

γ -Synergins: An EH Domain-containing Protein that Interacts with γ -Adaptin

Lesley J. Page, Penelope J. Sowerby, Winnie W.Y. Lui, and Margaret S. Robinson

Department of Clinical Biochemistry and Cambridge Institute for Medical Research, University of Cambridge, Cambridge CB2 2XY, England

Abstract. The AP-1 adaptor complex is associated with the TGN, where it links selected membrane proteins to the clathrin lattice, enabling these proteins to be concentrated in clathrin-coated vesicles. To identify other proteins that participate in the clathrin-coated vesicle cycle at the TGN, we have carried out a yeast two-hybrid library screen using the γ -adaptin subunit of the AP-1 complex as bait. Two novel, ubiquitously expressed proteins were found: p34, which interacts with both γ -adaptin and α -adaptin, and γ -synergins, an alternatively spliced protein with an apparent molecular mass of \sim 110–190 kD, which only interacts with γ -adaptin. γ -Synergins is associated with AP-1 both in the cytosol and on TGN membranes, and it is strongly

enriched in clathrin-coated vesicles. It binds directly to the ear domain of γ -adaptin and it contains an Eps15 homology (EH) domain, although the EH domain is not part of the γ -adaptin binding site. In cells expressing α -adaptin with the γ -adaptin ear, a construct that goes mainly to the plasma membrane, much of the γ -synergins is also rerouted to the plasma membrane, indicating that it follows AP-1 onto membranes rather than leading it there. The presence of an EH domain suggests that γ -synergins links the AP-1 complex to another protein or proteins.

Key words: AP-1 • γ -adaptin • clathrin • TGN • EH domain

THE coats on clathrin-coated vesicles are assembled from two components, clathrin and adaptor complexes or APs, both of which must be recruited from the cytosol onto the appropriate membrane. Two adaptor complexes have been identified on clathrin-coated vesicles, AP-1, which is associated with the TGN, and AP-2, which is associated with the plasma membrane. Both complexes are heterotetramers, consisting of two large subunits or adaptins (γ and β 1 in the case of AP-1, α and β 2 in the case of AP-2), a medium-sized subunit (μ 1 or μ 2), and a small subunit (σ 1 or σ 2) (Robinson, 1997).

A number of additional peripheral membrane proteins are now known to be involved in clathrin-coated vesicle formation at the plasma membrane, and most of these have been shown to bind either directly or indirectly to the COOH-terminal ear domain of the α -adaptin subunit of the AP-2 complex. These include amphiphysin, which binds directly to the α -adaptin ear (David et al., 1996), and

dynamins, which binds to amphiphysin and possibly also directly to the α -adaptin ear (Wang et al., 1995; David et al., 1996). Two other amphiphysin binding partners are synaptojanin (DeHeuvel et al., 1997) and endophilin, a protein that interacts with synaptojanin as well as with amphiphysin (Micheva et al., 1997). Dynamins is a GTPase that may be involved in the pinching off of the vesicle from the plasma membrane, whereas synaptojanin is an inositol 5-phosphatase. The exact functions of amphiphysin and endophilin are unknown, other than that they each have two or more binding partners. Another protein that binds directly to the α -adaptin ear is Eps15 (Benmerah et al., 1996; Iannolo et al., 1997), first identified as a substrate for the EGF receptor tyrosine kinase (Fazioli et al., 1993; Wong et al., 1995). Eps15 contains three copies of a motif known as an EH¹ (for Eps15 homology) domain (Wong et al., 1995), which has been shown to bind to sequences containing the tripeptide NPF (Salcini et al., 1997). One of the isoforms of synaptojanin contains three NPF sequences, making it a potential binding partner for Eps15, and there is more direct evidence for an interaction between the EH domains of Eps15 and the NPF-containing protein epsin (Chen et al., 1998). In addition, epsin has been shown to bind di-

L.J. Page and P.J. Sowerby contributed equally to this work.

Dr. Page's present address is Imperial Cancer Research Fund, Lincoln's Inn Field, London WC2A 3PX.

Address correspondence to Margaret S. Robinson, University of Cambridge, CIMR, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2XY, England. Tel.: 44-1223-330163. Fax: 44-1223-762322. E-mail: msr12@mole.bio.cam.ac.uk

1. *Abbreviations used in this paper:* BFA, brefeldin A; EH, Eps15 homology; EST, expressed sequence tag; GST, glutathione-S-transferase.

rectly to the α -adaptin ear (Chen et al., 1998). There is also some evidence that epsin binds to the EH domain-containing proteins Eps1 and Eps2, the vertebrate homologues of the *Drosophila* protein Dap160, recently identified as a dynamin binding partner (Roos and Kelly, 1998; Sengar et al., 1999). Thus, although clathrin and adaptors are the most abundant components of clathrin-coated vesicles, it is now apparent that there are many additional proteins involved in clathrin-coated vesicle formation, at least at the plasma membrane, which may act in an orderly sequence to drive the process forward.

Clathrin-coated vesicle formation is likely to be just as complex at the TGN as at the plasma membrane. However, so far no accessory molecules have been identified that interact either directly or indirectly with the AP-1 complex. Part of the reason may be that many of the proteins described above were originally purified and characterized as abundant components of nerve terminals, where AP-2 and clathrin are also concentrated because of the enormous amount of endocytosis that must take place in the nerve terminal to recycle synaptic vesicle components. In this study, we set out to identify novel AP-1 binding partners, specifically those that interact with the γ -adaptin subunit. We were particularly interested in proteins that might participate in the recruitment of AP-1 onto the TGN membrane. Experiments making use of chimeras between γ - and α -adaptin have shown that these subunits contain at least some of the targeting information (Robinson, 1993; Page and Robinson, 1995). We also hoped to find proteins that might participate in a complex network of interactions involving the AP-1 complex, similar to the one described for the AP-2 complex. The approach that we used was to carry out a yeast two-hybrid library screen, using full-length γ -adaptin as bait. Here we report the discovery of a novel γ -adaptin binding partner, which interacts with the COOH-terminal ear domain and may act as an adaptor adaptor, connecting the AP-1 complex to other proteins in the same way that amphiphysin, Eps15, and epsin are thought to connect the AP-2 complex to proteins such as dynamin and synaptojanin.

Materials and Methods

Library Screening

A Matchmaker rat brain cDNA library in pGAD10 was purchased from CLONTECH Laboratories and screened according to the manufacturer's instructions. The construct used as bait was full-length γ -adaptin in the vector pGBT9 (Page and Robinson, 1995). The colonies were allowed to grow for 8 d on plates lacking tryptophan, leucine, and histidine, and then replica plated and assayed for β -galactosidase activity. Positive colonies were grown in medium lacking tryptophan but containing leucine and histidine and tested for loss of the pGBT9- γ -adaptin plasmid, and then re-assayed for β -galactosidase activity. Only those colonies that tested negative were analyzed further.

Plasmid DNA was isolated from eight representative colonies, transformed into *Escherichia coli* TG2 cells, and analyzed by sequencing and Southern blotting (Sambrook et al., 1989). To find out whether the remaining yeast colonies contained the same plasmids as the test colonies, colony blots were prepared using Hybond-N⁺ nylon membranes. The membranes were placed on a plate of colonies, transferred (colony side up) onto filter paper soaked in 1 M sorbitol, 0.1 M sodium citrate, pH 7.0, 50 mM EDTA, and 15 mM DTT containing 2 mg/ml yeast lysis enzyme (ICN Biochemicals), and incubated overnight at 30°C. Lysis of the cells was completed by incubating the membranes on filter paper soaked with

10% SDS for 5 min. The membranes were treated with NaOH to denature the DNA, neutralized, and alkali fixed (Sambrook et al., 1989). Prehybridization, hybridization, and washing were carried out as previously described (Robinson, 1989, 1990).

