A Novel Adaptor-related Protein Complex

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Abstract. Coat proteins are required for the budding of the transport vesicles that mediate membrane traffic pathways, but for many pathways such proteins have not yet been identified. We have raised antibodies against p47, a homologue of the medium chains of the adaptor complexes of clathrin-coated vesicles (Pevsner, J., W. Volknandt, B.R. Wong, and R.H. Scheller. 1994. *Gene (Amst.).* 146:279–283), to determine whether this protein might be a component of a new type of coat. p47 coimmunoprecipitates with three other proteins: two unknown proteins of 160 and 25 kD, and β-NAP, a homologue of the β/β'-adaptins, indicating that it is a subunit of an adaptor-like heterotetrameric complex. However, p47 is not enriched in preparations of clath-rin-coated vesicles. Recruitment of the p47-containing complex onto cell membranes is stimulated by GTPγS and blocked by brefeldin A, indicating that, like other coat proteins, its membrane association is regulated by an ARF. The newly recruited complex is localized to non–clathrin-coated buds and vesicles associated with the TGN. Endogenous complex in primary cultures of neuronal cells is also localized to the TGN, and in addition, some complex is associated with the plasma membrane. These results indicate that the complex is a component of a novel type of coat that facilitates the budding of vesicles from the TGN, possibly for transporting newly synthesized proteins to the plasma membrane.

P ROTEINS are transported between the organelles of the secretory and endocytic pathways by means of carrier vesicles, which bud from a donor membrane and fuse with a target acceptor membrane. The process of vesicle budding is initiated by the recruitment of proteins from the cytosol onto the donor membrane, where they form a coat around the nascent vesicle. This coat may have a dual role: first, to act as a scaffold, enabling the membrane to bud; and second, to determine the composition of the vesicle by interacting with the cytoplasmic domains of selected transmembrane proteins, which in turn may bind to ligands on the other side of the membrane.

So far three types of coated transport vesicles have been characterized. Clathrin-coated vesicles, the first type to be identified, bud from the plasma membrane and the TGN. Their coats are made out of two components: clathrin, which forms the scaffold, and adaptors. The adaptors are heterotetrameric protein complexes, which attach the clathrin to the membrane and are thought to specify the vesicle membrane and contents (Pearse and Robinson, 1990). Different adaptors are found at the plasma membrane and at the TGN. More recently two other classes of coated transport vesicles have been identified that act at

earlier stages of the secretory pathway: coatomer- (or COPI-) coated vesicles, which bud from the Golgi stack, the intermediate compartment, and possibly the ER (Serafini et al., 1991; Bednarek et al., 1995); and COPII-coated vesicles, which bud from the ER (Barlowe et al., 1994). Although very weak similarities have been reported between two of the coatomer subunits and two of the adaptor subunits (Duden et al., 1991; Kuge et al., 1993), it is clear that there are no strong homologies between the three types of coats, and that the cell has evolved several mechanisms for forming transport vesicles.

One feature that all three classes of vesicles have in common, however, is that the recruitment of coat proteins onto the membrane is regulated by GTP-binding proteins. Thus, GTPγS greatly enhances the recruitment of both coatomer and TGN adaptors onto their respective membranes in vitro (Robinson and Kreis, 1992), and in both cases the small GTP-binding protein ARF1 has been implicated in this process (Stamnes and Rothman, 1993; Traub et al., 1993). The fungal metabolite brefeldin A (BFA)[1], which prevents the nucleotide exchange of ARF proteins (Donaldson et al., 1992; Helms and Rothman, 1992), blocks the recruitment of both coatomer and TGN

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1. *Abbreviations used in this paper.* BFA, brefeldin A; DTSSP, 3, 3'-dithio-bis[sulfosuccinimidylpropionate]; GST, glutathione-S-transferase; NRK, normal rat kidney.

adaptors in vitro and causes them to redistribute to the cytoplasm when added to living cells (Robinson and Kreis, 1992). The role of GTP-binding proteins in plasma membrane adaptor recruitment is less clear-cut in that GTPγS causes mislocalization of the adaptors to an endosomal compartment. This mislocalization can be blocked by pretreatment with BFA (Seaman et al., 1993), again suggesting the involvement of an ARF family member. In contrast, COPII recruitment is regulated not by an ARF but by another small GTP-binding protein, Sar1p (Barlowe et al., 1994).

