha$ and $\gamma\gamma\alpha$, were found to bind both to the

Table I. The β -Galactosidase Activities of Adaptor Subunits in
the Two-Hybrid System

pGAD424 insert	pGBT9 insert	Colony color	β -Galactosidase units
β	None	White	0.00
β	$\alpha\gamma\alpha^*$	Blue	38.56
β	$\gamma\gamma\alpha$	Blue	0.37
β	$\gamma\gamma\gamma$	Blue	0.52
β	AP50	Blue	102.00
β	AP47	Blue	25.35
β	AP17	White	0.00
β	AP19	White	0.00
β'	None	White	0.08
β'	$\alpha\gamma\alpha^*$	Blue	22.29
β'	$\gamma\gamma\alpha$	Blue	1.41
β'	$\gamma\gamma\gamma$	Blue	1.45
β'	AP50	Blue	20.08
β'	AP47	Blue	18.65
β'	AP17	White	0.01
β'	AP19	White	0.03
$\alpha\gamma\alpha$	None	White	0.01
$\alpha\gamma\alpha$	AP50	White	0.00
$\alpha\gamma\alpha$	AP47	White	0.00
$\alpha\gamma\alpha$	AP17	Blue	1.32
$\alpha\gamma\alpha$	AP19	White	0.01
$\gamma\gamma\alpha$	None	White	0.01
$\gamma\gamma\alpha$	AP50	White	0.02
$\gamma\gamma\alpha$	AP47	White	0.00
$\gamma\gamma\alpha$	AP17	White	0.00
$\gamma\gamma\alpha$	AP19	Blue	12.35

Yeast strains were grown in SD media lacking leucine and tryptophan and β -galactosi-
dase assays were performed on at least two independent colonies.

* $\alpha\gamma\alpha$ in the yeast vector pGBT9 produces a background β -galactosidase activity of
2.86 units.

β - and β' -adaptns and to the adaptor small chains, although apparently not to the medium chains, nor could any interactions be detected between the medium and small chains (not shown). In contrast to those interactions involving the β subunits, the interactions between α - and γ -adaptns and the small chains are entirely complex-specific. Thus, $\alpha\gamma$ interacts with AP17 but not with AP19, while $\gamma\alpha$ interacts with AP19 but not with AP17.

Discussion

Previous studies making use of α - and γ -adaptn chimeras have indicated that the major targeting determinant resides in the adaptor head rather than in the α and γ hinge or ear domains (Robinson, 1993). The present study reveals that a stretch of ~ 200 amino acids within the α - and γ -adaptn NH₂-terminal domain is important for the targeting of adaptors to the appropriate membrane. In the first series of constructs, the chimera N γ -132 was found to be recruited to the plasma membrane while N γ -331 was directed primarily to the TGN, suggesting that residues 132 to 331 are involved in targeting, but not residues 1-132 and 331-600. A similar conclusion was reached from the reverse series of constructs, where a region between residues 132 and 350 was found to be important. Any chimera made with a join between α - and γ -adaptn within this 200 amino acid stretch resulted in a cytoplasmic distribution, suggesting that this disrupted the targeting signal.

However, these findings do not necessarily mean that the 200-amino acid stretch itself contains the targeting signal. The NH₂-terminal domain of α - and γ -adaptn is the part of the protein that interacts with the other adaptor subunits, any one of which might contain targeting information, and an alternative explanation is that this stretch of sequence may be necessary for binding one or more of the other proteins. Thus, the subunit composition of the adaptor complexes assembled from the chimeric adaptns was determined. In the N γ series, essentially all of the constructs were found to associate preferentially with β -adaptn rather than with β' -adaptn, including those with a TGN distribution. Previous studies suggested but did not prove that the β subunits are not involved in adaptor localization. The present findings provide definitive evidence that β - and β' -adaptn do not contain any targeting information.

In contrast, the medium and/or small chains are strong candidates for subunits containing targeting signals, since the chimeras N γ -36 and N γ -132, which are targeted to the plasma membrane, were found to associate with AP50 and AP17, while N γ -331, N γ -566, and N γ -331 γ , which are targeted either primarily or exclusively to the TGN, interact with AP47 and AP19. This correlation between the presence of a particular medium and small chain and recruitment onto a particular membrane suggests that one or both of these subunits are likely to be involved in targeting, although a role for α/γ -adaptn NH₂-terminal sequence cannot be ruled out.

Although the major targeting determinant appears to reside in the adaptor head, the α - and γ -adaptn ears also contribute to targeting. This possibility was suggested by the original ear swap experiments, but the contribution of

the ears is more striking in the present study where different ears were placed on NH₂-terminal domain chimeras. Thus, N γ -331 with the α ear is found at the plasma membrane as well as at the TGN, while N γ -331 with the γ ear (N γ -331 γ) is found exclusively at the TGN; and N α -350 with the α ear has a patchy plasma membrane distribution, while with the γ ear (N α -350 γ) the membrane-associated fraction is found at the TGN. One possible explanation for these observations is that the ears may play a passive role in targeting, promoting adaptor-adaptor interactions and thus causing chimeric adaptors to bind to a particular membrane through interactions with endogenous adaptors. However, although self-association has been observed for plasma membrane adaptors *in vitro* (Beck and Keen, 1991), it seems to involve the head rather than the ear domains, and no such self-association has been observed for TGN adaptors. Alternatively, the ear domain might contain a weak but active targeting signal that takes over when a more dominant targeting signal is destroyed or weakened. This second possibility is supported by the observation that most of the NH₂-terminal domain chimeras appear to be impaired in their ability to associate with membranes, resulting in a more cytoplasmic distribution than that observed for either wild-type or ear-swapped adaptns. The fact that such chimeras, at least in the N γ series, are still capable of interacting with the medium and small chains suggests that the α - and γ -adaptn NH₂-terminal domains may contain intrinsic targeting information.