The constructs obtained from the library screen were all tested to determine whether they interacted with any of the other adaptor subunits (Page and Robinson, 1995), and the p34 clone was found to interact with α -adaptin as well as γ -adaptin. To determine whether the binding sites for p34 on the two adaptins mapped to their NH₂-terminal domains, both constructs were digested with ApaI, which cuts just upstream from the hinge, end repaired, and digested with EcoRI, which cuts at the 5' end, and ligated into pGBT9 digested with EcoRI and SmaI. The resulting constructs were cotransformed with the p34 clone (in pGAD424) into host cells, and the colonies were assayed for β -galactosidase activity.

To obtain the complete coding sequence of γ -synergin, a probe was prepared from the clone obtained in the two-hybrid library screen by random priming and was used to screen a rat brain cDNA library in λ gt10 (CLONTECH Laboratories). Library screening, subcloning, and characterization of the clones were all carried out as previously described (Robinson, 1989, 1990). The expressed sequence tag (EST) database was also searched and three human cDNAs were identified (clone I.D. numbers 510825, 565380, and 51509). These three clones were obtained from the IMAGE Consortium and sequenced in their entirety in both directions, using oligonucleotide primers. In addition, a search of the nonredundant database revealed a partial human genomic sequence for γ -synergin (accession number AC004099), which enabled us to confirm the EST sequences and to identify the exon-intron boundaries, including the alternative splice sites.

Northern Blotting

A rat multiple tissue Northern blot was purchased from CLONTECH Laboratories and probed according to the manufacturer's instructions. The probe for p34 was the insert from one of the clones from the two-hybrid screen, containing the full coding sequence, whereas the probe for γ -synergin was the insert from the single clone identified in the two-hybrid screen, which encodes the NH₂-terminal portion of the protein (see below). Both inserts were labeled with [³²P]dCTP by random priming (Sambrook et al., 1989).

Antibody Production

Antibodies were raised against a GST- γ -synergin fusion protein, using the expression vector pGEX3X (Pharmacia). PCR was used to introduce SmaI and EcoRI sites into the insert from the original rat γ -synergin clone (corresponding to amino acids 168–786 of the full-length protein, but missing amino acids 197–274, presumably because the protein is alternatively spliced) so that it could be expressed in the appropriate reading frame. The construct was soluble and was purified on GSH-Sepharose (Pharmacia) according to the manufacturer's instructions. Immunization and affinity purification of the resulting antisera were carried out as previously described (Page and Robinson, 1995). In brief, two rabbits were injected with 0.5 mg of 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 preimmune and immune sera were tested on blots of the fusion protein. Both immune sera were found to give a strong signal, whereas no signal was obtained with the preimmune sera. The antisera were absorbed with GST and affinity-purified with GST-fusion protein, and then the affinity-purified antisera were absorbed again with GST (Page and Robinson, 1995).

Immunoprecipitation and Western Blotting

Immunoprecipitation of coat proteins from rat liver cytosol was carried out under nondenaturing conditions as previously described (Page and Robinson, 1995; Seaman et al., 1996). The antibodies included affinity-purified anticlathrin heavy chain, anti- γ -adaptin, and anti- α -adaptin (Ball et al., 1995; Seaman et al., 1996; Simpson et al., 1996), and the affinity-purified antibody against γ -synergin described above. Western blotting was carried out as previously described (Robinson and Pearce, 1986). The affinity-purified anti- γ -synergin was used at 1:500.

Immunofluorescence

Madin Darby bovine kidney cells were grown on multiwell test slides,

fixed with methanol and acetone, and prepared for immunofluorescence as previously described (Robinson, 1987). For some experiments, before fixation the cells were treated with 5 μ g/ml brefeldin A (Sigma Chemical Co.), and then either fixed immediately or washed in fresh medium and allowed to recover. To examine the distribution of γ -synergin in cells expressing the chimeric adaptin $\gamma\gamma\alpha$, stably transfected Rat1 cells (Robinson, 1993) were fixed as above. The fixed cells were incubated with mouse anti- γ -adaptin mAb100/3 (Sigma Chemical Co.) (Ahle et al., 1988), which interacts both with the endogenous (nonrodent) γ -adaptin in the MDBK cells and with the chimeric construct in the transfected Rat1 cells, together with affinity-purified rabbit anti- γ -synergin, followed by fluorescein-labeled donkey anti-rabbit IgG and Texas red-labeled sheep anti-mouse IgG, both obtained from Amersham. The slides were examined in a Zeiss Axioplan fluorescence microscope.

Identification of Binding Sites

The binding sites on γ -adaptin for γ -synergin and on γ -synergin for γ -adaptin were identified using both the yeast two-hybrid system (described above) and GST pull-down experiments. The γ -adaptin ear GST fusion protein has already been described (Seaman et al., 1996) and the α -adaptin ear GST fusion protein (Owen et al., 1999), used as a control, was a gift from David Owen (MRC Laboratory of Molecular Biology, Cambridge, UK). The γ -synergin GST fusion proteins were constructed from the clone isolated in the original two-hybrid library screen. The construct GST- γ s1 contains the rat sequence corresponding to amino acids 168–517 of the human sequence (but missing amino acids 197–274, presumably because of alternative splicing), the construct GST- γ s2 contains the rat sequence corresponding to amino acids 385–661 of the human sequence, and the construct GST- γ s3 contains the rat sequence corresponding to amino acids 518–786 of the human sequence. The construct GST-EH contains the EH domain and several amino acids on either side of it, corresponding to amino acids 188–390 of the human sequence (but missing amino acids 197–274). All of the constructs were soluble and were prepared as previously described (Page and Robinson, 1995). For the GST pull-down experiments, 1 ml of rat liver cytosol prepared in PBS containing 0.1% NP-40 and a protease inhibitor cocktail (Complete Mini; Boehringer Mannheim) at a protein concentration of 1.5 mg/ml was first pre-cleared by incubating for 8 h at 4°C on a rotating wheel with 10 μ g GST and 60 μ l of a 50% suspension of glutathione-Sepharose 4B (Pharmacia). The samples were incubated with 10 μ g of the appropriate fusion protein and 60 μ l of the 50% suspension of glutathione-Sepharose overnight at 4°C. The beads were pelleted, washed five times in PBS containing 0.1% NP-40, eluted with SDS-PAGE sample buffer, and subjected to SDS-PAGE and Western blotting.

A blot overlay assay was used to demonstrate that γ -adaptin and γ -synergin bind directly to each other. First, the insert from the GST- γ s3 construct was ligated in-frame into the vector pQE30 to introduce a histidine tag at the NH₂ terminus (Qiagen). Expression and purification of the resulting His- γ s3 construct were carried out as instructed by the manufacturer. A control construct, His-DHFR (supplied with the kit), was also expressed and purified. Equivalent amounts of the two His-tagged fusion proteins were subjected to SDS-PAGE and blotted onto a nitrocellulose membrane, and the blot was blocked with 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20, 0.5% BSA, 3 μ M reduced glutathione for 30 min. This buffer was used in all the following steps. The blot was incubated with 10 nM GST or γ -ear-GST for 45 min, washed for 30 min, and then labeled as described above using anti-GST followed by ¹²⁵I-protein A.

Results

Isolation of Clones Expressing γ -Adaptin Interacting Proteins

Full-length mouse γ -adaptin cDNA was used as bait to screen a yeast two-hybrid library containing inserts derived from rat brain cDNA. Out of $\sim 6 \times 10^6$ transformants, 62 clones were isolated that exhibited a strong and specific interaction between the γ -adaptin construct and the library construct. To determine the identity of each of the 62 clones, both colony blotting and sequencing were carried out. The results are shown in Table I.

Table I. List of Proteins Identified in the Two-hybrid Screen

Proteins identified in yeast two-hybrid screen	Number isolated
σ 1A	6
σ 1B	6
β -spectrin	11
p34	38
γ -synergin	1

A combination of colony blotting and/or sequencing was used to determine the identity of each colony.