Although these three types of coats are well characterized, there are many other membranes and pathways for which coats have not yet been identified. In particular, both the TGN and endosomes are major sorting stations in the cell: in both compartments different proteins are packaged into different populations of vesicles and sent to different destinations, but so far only the clathrin-coated vesicles, which mediate the pathway from the TGN to prelysosomes, have been identified and characterized. Intriguingly, however, some of the sorting signals that function in the TGN and endosomes have been identified, including those that direct proteins from both compartments to the basolateral plasma membrane (Matter and Mellman, 1994), from endosomes to the TGN, and from endosomes to lysosomes (Luzio and Banting, 1993); and these have been found to bear a resemblance to internalization signals, which are thought to function at the plasma membrane by binding to adaptors (Pearse and Robinson, 1990). Thus, there may be adaptor-related proteins associated with other types of transport vesicles, even though ultrastructural studies indicate that such vesicles are not coated with clathrin.

The adaptors are composed of four subunits: an α- or γ-adaptin (α for the plasma membrane; γ for the TGN), a β-type adaptin (β or β'), a medium chain (AP50 or AP47), and a small chain (AP17 or AP19). Recently cDNAs have been cloned that encode proteins with homology to two of the adaptor subunits. Homologues of the adaptor medium chains were cloned and sequenced by Pevsner et al. (1994), initially by using an antiserum raised against a synaptic vesicle preparation from the electric ray to screen an electric organ cDNA expression library. One of the clones, ray p47, was subsequently used to screen a rat brain cDNA library, resulting in the isolation of rat p47A and p47B. Northern blotting revealed that p47B is only expressed in brain and spinal cord, while p47A is expressed more widely. The three p47 proteins are ~80% identical to each other and are all 28–30% identical to the adaptor medium chains. Thus, since the two medium chains are 40% identical to each other, the p47 proteins are more distantly related members of the family. A homologue of the β- and β'-adaptin subunits of the two adaptor complexes has also been cloned and sequenced, in this case by screening a human brain cDNA library with an autoimmune antiserum. This protein, β-NAP (for neuronal adaptin-like protein), is ~28% identical to each of the β subunits, which in turn are 84% identical. Like p47B, β-NAP is tissue specific in its expression, being found exclusively in neurons and neuroendocrine cells. An antiserum was raised against recombinant β-NAP, and the protein was found to be present both in the cytosol and associated with membranes, consis-

tent with a possible role as a coat component (Newman et al., 1995).

We have raised an antiserum against recombinant p47 and used it in conjunction with the recently described antiserum against β-NAP to find out whether the proteins have the characteristics one would expect if they are subunits of a novel adaptor-like complex. We find that p47 and β-NAP are part of the same complex and that this complex, unlike a conventional adaptor, is not associated with clathrin. We can reconstitute the recruitment of the complex onto cell membranes in a GTP-dependent manner. Biochemical and ultrastructural studies indicate that these membranes are a subcompartment of the TGN. These results indicate that the complex is a component of a new type of coat and that this coat facilitates the budding of vesicles from the TGN for transport to another membrane compartment of the cell.

Materials and Methods

Production of Antibodies against p47

Plasmids containing cDNAs encoding full-length rat p47A and p47B were generously provided by Jonathan Pevsner (Stanford University, Palo Alto, CA). Most DNA manipulations were carried out as described by Sambrook et al. (1989). Two degenerate PCR primers were designed to amplify p47A and p47B incorporating BamHI, EcoRI, and SmaI restriction sites. The sequences of the primers were:

Forward: 5' CGC GGG ATC CCC ATG ATC/T CAC AGT CTT/A TT 3'.
Reverse: 5' GGA CCC GGG AAT TCA G/TGT C/TCG/T G/CAC TTG GAA 3'.

The PCR products were ligated into pGEX-3X, using the BamHI and EcoRI sites for p47A and the BamHI and SmaI sites for p47B (which contains an internal EcoRI site). MC1061 cells were transformed with the two plasmids, and expression of fusion proteins was induced. Both fusion proteins were found to be insoluble, so they were purified from an inclusion body preparation as previously described (Page and Robinson, 1995).

Rabbits were injected subcutaneously along the flank with 0.5 mg fusion protein (either individual fusion proteins or a mixture of the two) in Freund's complete adjuvant and boosted with the same amount of protein in Freund's incomplete adjuvant after 2 wk and then after an additional 6 wk. The rabbits were bled 10 d after the final boost. The specificity of the antisera was tested on Western blots of the two fusion proteins, glutathione-S-transferase (GST) alone, and brain homogenate. Only one of the rabbits, which had been injected with a mixture of the two fusion proteins, was found to have responded, and its serum was absorbed with GST and affinity purified with a mixture of the two fusion proteins (see Page and Robinson, 1995).