Although most of the chimeras showed at least some membrane association, those made with a join between amino acids ~ 130 and 330 – 350 appeared to be completely cytoplasmic. This suggests either that such constructs are missing a targeting signal in this region, or that they are unable to fold correctly and/or to coassemble with one or more of the other subunits. In the blot shown in Fig. 4 of the N γ series, none of the other subunits could be detected in the immunoprecipitate of the cytoplasmic construct N γ -190, but this result must be interpreted with caution since the precipitate also contained relatively little of the construct itself. Attempts to label blots of immunoprecipitates from the supernatant/cytosol fraction rather than the pellet/membrane fraction generally resulted in very high background, but AP19 was clearly present (not shown), and our inability to detect any of the other subunits unambiguously may simply be a reflection of the quality of the antibodies (the adaptor subunits are very poor antigens and these are the first such antibodies so far described).

Three of the constructs in the N α series, N α -350, N α -430, and N α -566, had an unexpected distribution: they were found in only a subset of the plasma membrane coated pits, which tended to form linear arrays. This sort of linear pattern has also been observed in nontransfected cells, where the lines have been shown to correspond to stress fibers (Anderson et al., 1978), but in the transfected cells these linear arrays were often devoid of endogenous adaptors. The exclusion of endogenous adaptors from the coated pits containing the constructs suggests that some sort of competition might be occurring. For instance, the chimeric adaptors may compete with endogenous adaptors for a specific receptor or docking site, or they may preferentially self-associate. Alternatively, they may be impaired in their ability to cycle on and off the membrane

and the coated pits containing the chimeras may be old pits unable to pinch off as coated vesicles and/or to uncoat.

As well as providing information about targeting signals, the construction of chimeras has also extended our knowledge of how the adaptor subunits interact with each other. Thus, we have identified amino acids 132–331 as a sequence involved in the binding of the medium and/or small chains, since the construct N γ -132 associates with AP50 and AP17, while N γ -331 associates with AP47 and AP19. We have also identified amino acids 566–594 as a sequence involved in the binding of the β subunit, since N γ -566 preferentially associates with β -adaplin while $\gamma\gamma\alpha$ preferentially associates with β' -adaplin. Interestingly, this sequence is immediately downstream from an 11-amino acid sequence that is identical in α - and γ -adaplin. Possibly the two sequences together constitute an important part of the β binding domain, the first sequence being involved in general binding and the second sequence providing some specificity. It is clear, however, that β - and β' -adaplin behave somewhat promiscuously. Immunoprecipitates not only of the NH₂-terminal domain chimeras, but also of the constructs $\alpha\gamma\alpha$ and $\gamma\gamma\alpha$, and even of wild-type α - and γ -adaplin in pig brain cytosol (not shown), show a certain amount of coprecipitation of the wrong β subunit.

The yeast two-hybrid approach has allowed us to investigate interactions between adaptor subunits more directly, using a more defined system. We were able to confirm that β - and β' -adaplin bind promiscuously, not only to α - and γ -adaplin but also to the adaptor medium chains. We were also able to demonstrate complex-specific interactions between the α/γ subunits and the adaptor small chains. Thus, we can now begin to refine the schematic model shown in Fig. 1 to include specific protein–protein interactions. An earlier study making use of reversible cross-linkers suggested that the 100-kD proteins (i.e., adaptins) interact with both the medium and the small chains, but at that time it was not possible to distinguish between the different adaptins (Virshup and Bennett, 1988). More recently a model has been proposed, based on relative sequence homologies, in which the β subunits bind to the small chains while the α/γ subunits bind to the medium chains (Nakayama et al., 1991). Our results show that in fact the opposite is the case. However, we cannot rule out the possibility that there may be additional interactions that cannot be detected using the two-hybrid system due to conformational differences between isolated subunits and the adaptor complex as a whole. Indeed, it seems likely that the medium and small chains must interact with each other to account for our immunoprecipitation results, since AP50 was always found to be coincident with AP17, and AP47 with AP19.

In conclusion, our results show that there must be more than one targeting signal in the adaptor complex. The β subunits, which are to some extent interchangeable, can be ruled out; but the α - and γ -adaplin ears play more of a role in targeting than was previously suspected, and there is also a targeting signal in the head that is likely to involve the medium and/or small chains, and possibly also the NH₂-terminal domains of the α - and γ -adaplin. These findings can be correlated with studies on adaptor targeting making use of in vitro reconstitution systems. It is clear from such studies that adaptor recruitment is not a simple

binding event. Energy is required, and there is evidence that members of the ARF family of proteins are involved, possibly by activating the putative docking proteins (Robinson and Kreis, 1992; Traub et al., 1993; Stamnes and Rothman, 1993; Seaman et al., 1993). Membrane proteins known to be concentrated in clathrin-coated pits and vesicles, such as the mannose-6-phosphate receptor, are also likely to play a role (Le Borgne et al., 1993). Recent cross-linking experiments suggest that the docking proteins may consist of more than one subunit (Seaman, 1995; Seaman and Robinson, unpublished observations). Thus, it is possible that different targeting signals on the adaptor complexes interact with different components of the cellular machinery involved in adaptor recruitment. The yeast two-hybrid system has the potential to be used not only to investigate interactions between known proteins such as neighboring adaptor subunits, but also to look for unknown binding partners. We are currently using this method to try to identify novel proteins that interact with each of the adaptor subunits.

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