Six of the clones were found to contain plasmids encoding σ 1 (σ 1A), the small chain subunit of the AP-1 adaptor complex. We have previously shown that σ 1 and γ -adaptin interact strongly in the yeast two-hybrid system, so the isolation of a σ 1-encoding plasmid confirmed that the library screen had worked (Page and Robinson, 1995). Another six plasmids were found to encode a protein closely related to σ 1, with 87% amino acid identity. This protein has been independently identified by Takatsu et al. (1998) and named σ 1B. Eleven plasmids were found to encode β -spectrin. The significance of this interaction is at present unclear (see Discussion). The other 39 plasmids were found to encode two unknown proteins, and these cDNAs were subjected to further analysis.

The first of the two unknown cDNAs was isolated with very high frequency in the two-hybrid screen, accounting for more than half of all the clones. A representative clone with an insert of ~ 2.5 kb was sequenced and was found to encode a protein of 315 amino acids with a deduced size of ~ 34 kD (p34) (Fig. 1 a). There are several mammalian ESTs in the database encoding p34, but no homologues were found that might help to establish the protein's function. Northern blotting demonstrated that p34 is expressed ubiquitously and that the mRNA has a size of ~ 2.75 kb (Fig. 1 b). Unlike most of the other proteins identified in the screen, p34 was found to interact not only with γ -adaptin but also with α -adaptin in the two-hybrid system, and this interaction was mapped to the NH₂-terminal domains of the two adaptins (Fig. 1 c). Attempts were made to raise antisera against p34, but unfortunately the protein proved to be a very poor antigen. Thus, although two different domains were expressed as fusion proteins for antibody production, and although the resulting antisera were affinity-purified, all of the antisera labeled multiple bands on Western blots. However, one of the antisera labeled a band of around the expected size (~ 37 kD), and this protein could be immunoprecipitated in substoichiometric amounts with cytosolic AP-1 and AP-2, suggesting that the interactions detected in the two-hybrid system are physiologically relevant (data not shown). But because we were looking for proteins that interact specifically with the AP-1 complex, p34 was not characterized further.

γ -Synergin: Sequence and Splicing

The other unknown cDNA came up with the least frequency in the library screen, accounting for only one of the clones. Northern blotting showed that the transcript is expressed ubiquitously and has a size of ~ 4.4 – 5.6 kb, with bands of different mobilities labeled in different tissues

a

MAAGVPCALV	TSCSATFTGD	RLVQHILGTE	DAVVEATSSD	AVRFYPWTID	NKYSAEVNL	60
CVVPSKCRVT	AEIAEAVQAF	VVYFDSTQKS	GLDSVSSWLP	LAETWLPEVM	ILVCDRVCED	120
GINRQQAQEW	CIKHGFELVE	LCPEELPEED	DDFPESTGVK	RIVQALNANV	WSNVVMKNDR	180
SQGFSLNLSL	AGASRSVGS	ESCQCEQEPS	PTAERTESLP	GHRSGACGPA	GAQVDSIVDP	240
MLDLDIQELA	SLTTGGGDLE	NFERLFSKLG	EMKDKAATLP	HEQRKLHAEK	VAKAFWMAIG	300
GDRDEIEGLS	SDDEH					315

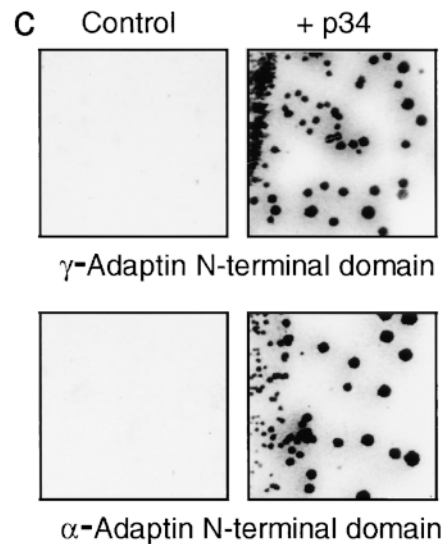
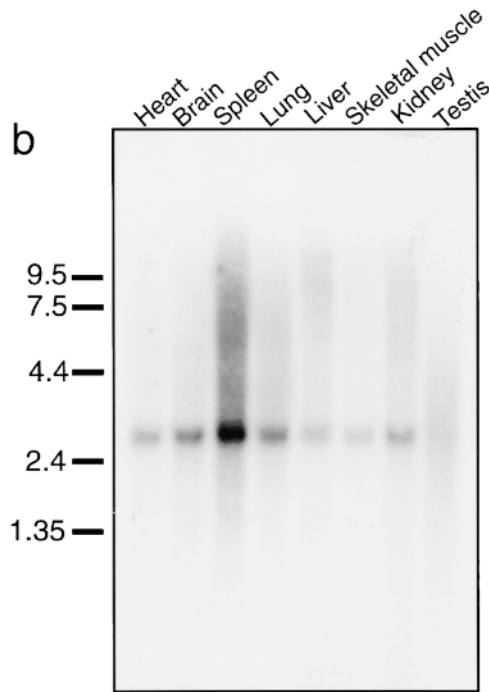


Figure 1. Characterization of p34. (a) Protein sequence deduced from the cDNA sequence. The identification of the initiator methionine is based on two observations: first, it is preceded by a Kozak sequence; and second, none of the clones, either from the original library screen or from a subsequent screen of a λ gt10 cDNA library, extended any further in the 5' direction. (b) Northern blot. A rat multiple tissue Northern blot was probed with the insert from one of the clones isolated in the two-hybrid library screen, which contains the complete coding sequence for p34. (c) p34 interacts with both γ and α in the two-hybrid system. The NH_2 -terminal domains of both γ -adaptin and α -adaptin were cloned into the two-hybrid bait vector pGBT9 and coexpressed with either the prey vector pGAD424 with no insert (control) or with the prey vector pGAD10 containing the coding sequence for p34. Colonies were assayed for the expression of β -galactosidase. Cells expressing p34 in pGAD10 alone produced no detectable β -galactosidase.

(Fig. 2 a). The original clone contained an insert of only ~ 1.6 kb and the sequence appeared to be all open reading frame, so this clone was used as a probe to screen a rat brain λ gt10 cDNA library to try to obtain a full-length sequence. Three additional clones were isolated and sequenced (Fig. 2 b). A comparison of the four sequences revealed that the mRNA is alternatively spliced, consistent with the heterogeneity seen on the Northern blot. A putative start was identified in one of the clones, but none of the clones had an in-frame stop codon at the 3' end. However, when the EST database was searched with the rat sequences, three human sequences were found, and from the corresponding cDNAs it was possible to assemble a contiguous human open reading frame. The nonredundant database was also searched with both the rat and the human sequences, and the human genomic sequence encoding the 3' end of the mRNA was found. Fig. 2 c shows the genomic structure, including the alternative splice sites.

An analysis of the open reading frame revealed that the protein contains an EH domain at amino acids 295–377 (Figs. 2 d and 3 b). EH domains, which bind to proteins containing the sequence NPF, have now been found in a large number of proteins, including the mammalian proteins Eps15, Ese1, and Ese2, and the yeast proteins End3p

and Pan1p, all of which are involved in endocytosis (Di Fiore et al., 1997). γ -Adaptin contains no NPF sequences, so it is likely that the novel protein serves as a linker between γ -adaptin and some other unidentified protein. We propose that this protein be called γ -synergin (from the Greek, synergos, meaning partner or workmate).