Western Blotting

Western blots were prepared as previously described (Robinson and Pearse, 1986). The samples that were blotted included tissue and cell homogenates, high speed supernatants and pellets of a brain homogenate, purified clathrin-coated vesicles, and samples from a Superose 6 gel filtration column. The homogenates were prepared by homogenizing the samples in cytosol buffer (25 mM Hepes-KOH, pH 7.0, 125 mM potassium acetate, 2.5 mM magnesium acetate, 1 mM dithiothreitol, and 1 mg/ml glucose), diluting them to a concentration of 3 mg protein per ml, mixing them with an equal volume of 2× sample buffer and boiling for 2 min, and then sonicating and clarifying them by centrifugation before SDS-PAGE. The high speed supernatants and pellets were prepared by homogenizing 1 g of pig brain in 4 ml cytosol buffer, centrifuging at 50,000 rpm for 20 min at 4°C in a TL Ultracentrifuge using a TL100.3 rotor (Beckman Instruments, Inc., Palo Alto, CA), and then resuspending the pellet in 4 ml cytosol buffer to give equal dilutions of supernatant and pellet. Clathrin-coated vesicles were kindly provided by Matthew Seaman (University of Cambridge) and were purified as described by Manfredi and Bazari (1987). Gel filtration of 3 ml pig brain cytosol (prepared as described above) was carried out on a

Superose 6 100-ml column as described by Stamnes and Rothman (1993). The column was calibrated using 2 mg each of the following standards: blue dextran (2000 kD), thyroglobulin (669 kD), apoferritin (443 kD), BSA (66.2 kD), ovalbumin (45 kD), carbonic anhydrase (29 kD) and cytochrome C (12.4 kD), all obtained from Sigma Chemical Co. (St. Louis, MO).

Blots were probed with anti-p47, the species-specific γ-adaptin antibody mAb100/3 (Sigma Chemical Co.), anti-β-NAP (Newman et al., 1995), and the tissue-specific α-adaptin antibody A706-727 (Ball et al., 1995). The blots were then labeled with ^{125}I-protein A (Amersham Intl., Little Chalfont, UK), either directly, or in the case of mAb100/3, after a second incubation with rabbit anti–mouse IgG (Sigma Chemical Co.).

Immunoprecipitation

Immunoprecipitations were carried out on pig brain cytosol, prepared as described above, and on cytosol from metabolically labeled AtT20 cells. The AtT20 cells were grown until 70% confluent, washed in PBS, and grown for 15–18 h in methionine-free medium with 10% dialyzed FCS, 5 mM L-glutamine, 50 IU/ml penicillin, and 50 mg/ml streptomycin to which 1 mCi Tran[35]S label (ICN Flow Laboratories, High Wyncombe, UK) had been added. The cells were harvested by trypsinizing, washed three times in cytosol buffer, homogenized, and then cytosol was prepared as described above.

For some experiments the cytosol samples were cross-linked and denatured before immunoprecipitation. This was carried out by first diluting 150 μl of cytosol with 150 μl cross-linking buffer (25 mM Hepes-NaOH, pH 7.4,1 mM MgCl$_2$, 0.25 M sucrose). 3,3'-dithiobis[sulfosuccinimidylpropionate] (DTSSP) was added from a freshly made 100 mM stock solution in dimethylformamide to a final concentration of 2 mM. The mixture was incubated at room temperature for 30 min, after which SDS was added to 1%, and the sample was boiled for 2 min. The sample was then diluted with 1200 μl RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% NP-40, and 0.5% deoxycholate) before immunoprecipitation. Native immunoprecipitations were carried out in PBS containing 0.1% NP-40, using 1350 μl buffer for 150 μl cytosol.

All samples were precleared twice by incubation for 1 h on a rotating wheel with 30 μl of a 50% suspension of Protein A–Sepharose. The Sepharose was then pelleted, and the appropriate antibody was added to the supernatant for 1 h, followed by Protein A–Sepharose for an additional hour. The beads were pelleted and washed five times before eluting by resuspending the beads into 2× sample buffer and boiling. Cross-linked samples were washed in RIPA buffer containing 0.1% SDS, while native immunoprecipitations were washed in PBS containing 0.1% NP-40.

Recruitment

Recruitment experiments were carried out both on normal rat kidney (NRK) cells and on membrane fractions. Methods for permeabilizing the NRK cells, incubating them with pig brain cytosol under various conditions, and preparing them for immunofluorescence after methanol/acetone fixation or for Western blotting have already been described (Robinson and Kreis, 1992; Seaman et al., 1993). All samples contained 100 μM EGTA. BFA was used at a final concentration of 100 μg/ml and GTPγS at 100 μM. The ATP regenerating system consisted of 5 mM creatine phosphate, 80 μg/ml creatine phosphokinase, and 1 mM ATP, while the ATP depletion system consisted of ~1 U hexokinase with glucose added to a final concentration of 50 mM. For quantitative Western blotting, the radioactivity of each band was analyzed with a FujiX Bas2000 Bio-Imaging Analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan). The experiments were repeated three times, and the results were averaged.

Immunolocalization

Primary antibodies used for the immunofluorescence experiments included those mentioned above, a new anti–β-NAP antibody that we prepared as previously described (Newman et al., 1995) and used to label primary neuronal cells, and a mouse antibody specific for brain α-adaptin, MC4 (Seaman et al., 1993). Fluorescein-labeled donkey anti–rabbit IgG and Texas red–labeled sheep anti–mouse IgG were obtained from Amersham Intl. Both NRK cells, permeabilized and incubated with pig brain cytosol as described above, and primary cultures of cerebellar granule neurons were examined by immunofluorescence. The cerebellar cells were generously provided by A. Randall and J. Mellor (Medical Research Council Laboratory of Molecular Biology, Cambridge, UK). They were prepared from p4 mouse cerebellum, essentially as described by Randall

and Tsien (1995), except that the culture medium was supplemented with an additional 20 mM K$^+$ to promote granule cell survival. Both types of cells were examined using either a fluorescence microscope (Axioplan; Carl Zeiss, Inc., Thornwood, NY) or a confocal microscope (MRC1000; Bio-Rad Laboratories, Richmond, CA).

Two additional primary antibodies were used for immunogold EM: a rabbit anti-γ-adaptin and a rabbit anti-clathrin heavy chain, both raised against GST fusion proteins. The sequences that were amplified by PCR for cloning into pGEX were those encoding amino acids 595–703 for mouse γ-adaptin (the hinge), and amino acids 1545–1675 for rat clathrin heavy chain (the COOH-terminal variable domain). The γ-adaptin construct was soluble and purified by affinity chromatography, while the clathrin construct was insoluble and electrophoretically purified from inclusion bodies. Purification of both was carried out as previously described (Page and Robinson, 1995). The rabbits were injected, boosted, and bled, and their serum was affinity purified as described above.

Electron Microscopy

For immunogold localization of newly recruited proteins, NRK cells were permeabilized by immersion into liquid N$_2$ before incubation with pig brain cytosol as previously described (Seaman et al., 1993). At the end of the incubation, the cells were fixed for 30 min with 0.05% glutaraldehyde in cytosol buffer minus the DTT, scraped, pelleted, and embedded in 10% gelatin. For studies on endogenous proteins, cerebellar cells were fixed with 0.05% glutaraldehyde before scraping, pelleting, and embedding in 10% gelatin. Both sets of cells were then prepared for ultrastructural immunocytochemistry essentially as outlined by Griffiths (1993). Briefly, the embedded cell pellets were infused with 2.1 M sucrose in PBS overnight at 4°C, and then frozen on aluminium stubs in liquid nitrogen. Frozen ultrathin sections were cut using a Reichert Ultracut S ultramicrotome equipped with an FCS cryochamber attachment (Leica, Milton Keynes, UK). Sections were collected and labeled using the protein A–gold technique (Slot and Geuze, 1983). Double labeling was performed using the sequential labeling methodology of Slot et al. (1991). The sections were then contrasted by embedding them in freshly prepared 1.8% methyl cellulose/0.3% uranyl acetate (Tokuyasu, 1978), allowed to air dry, and observed in a transmission electron microscope (CM100; Philips Electronic Instruments, Inc., Mahwah, NJ).

Fractionation

Fractionation of rat liver membranes was carried out essentially as described by Branch et al. (1987), using 15 g of rat liver homogenized in STM (40 ml of cold 0.25 M sucrose, 10 mM N-tris(hydroxymethyl)-methyl-2-aminoethane-sulfonic acid, pH 7.4, 1 mM MgCl$_2$). A postmitochondrial supernatant was prepared by centrifuging the homogenate at 1,500 g for 10 min, and 5-ml samples were then loaded onto 34-ml linear gradients prepared from 0.25 M sucrose, 10 mM TES, pH 7.4, 1 mM EDTA, and 45% Nycodenz in 10 mM N-tris(hydroxymethyl)-methyl-2-aminoethane-sulfonic acid, pH 7.4, 1 mM EDTA. The gradients were centrifuged in a vertical rotor (VTi50; Beckman Instruments, Inc.) at 206,000 g for 1 h, and 1-ml fractions were collected by upward displacement. For recruitment experiments, 400-μl aliquots of the fractions were diluted threefold with STM and collected by pelleting at 90,000 g (50,000 rpm) for 15 min in a Beckman TL100.2 rotor. Membrane pellets were resuspended in 50 μl cytosol (clarified by centrifugation for 15 min at 350,000 g just before use) with appropriate additions and incubated for 10 min at 37°C before washing by 20-fold dilution with cold STM and pelleting as before. Pellets were then resuspended in 50 μl SDS-PAGE sample buffer, and 10-μl aliquots were analyzed by Western blotting.