A schematic diagram of γ -synergin is shown in Fig. 3 a, indicating the positions of the EH domain, some of the alternative splice sites, and the γ -adaptin-binding domain (see below). Fig. 3 b shows an alignment of the EH domains from γ -synergin, Eps15, Ese1, End3p, and Pan1p. The EH domain of γ -synergin can be seen to contain all of the highly conserved amino acids found in other, well characterized EH domains. However, apart from the EH domain, γ -synergin shows no significant sequence homology to any other proteins in the database, and it does not share any of the other features found in the α -adaptin binding partner Eps15 such as a coiled-coil domain or a proline-rich region.

γ -Synergin: Association with AP-1 In Vivo

To learn more about the function of γ -synergin, the original clone identified in the two-hybrid library screen was

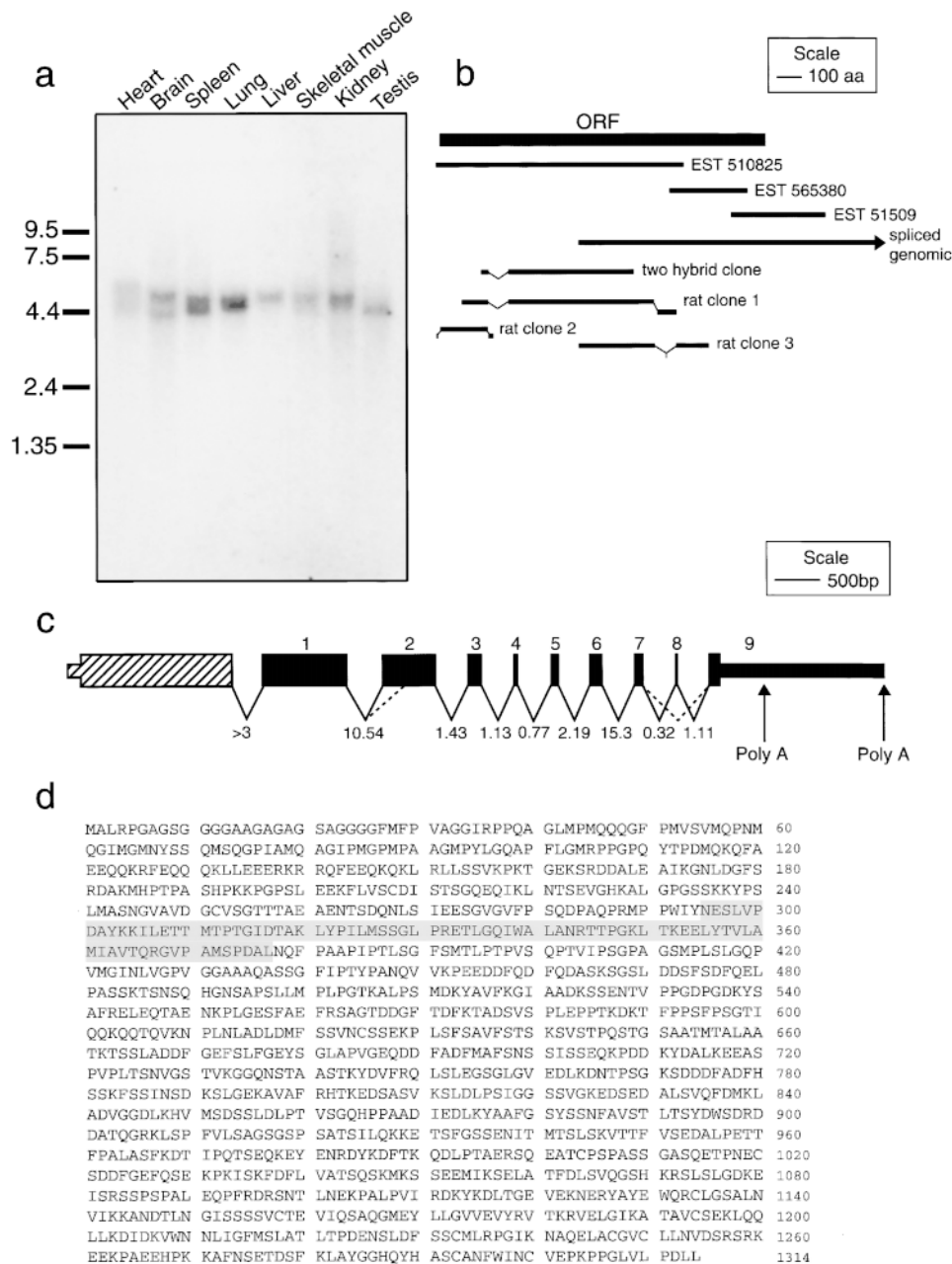


Figure 2. Characterization of γ -synergin at the nucleic acid level. (a) Northern blot. A rat multiple tissue Northern blot was probed with the insert from the clone isolated in the two-hybrid library screen. Different sized bands are labeled in different tissues, indicative of alternative splicing. (b) Diagram of the protein sequence of γ -synergin, showing the open reading frame, the three human ESTs used in the assembly of the full-length sequence, the partial genomic sequence (after splicing), the original clone isolated in the two-hybrid library screen, and the three clones isolated from a subsequent screen of a rat brain λ gt10 cDNA library. The rat clones isolated differ from the human sequence near their NH₂ termini in missing amino acids 197–274. Although there is no genomic sequence available for this region, this difference is likely to be due to alternative splicing. The 3' end of rat clone 1 has sequence homologous to the human intron between exons 1 and 2 (see c) and is, therefore, either an incompletely spliced precursor or an alternatively spliced mRNA. Rat clone 3 uses the alternative splice site between exons 1 and 2 indicated in c with an additional four amino acids that could be the result of an additional exon or a difference between the rat and human sequence at splice junctions. (c) Diagram of the human γ -synergin gene. Exon and intron junctions are shown for the 3' region where the genomic sequence is known. Hatched regions represent sequence derived solely from cDNA clones. The sizes of

the predicted introns (in kb) are indicated. The two alternative polyadenylation sites were determined from human ESTs in the database. (d) Deduced protein sequence of human γ -synergin. Amino acid 1,123 is ambiguous because of a single base difference between the submitted genomic sequence and our own cDNA sequence, and is either an E in the former or a K in the latter. The identification of the putative initiator methionine is based on the observation that it is preceded by a Kozak sequence and on the finding that there are three short (<500 bp), internally primed γ -synergin sequences in the EST database, none of which extends any further in the upstream direction than EST 510825. The EH domain of the protein is highlighted. The rat and human sequences have 85% amino acid identity overall, and 98% identity in the EH domain. These sequence data are available from GenBank/EMBL/DDBJ under accession numbers AF169548 (human sequence) and AF169549 (rat sequence).

expressed as a fusion protein for antibody production. Fig. 4 shows a Western blot of equal protein loadings of homogenate from both brain and liver as well as various subcellular fractions from liver probed with the affinity-purified antibody. Two bands are labeled in the brain of \sim 110 and \sim 150 kD, whereas in the liver, a single band is labeled of \sim 190 kD. This is consistent with the Northern blot (Fig. 2 a) in which a single band was labeled in liver, whereas two bands were labeled in brain, indicating that the different

protein species might represent different spliced variants, although we cannot rule out the possibility that the differences might also be due to proteolysis. γ -Synergin is found in both a high speed supernatant and membrane-containing pellet, indicating that it is peripherally associated with membranes. It is somewhat enriched in a TGN-enriched fraction from liver and it is strongly enriched in liver clathrin-coated vesicles.