Results

Distribution of p47

To raise antisera against p47, rabbits were injected with rat p47A and rat p47B expressed as GST fusion proteins. Like their homologues, the adaptor medium chains, the p47 proteins were found to be extremely poor antigens: only one of the rabbits responded, and the resulting antiserum only recognized SDS-denatured p47.
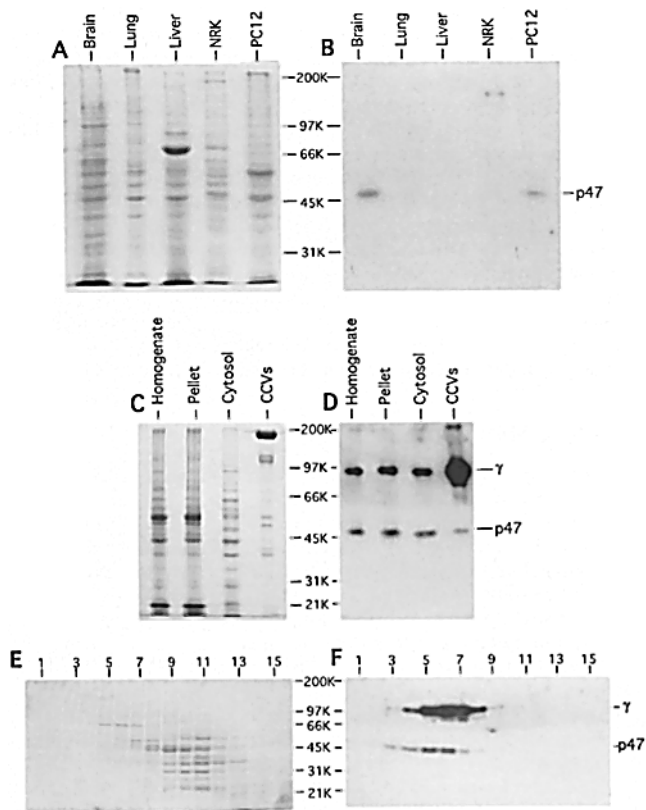
Figure 1. Characterization of p47 by Western blotting. (*A* and *B*) Gel (*A*) and blot (*B*) showing equal protein loadings of various tissue and cell homogenates. The blot was probed with anti-p47. The most intensely labeled 47-kD band is in brain followed by the neuroendocrine cell line PC12. The higher molecular weight band labeled in the NRK cell lane is presumably an unrelated cross-reacting protein. (*C* and *D*) Gel (*C*) and blot (*D*) showing whole pig brain homogenate, a high speed pellet containing membranes, a high speed supernatant containing cytosol, and purified clathrin-coated vesicles (*CCVs*). The blot was cut in half, and the top half was probed with anti-γ-adaptin, the bottom half with anti-p47. Both proteins partition about equally between membranes and cytosol, but whereas γ-adaptin is enriched in clathrin-coated vesicles, p47 is depleted. (*E* and *F*) Gel (*E*) and blot (*F*) of fractions (pooled groups of six) from a Superose 6 gel filtration column. The blot was cut in half, and the top half was probed with anti-γ-adaptin, the bottom half with anti-p47. p47 elutes slightly ahead of γ-adaptin with an apparent size of ~400 kD.

Fig. 1, *A* and *B*, shows a gel and Western blot of different tissues and cells, probed with affinity-purified anti-p47. A 47-kD band is detectable in brain homogenate and in the neuroendocrine cell line PC12. Longer exposure revealed a 47-kD band in lung and a much weaker 47-kD band in NRK cells (data not shown). Thus, the antigen shows the tissue distribution expected from Northern blotting, since the antiserum recognizes both isoforms: p47B, which is only expressed in brain; and p47A, which is expressed more widely but which is particularly abundant in brain and lung. Formal proof that the antibody is specific for p47 was obtained by immunoprecipitating the protein from pig brain cytosol after SDS denaturation (see Fig. 2) and sequencing the 47-kD band. The first seven residues were able to be determined, and with the exception of an iso-

leucine for leucine substitution at position 7 (possibly due to the difference in species), there was a complete match with the sequences deduced from the two p47 cDNAs.