The association between γ -synergin and γ -adaptin was

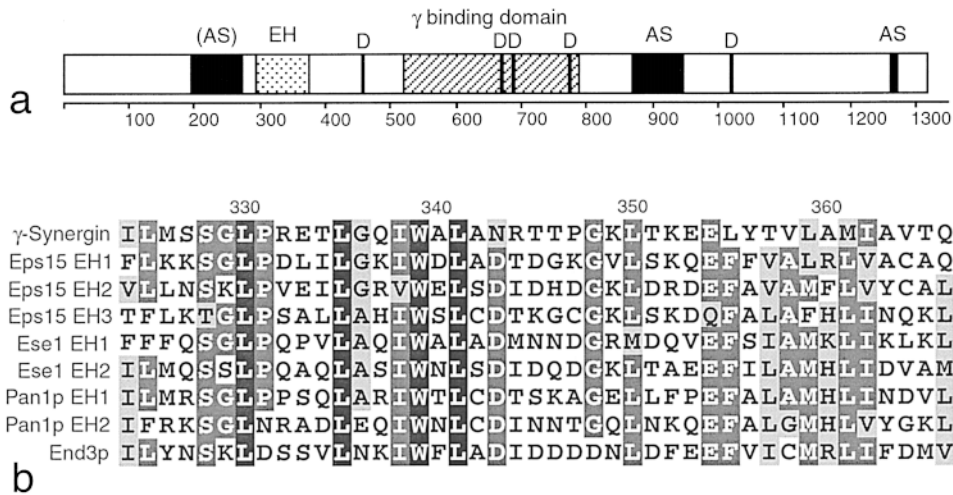


Figure 3. Characteristics of γ -synergin. (a) The schematic diagram shows the positions of the alternative splice sites (AS), EH domain, γ -adaplin-binding domain, and DDFD/EXF sequences (D). (b) Comparison of the EH domain of γ -synergin with that of several well characterized EH domain-containing proteins: the mammalian proteins Eps15 and Ese1, and the yeast proteins Pan1p and End3p.

confirmed by immunofluorescence microscopy. Double labeling of MDBK cells with anti- γ -synergin and anti- γ -adaplin revealed a striking degree of colocalization of the two proteins (Fig. 5, a and b). Next, we investigated whether the membrane association of γ -synergin is affected by the drug brefeldin A (BFA). This drug causes

ARF to dissociate rapidly from membranes (Donaldson et al., 1992; Helms and Rothman, 1992), leading to the dissociation of other peripheral membrane proteins whose membrane association is ARF-dependent, such as the AP-1 adaptor complex (Robinson and Kreis, 1992). In Fig. 5, c and d, MDBK cells were treated with BFA for 2 min, and then double labeled for γ -synergin (c) and γ -adaplin (d). Both proteins can be seen to have redistributed to the cytoplasm. To examine the behavior of the two proteins upon BFA washout, we treated cells with the drug for 30 min, and then allowed them to recover for 2 min (Fig. 5, e and f). Both proteins can be seen to have reassociated with the membrane, which now has a more tubular appearance as a result of the BFA treatment. Thus, γ -synergin, like the AP-1 adaptor complex, appears to associate with the TGN membrane in an ARF-dependent manner.

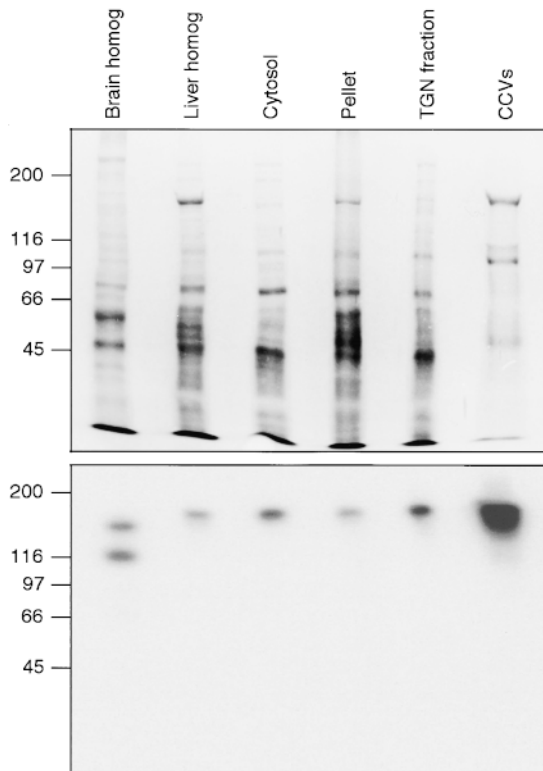


Figure 4. SDS-PAGE and Western blot probed with anti- γ -synergin. The samples, from left to right, are: pig brain homogenate, rat liver homogenate, rat liver high speed supernatant (cytosol), rat liver high speed pellet, rat liver TGN-enriched fraction, and rat liver clathrin-coated vesicles. The blot shows that γ -synergin is a peripheral membrane protein that is highly enriched in clathrin-coated vesicles. The different patterns seen in brain and liver may be due to alternative splicing.

The immunofluorescence data demonstrate that γ -synergin is associated with AP-1 on TGN membranes. To find out whether the two proteins are also associated in the cytosol, immunoprecipitations were carried out under non-denaturing conditions (Fig. 6). Rat liver cytosol was immunoprecipitated with anti- γ -adaplin followed by protein A-Sepharose, and Western blots were probed with anti- γ -synergin or anti- γ -adaplin. As a control, cytosol was also immunoprecipitated with anti- α -adaplin. Cytosolic γ -synergin was found to coprecipitate with γ -adaplin but not with α -adaplin. Thus, γ -synergin is associated with AP-1 in the cytosol as well as on membranes.

Identification of Binding Domains

To identify the domain on γ -adaplin that binds to γ -synergin and the domain on γ -synergin that binds to γ -adaplin, two approaches were used: yeast two-hybrid analysis and GST pulldown experiments. The NH_2 -terminal domain construct of γ -adaplin, which was found to interact with p34 in the two-hybrid system (Fig. 1 c), did not interact with γ -synergin nor did a γ -adaplin construct with the α -adaplin ear, indicating that γ -synergin binds to the ear domain of γ -adaplin (data not shown). This was confirmed using GST fusion proteins to isolate binding partners in rat liver cytosol, followed by Western blotting and probing with anti- γ -synergin. Fig. 7 shows that GST fused to the

γ -Synergin

γ -Adaptin

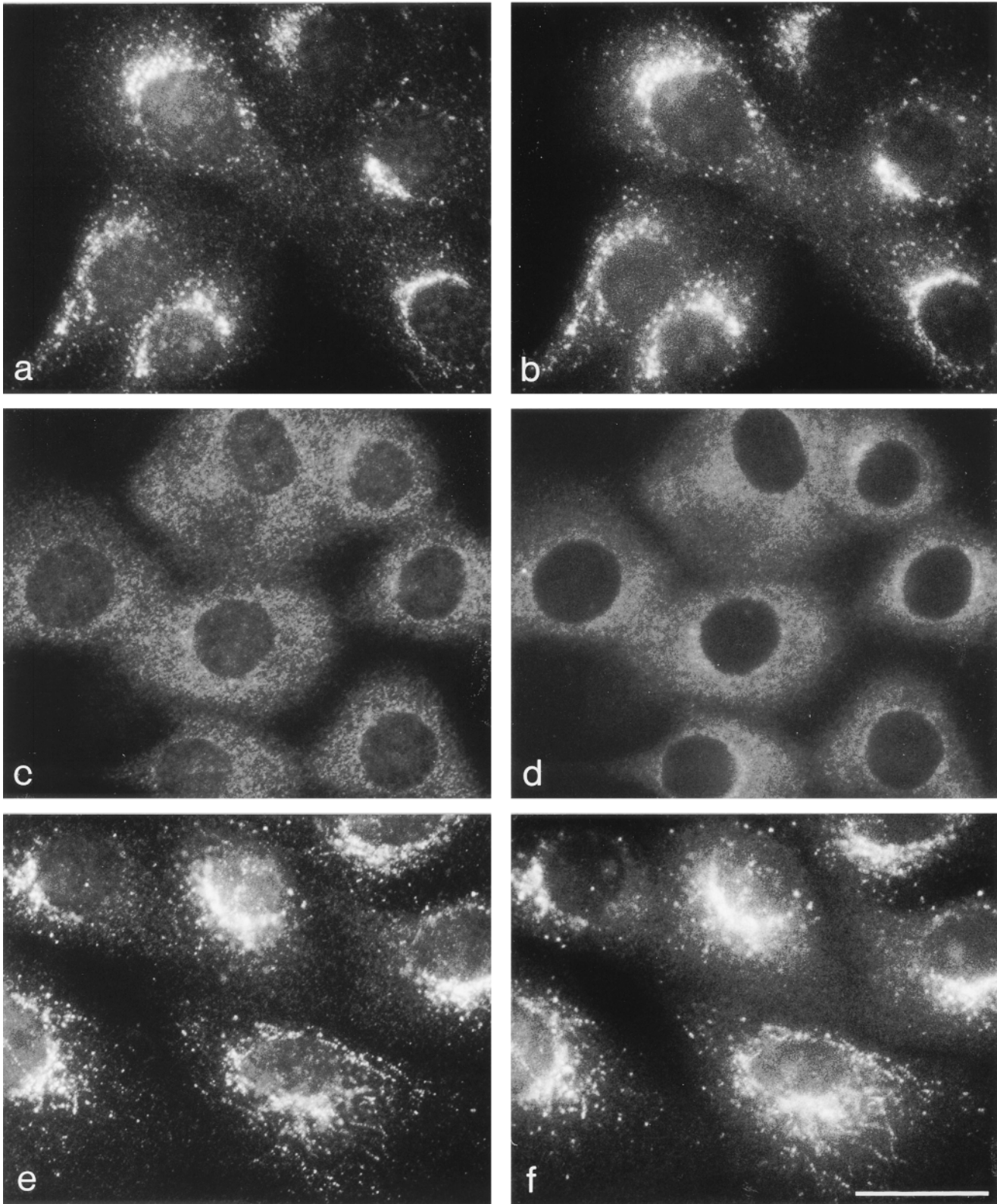


Figure 5. γ -Synergin colocalizes with γ -adaptin by immunofluorescence. MDBK cells were double labeled with anti- γ -synergin (a, c, and e) and anti- γ -adaptin (b, d, and f) either under control conditions (a and b), after incubating with brefeldin A for 2 min (c and d), or after incubating with brefeldin A for 30 min and allowing the cells to recover for 2 min (e and f). Bar, 20 μ m.

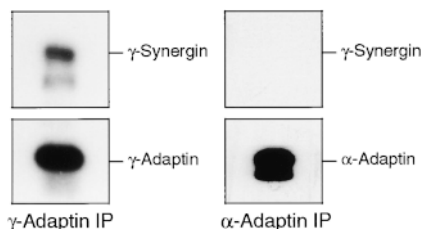


Figure 6. γ -Synergin is associated with AP-1 in the cytosol. Rat liver cytosol was immunoprecipitated with either anti- γ -adaptin or anti- α -adaptin, and Western blots were probed as indicated with either anti- γ -synergin, anti- γ -adaptin, or anti- α -adaptin. The lower molecular mass band (~ 165 kD) labeled with the anti- γ -synergin antibody is probably a breakdown product of the protein.

γ -adaptin ear binds γ -synergin, whereas GST alone or GST fused to the α -adaptin ear do not.

The same general strategy was used to identify the domain of γ -synergin that binds to γ -adaptin. Four sets of constructs were made from the original γ -synergin clone isolated in the two-hybrid library screen: one containing just the EH domain (GST-EH); one containing the NH₂-terminal half, including the EH domain (GST γ -1, corresponding to amino acids 168–517 of the human sequence but missing amino acids 197–274, presumably because of alternative splicing); one containing the middle portion of the protein (GST γ -2, amino acids 385–661); and one containing the COOH-terminal half (GST γ -3, amino acids 518–786). Only the construct containing the more COOH-terminal portion of γ -synergin (GST γ -3) bound γ -adaptin in the GST pulldown experiments (Fig. 7 b), and this interaction was confirmed using the two-hybrid system (data not shown).

The GST pulldown experiments were carried out using whole cytosol, and, thus, they cannot distinguish between a direct interaction between γ -synergin and γ -adaptin and an indirect one, mediated by another protein or proteins. The ability of the two proteins to interact in the yeast two-hybrid system strongly suggests that the interaction is direct, but to prove this formally, we also carried out Western blot overlay experiments, a technique that has been used to demonstrate that the α -adaptin ear domain binds directly to proteins such as amphiphysin and epsin (Chen et al., 1998; Owen et al., 1999). For these experiments, the portion of γ -synergin that contains the γ -adaptin-binding domain, amino acids 518–786, was expressed as a histidine-tagged construct (His- γ 3) and purified on a nickel affinity column. A control histidine-tagged construct was also expressed and purified (His-control). The two constructs were subjected to SDS-PAGE, blotted, and probed either with GST alone followed by anti-GST, with GST- γ ear followed by anti-GST, or with anti- γ -synergin. Fig. 7 c shows that the GST- γ ear construct, but not GST alone, binds to the His- γ 3 band on the Western blot. Thus, the interaction between γ -adaptin and γ -synergin must be a direct one. The binding site on γ -synergin for γ -adaptin is indicated in Fig. 3 a.

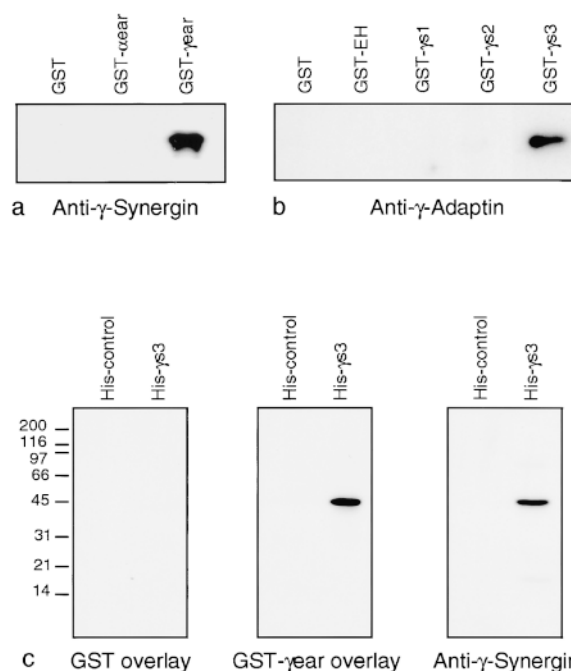


Figure 7. Identification of binding sites on γ -synergin and γ -adaptin. (a, left to right) Rat liver cytosol was incubated with GST alone, with a GST fusion protein containing the α -adaptin ear domain, or with a GST fusion protein containing the γ -adaptin ear domain, followed by glutathione-Sepharose. The Western blot was probed with anti- γ -synergin. The γ -synergin can be seen to bind specifically to the ear domain of γ -adaptin. (b, left to right) Rat liver cytosol was incubated with GST alone, with a GST fusion protein containing the EH domain of γ -synergin, with a GST fusion protein containing amino acids 168–517 of γ -synergin (but missing amino acids 197–274, presumably because of alternative splicing) (GST- γ 1), with a GST fusion protein containing amino acids 385–661 of γ -synergin (GST- γ 2), or with a GST fusion protein containing amino acids 518–786 of γ -synergin (GST- γ 3), followed by glutathione-Sepharose. The Western blot was probed with anti- γ -adaptin. Only the GST- γ 3 construct pulls down γ -adaptin. (c) Two histidine-tagged constructs, one containing the same domain of γ -synergin as the fusion protein γ 3 (His- γ 3) and a control construct, histidine-tagged DHFR (His-control), were subjected to SDS-PAGE followed by Western blotting. The blots were probed with either GST followed by anti-GST, GST coupled to the γ -adaptin ear domain (GST- γ ear) followed by anti-GST, or anti- γ -synergin. A band of ~ 45 kD, corresponding to His- γ 3, can be seen to be labeled in the GST- γ ear overlay.