We next examined the subcellular distribution of p47 to find out whether it was similar to that of adaptor complexes. The adaptors cycle on and off the membrane and thus exist both as a cytosolic pool and in a membrane-associated form, and they are highly enriched in preparations of clathrin-coated vesicles. The gel in Fig. 1 *C* contains equal protein loadings of pig brain homogenate, a high speed pellet containing membranes, a high speed supernatant containing cytosol, and purified pig brain clathrin-coated vesicles. A Western blot of these samples was probed for the γ-adaptin subunit of the TGN adaptor complex (*upper half*), and for p47 (*lower half*) (Fig. 1 *D*). Both proteins partition in a similar way between membranes and cytosol. However, whereas γ-adaptin is greatly enriched in the coated vesicle sample (50-fold increase in signal over that in the homogenate), p47 is depleted (four-fold decrease). Thus, although p47 is related to the adaptor medium chains, it does not appear to be associated with clathrin. p47 is also not enriched in a partially purified synaptic vesicle preparation, although it is detectable (data not shown), which is somewhat surprising in view of the fact that the original antiserum used to screen the ray expression library was raised against such a preparation.

To find out whether p47 is a component of an adaptor-sized protein complex, pig brain cytosol was subjected to gel filtration on a Superose 6 column, and the fractions were blotted and probed for both γ-adaptin and p47. Fig. 1, *E* and *F*, shows that p47 elutes slightly ahead of γ-adaptin. Calibration of the column indicated that p47 runs with an apparent size of ~400 kD, while on the same column, γ-adaptin runs with an apparent size of ~280 kD, close to its actual size of 263 kD as part of the TGN adaptor complex.

p47 and β-NAP Are Part of the Same Complex

Are the other subunits in the p47-containing complex also related to adaptor subunits? Two observations suggested that β-NAP might be one of the subunits of such a complex. First, it too shows ~30% identity to its adaptor homologue; and second, its pattern of expression is similar to that of p47B (Newman et al., 1995). Thus, we carried out experiments to determine whether β-NAP and p47 coimmunoprecipitate. First, we immunoprecipitated pig brain cytosol under nondenaturing conditions with an antiserum raised against a β-NAP fusion protein and showed by Western blotting that p47 coprecipitates (Fig. 2 *A*). Second, we treated the cytosol with the reversible cross-linker DTSSP, denatured it by boiling in SDS, and immunoprecipitated with anti-p47. Under these conditions β-NAP coprecipitates, but only in the presence of cross-linker (Fig. 2 *B*). Thus, the complex contains both an adaptor medium chain homologue and a β-adaptin homologue.

As a first step towards identifying the remaining subunits of the complex, we carried out immunoprecipitations on metabolically labeled AtT20 cells, a neuroendocrine cell line in which both p47 and β-NAP are strongly expressed. Under denaturing conditions, anti-p47 was found to bring down three additional bands in a cross-linker dependent manner: a ~120-kD band, which is likely to be β-NAP; a ~160-kD band; and a ~25-kD band (Fig. 2 *C*).
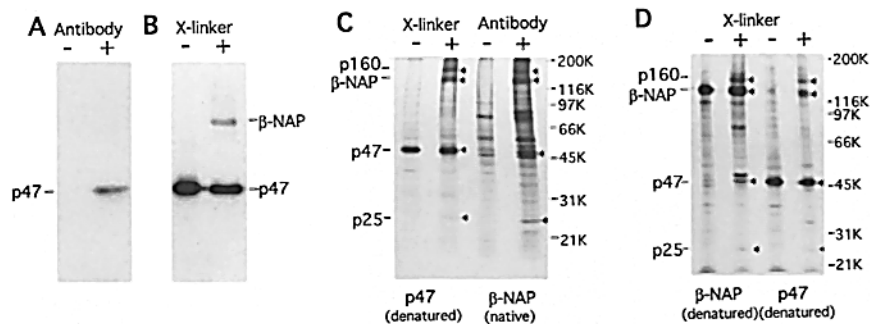
Figure 2. Composition of the p47-containing complex. (*A*) Pig brain cytosol was immunoprecipitated with anti-β-NAP under nondenaturing conditions, and the blot was then probed with anti-p47. p47 coimmunoprecipitates with β-NAP. (*B*) Pig brain cytosol was treated with the cross-linker DTSSP, boiled in SDS, and immunoprecipitated with anti-p47. A reducing gel was run to reverse the cross-linking, and the blot was cut in half and probed with anti-β-NAP (*top*) and anti-p47 (*bottom*). β-NAP coprecipitates with p47 in a cross-linker–dependent manner. (*C*) Cytosol was prepared from metabolically labeled AtT20 cells and immunoprecipitated either with anti-p47 after prior cross-linking and denaturation, as in *B*, or with anti-β-NAP under nondenaturing conditions, as in *A*. Proteins of ~160 kD (p160), ~120 kD (β-NAP), and ~25 kD (p25) coprecipitate with p47 in the presence of cross-linker. These same bands can be seen to coprecipitate with anti-β-NAP under native conditions, although additional bands are also seen. (*D*) Labeled cytosol was prepared as in *C* and immunoprecipitated with either anti-β-NAP or anti-p47 after cross-linking and boiling in SDS. The four bands that consistently coprecipitate in a cross-linker–dependent manner are p160, β-NAP, p47, and p25.