γ -Synergin Follows AP-1 onto the Membrane

We have previously shown that the COOH-terminal ear domains of γ - and α -adaptin contain weak targeting signals for recruitment onto the TGN and plasma membranes, respectively (Robinson, 1993; Page and Robinson, 1995). Thus, a construct containing mostly α -adaptin, but with the γ -adaptin COOH-terminal domain, coassembles with the three subunits normally found in the AP-2 complex and is mainly associated with the plasma membrane, although a small fraction is seen on the TGN. Similarly, a construct containing mostly γ -adaptin, but with the α -adaptin COOH-terminal domain, coassembles with the subunits

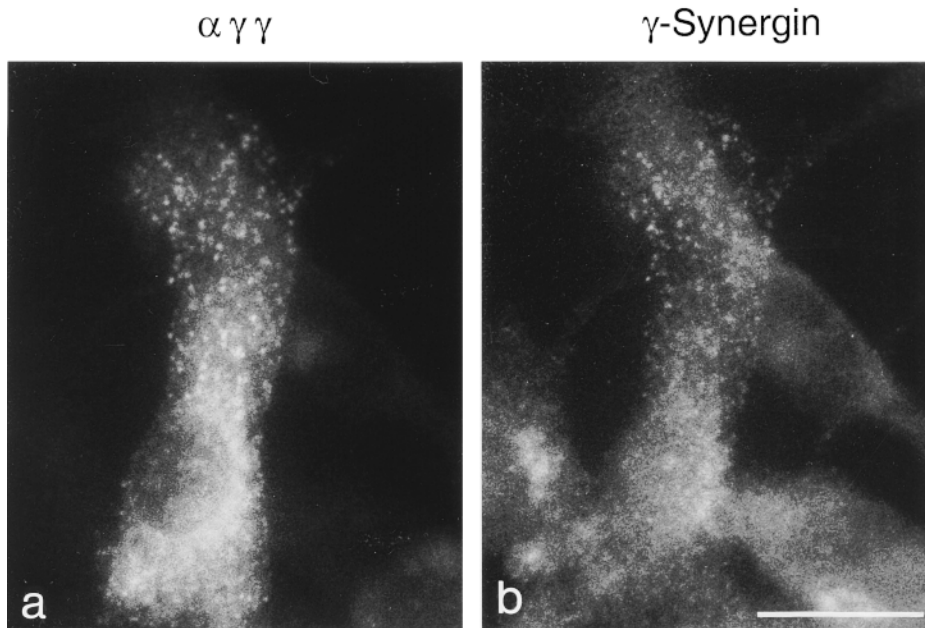


Figure 8. Localization of γ -synergin in cells expressing an adaptin chimera. Cells expressing a construct consisting of the NH₂-terminal domain of α -adaptin, the hinge domain of γ -adaptin, and the ear domain of γ -adaptin ($\alpha\gamma\gamma$) were double labeled with an antibody specific for the construct (a) and anti- γ -synergin (b). In the transfected cell, much of the γ -synergin has been redirected to the plasma membrane where it colocalizes with the construct. Bar, 20 μ m.

normally found in the AP-1 complex and is mainly associated with the TGN, although a small fraction is seen on the plasma membrane.

We and others have long been interested in identifying the membrane docking sites for coat proteins, and although it is clear that γ -synergin cannot be the only docking site for AP-1, since the α -adaptin chimera with the γ -adaptin ear goes mainly to the plasma membrane, it is possible that it might participate in AP-1 recruitment. Alternatively, γ -synergin may be localized to the TGN because of its association with AP-1 rather than vice versa. To distinguish between these two possibilities, i.e., to determine whether γ -synergin leads AP-1 onto the TGN or follows it there, we examined the distribution of γ -synergin in cells expressing a chimera consisting of the α -adaptin NH₂-terminal domain with the γ -adaptin hinge and ear ($\alpha\gamma\gamma$). If γ -synergin helps to recruit AP-1, we would expect its distribution to be unchanged in such cells. However, if AP-1 recruits γ -synergin, we would expect some of the γ -synergin to be rerouted to the plasma membrane. Fig. 8 clearly shows that the latter is the case. In the transfected cell expressing the chimeric adaptin, much of the γ -synergin labeling shows the characteristic punctate plasma membrane pattern (b), colocalizing with the chimera (a). Thus, γ -synergin follows AP-1 onto the appropriate membrane rather than leading it there.

Discussion

The yeast two-hybrid system has proved to be a powerful way of investigating protein-protein interactions that may be difficult to study by more conventional biochemical methods. Among the advantages of the two-hybrid system are that it can detect interactions that may occur only transiently in the cell, and that it can be used not only to identify but also to clone the binding partners of a protein of interest. Its disadvantages are that it sometimes fails to pick up protein-protein interactions that normally occur

in the cell, while at the same time revealing interactions that may occur in the two-hybrid system, but not under more physiological conditions. In the present study, we have used this approach to search for γ -adaptin binding partners and have cloned cDNAs encoding five different proteins: σ 1A, σ 1B, β -spectrin, p34, and γ -synergin.

The cloning of σ 1A acts as a positive control, since we previously showed that it interacts strongly with γ -adaptin in the two-hybrid system (Page and Robinson, 1995). The finding that σ 1B also binds to γ -adaptin is consistent with the findings of Takatsu et al. (1998), who also found that both isoforms of σ 1 can interact with the same isoform of γ . Northern blotting reveals that both isoforms of σ 1 are expressed ubiquitously (Takatsu et al., 1998, and our own unpublished results). Although we attempted to raise monospecific antibodies that recognize σ 1B but not σ 1A by immunizing and cross-absorbing with different fusion proteins, so far we have not succeeded, presumably because of the high degree of homology between the two proteins. Thus, at present we do not know whether there are any functional differences between the two σ 1 isoforms.

In our previous study in which we investigated interactions between neighboring adaptor subunits using the two-hybrid system, we found that γ -adaptin binds not only to σ 1, but also to β 1 and (to a lesser extent) to β 2 (Page and Robinson, 1995). However, we did not pick up either of the two β -adaptins in the library screen. This appears to be because under the more stringent conditions used to screen a two-hybrid library, the interaction between γ -adaptin and β 1 or β 2 is not strong enough to produce a signal (data not shown). Another potential γ -adaptin binding partner is p75, which can be cross-linked to membrane-associated γ -adaptin (Seaman et al., 1996). However, none of the proteins that we identified in the screen has a molecular mass of \sim 75 kD. It is not clear why we failed to clone this protein, but one possibility is that the interaction between p75 and γ -adaptin does not occur

when the two are expressed as fusion proteins in yeast (e.g., if p75 only interacts with γ -adaptin when it is incorporated into the AP-1 complex). Alternatively, if p75 is an integral membrane protein, the presence of a transmembrane domain may prevent it from entering the nucleus, which is where it must be to be detected by the two-hybrid system.

The cloning of β -spectrin was unexpected and it is not yet clear whether its interaction with γ -adaptin is physiologically relevant. Although spectrin was initially assumed to be associated only with the plasma membrane, a number of immunofluorescence studies using certain antibodies against erythrocyte β -spectrin have indicated that an isoform of the protein is associated with the Golgi apparatus, and recently a novel member of the β -spectrin family, β III spectrin, was cloned and localized to the Golgi region of the cell (Stankewich et al., 1998). However, sequencing indicates that the β -spectrin isoform that we cloned as a γ -adaptin binding partner is not β III spectrin but β II spectrin, which has been localized to the plasma membrane. Future studies should show whether γ -adaptin can bind to β III spectrin as well as to β II spectrin, and whether the two proteins can associate with each other in the cell.

The protein that came up most frequently in the screen, p34, is unusual in that it interacts with both γ -adaptin and α -adaptin. This interaction was mapped to the NH₂-terminal domains of the two adaptins, which is where γ and α show the most homology, although even here they are only 32% identical. Another clue as to the function of p34 comes from the observation that it can be coimmunoprecipitated with soluble adaptor complexes, both AP-1 and AP-2, although it is not enriched in purified clathrin-coated vesicles. This suggests that p34 may play some sort of chaperone role. For instance, it could help to prevent the soluble adaptors from coassembling with soluble clathrin, or it could participate in uncoating by helping to remove the adaptors from the coated vesicle. Another possibility is that p34 may aid in the recruitment of soluble adaptors onto the membrane. However, it is clear that it cannot be involved in the specificity of adaptor recruitment, since it appears to interact equally well with both adaptor complexes.

Potentially the most interesting of the proteins that we isolated is γ -synergin. This protein colocalizes with AP-1 by immunofluorescence, and it can be coimmunoprecipitated with cytosolic AP-1. It binds specifically to the COOH-terminal ear domain of γ -adaptin, the same domain that, on α -adaptin, binds to at least three different partners. The COOH-terminal ear domains of both γ and α have also been implicated in the recruitment of the AP-1 and AP-2 complexes onto their respective membranes, although the major targeting information appears to reside in the adaptor heads. However, γ -synergin is a peripheral membrane protein, not an integral membrane protein; its sensitivity to BFA indicates that it associates with the TGN in an ARF-dependent manner, and we have previously shown that the only soluble proteins required for AP-1 recruitment are the AP-1 itself and ARF-1 (Seaman et al., 1996). In addition, in cells expressing α -adaptin with the γ -adaptin ear, a construct that goes mainly to the plasma membrane, a substantial amount of the γ -synergin also goes to the plasma membrane. These observations indicate that γ -synergin is recruited onto the membrane

through its interaction with AP-1 rather than vice versa, and, thus, that it does not play any part in targeting the AP-1 complex to the appropriate membrane.

What, then, is the function of γ -synergin? The presence of an EH domain indicates that, like Eps15 (the first EH domain-containing protein to be characterized) it is an adaptor for an adaptor. Eps15 interacts with the ear domain of the α -adaptin subunit of the AP-2 complex (Benmerah et al., 1995) although, like γ -synergin, its adaptin binding site is distinct from its EH domain. The α -adaptin binding site on Eps15 is quite large, comprising over 100 amino acids and including multiple repeats of the tripeptide DPF (Benmerah et al., 1998). The γ -adaptin binding site on γ -synergin shows no homology to the α -adaptin binding site on Eps15, and no DPF sequences are present in γ -synergin. However, it may be relevant that there are five repeats of the sequence DDFXD/EF, three of which (at positions 668–673, 689–694, and 774–779) are within amino acids 518–786, which we mapped as the γ -adaptin binding site (Fig. 3 a, the repeats are marked D). The other two copies of this sequence are outside of this region (456–461 and 1022–1027); however, preliminary evidence suggests that the true γ -adaptin binding site may encompass more than amino acids 518–786. Although only the construct containing this sequence interacted with the γ -adaptin ear domain in GST pulldown experiments, when interactions between γ -adaptin and γ -synergin were assayed using the two-hybrid system, clones containing amino acids 168–517 and 385–661 as well as 518–786, but not the clone containing the EH domain alone, produced positive results (data not shown). We now intend to investigate whether the DDFXD/EF sequence is required for γ -adaptin binding.

How much of the γ -adaptin and γ -synergin in the cell are associated with each other? Western blots of AP-1 immunoprecipitated from cytosol under nondenaturing conditions show that γ -synergin coprecipitates (Fig. 6); however, under conditions where AP-1 subunits can be seen by Coomassie blue staining, no γ -synergin band can be seen, indicating that the interaction is substoichiometric (Sowerby, P.J., and M.S. Robinson, unpublished observation). When we immunoprecipitate γ -synergin under nondenaturing conditions, we are unable to detect any γ -adaptin by Western blotting (Sowerby, P.J., and M.S. Robinson, unpublished observations). This is presumably because the anti- γ -synergin antibody, which was raised against the portion of the protein that we isolated in the two-hybrid screen, binds to the same site on γ -synergin as γ -adaptin. Thus, it is clear that although some of the AP-1 and γ -synergin are associated with each other in the cytosol, there are also unoccupied pools of both proteins. At present, we do not know whether the γ -synergin and AP-1 that are associated with each other are stably bound, or whether the interaction is more dynamic.

In addition to its association with γ -adaptin, we would predict that γ -synergin has at least one additional binding partner, which would interact with its EH domain. So far, attempts to screen a yeast two-hybrid library with the EH domain of γ -synergin have been unsuccessful, nor have we identified any candidates by GST pulldown or blot overlay experiments using the γ -synergin EH domain. However, when we have carried out GST pulldown experiments with

the γ -adaptin ear construct, we find that other proteins come down in addition to γ -synergin (Liu, W.W., P.J. Sowerby, and M.S. Robinson, unpublished observations), which could interact either directly with γ -adaptin or indirectly, via γ -synergin or another γ ear binding partner. We now intend to identify and characterize these proteins, to see whether they are related to any of the AP-2 interacting proteins, or whether they contain NPF sequences. One potential (indirect) γ -adaptin binding partner might be an isoform or homologue of dynamin, such as dynamin 2, which has been implicated in trafficking from the TGN (Cao et al., 1998; Jones et al., 1998; but see also Altschuler et al., 1998). However, the lack of sequence homology between the γ ear and the α ear (Robinson, 1990) suggests that there may also be binding partners that are entirely specific for one or the other of the two AP complexes.

Some of the proteins associated either directly or indirectly with AP-2, including Eps15 (Benmerah et al., 1998), amphiphysin (Wigge et al., 1997), dynamin (Damke et al., 1994), and epsin (Chen et al., 1998), have been used to create dominant negative mutants by overexpressing truncated or mutated forms of the protein. Thus, for instance, a construct consisting of just the α -adaptin binding portion of Eps15 is a potent inhibitor of clathrin-mediated endocytosis (Benmerah et al., 1998). It should be possible to use the same strategy with γ -synergin. Preliminary experiments in which we transiently transfected cells with a truncated form of γ -synergin, consisting of amino acids 168–786 (the original two-hybrid clone), indicate that this construct is toxic to cells, so we are now developing inducible systems. These studies should help to define both the role of γ -synergin and the role of the AP-1 pathway in general. Although there is abundant evidence that AP-1 is involved in the trafficking of newly synthesized lysosomal proteins from the TGN to an endosomal or prelysosomal compartment, it may have other functions as well. AP-1 has been localized not only to the TGN, but also to early/recycling endosomes (Futter et al., 1998) and (in cells with a regulated secretory pathway) to immature secretory granules (Dittie et al., 1996; Klumperman et al., 1998), and it has been proposed that it may participate not only in trafficking to prelysosomes, but also in transcytosis (Futter et al., 1998), the recycling of proteins from the endosome back to the plasma membrane (Futter et al., 1998), transport from the early endosome to the TGN (Mallard et al., 1998), and the removal of nongranule proteins from the immature secretory granule (Dittie et al., 1996; Klumperman et al., 1998). Another possibility is that the AP-1 pathway may be used to transport not only lysosomally directed proteins, but also some of the proteins destined for the plasma membrane, from the TGN to an endosomal compartment. It has also been proposed that AP-1 may play a specialized role in polarized epithelial cells, and interestingly there is an isoform of the μ 1 subunit, μ 1B, which is expressed exclusively in epithelial cells and tissues (Ohno et al., 1999). So far, most of our knowledge of the AP-1 pathway has come from immunolocalization and in vitro binding experiments. If we can inactivate the AP-1 pathway experimentally, it should be possible to carry out the same types of functional studies, making use of living cells, that have been so informative in the case of AP-2 and AP-3.

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