These same bands were found to coimmunoprecipitate with anti-β-NAP under native conditions, although a number of additional bands came down as well (Fig. 2 *C*). A simpler pattern was obtained when anti-β-NAP was used to immunoprecipitate after cross-linking and denaturing in SDS, and again proteins of 160, 47, and 25 kD coprecipitated, as well as bands of ~50 and ~80 kD (Fig. 2 *D*). Thus, there are four proteins common to all three immunoprecipitations, and although we cannot rule out the possibility that the extra bands that come down with anti-β-NAP are additional components of the complex, we favor a model where the p47 complex, like an adaptor complex,

is composed of four subunits: two large (β-NAP and p160), one medium (p47), and one small (p25). This would give the complex a native molecular mass of ~350 kD, which is somewhat less than its apparent molecular mass by gel filtration of ~400 kD, but which could be explained if the complex is nonglobular.

Recruitment of the Complex onto Membranes

If the function of the p47 complex is to act as part of a vesicle coat, its association with membranes should be regulated by a small GTP-binding protein. To test this possibil-
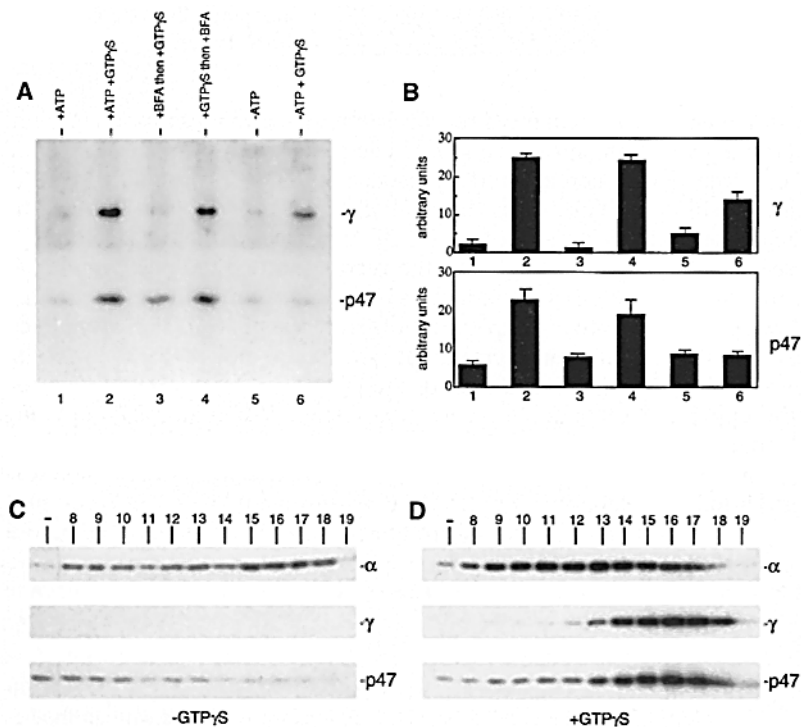

Figure 3. Recruitment of the p47-containing complex onto membranes. (*A*) Immunoblot of permeablilized NRK cells incubated with pig brain cytosol under various conditions, cut in half and probed for newly recruited γ-adaptin (*top*) and p47 (*bottom*). Each lane contains pooled samples from three experiments. (*B*) Quantification of the recruitment. Blots similar to the one in *A* were prepared from three separate experiments, and the signals were quantified using the phosphoroimager. The numbers refer to the six conditions shown in *A*. Recruitment of both proteins is stimulated by GTPγS and blocked by brefeldin A. However, whereas GTPγS partially stimulates γ-adaptin recruitment even in the absence of ATP, recruitment of p47 is completely ATP dependent. (*C* and *D*) A rat liver postmitochondrial supernatant was fractionated on a 0–45% Nycodenz gradient, and 36 fractions were collected and incubated with pig brain cytosol, ATP, and a regenerating system, either without (*C*) or with (*D*) GTPγS, centrifuged, and assayed by Western blotting for coat protein recruitment. Blots of fractions from the central portion of the gradient (the light end is on the left) are shown labeled with antibodies against newly recruited α-adaptin, γ-adaptin, and p47. Both α-adaptin and p47 have a tendency to

precipitate even in the absence of membranes, as shown by the control lane (−) in which no membranes were added. With the exception of α-adaptin, recruitment above background in the absence of GTPγS is not detectable. However, specific recruitment of all three proteins can be seen in the presence of GTPγS, and the membranes that recruit p47 cofractionate with the membranes that recruit γ-adaptin but not with the membranes that recruit α-adaptin.
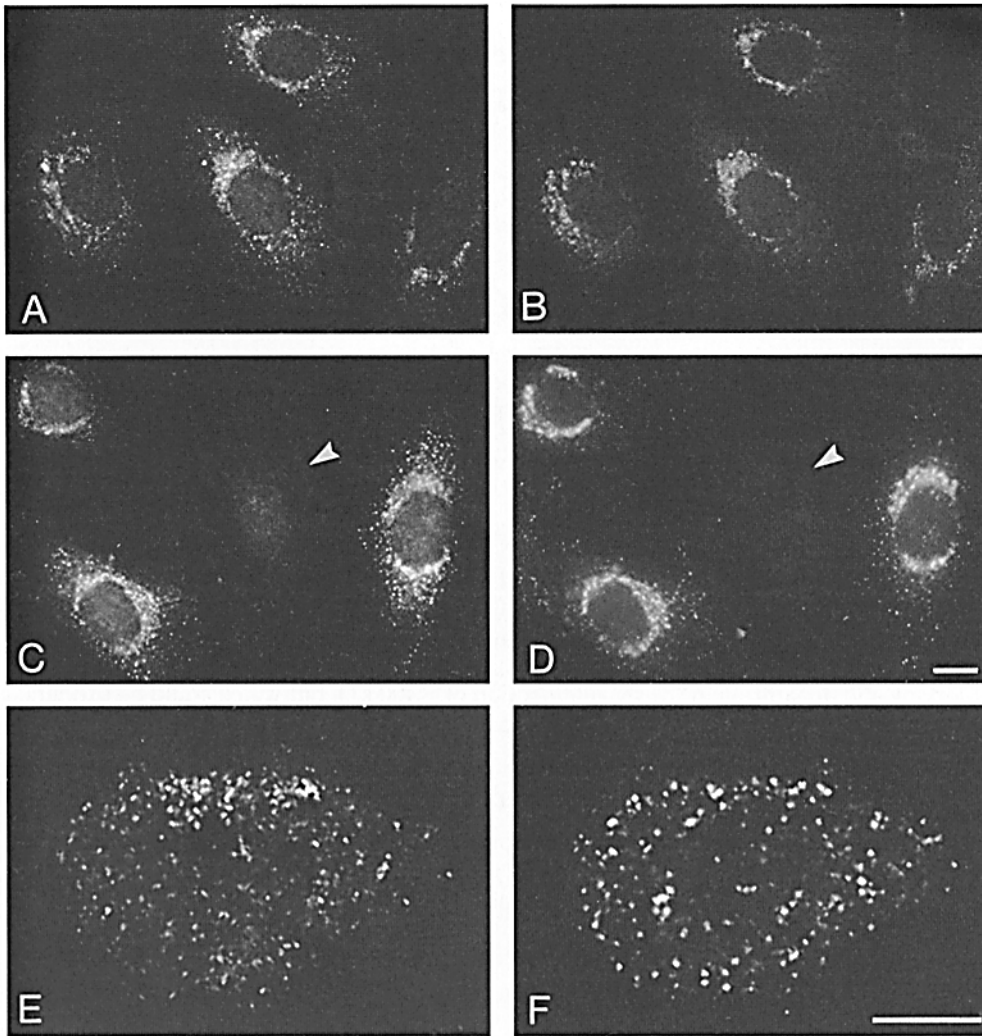
Figure 4. Localization of newly recruited β-NAP by immunofluorescence. (*A–D*) Conventional fluorescence micrographs of β-NAP recruited onto NRK cell membranes in the presence of GTPγS (*A* and *C*), double labeled for newly recruited α-adaptin (*B*) and γ-adaptin (*D*). The arrowheads indicate a nonpermeabilized cell that serves as a control. All three types of coats are recruited onto perinuclear membranes (α-adaptin is mistargeted to an endosomal compartment in the presence of GTPγS), but the fine details of the labeling are different. (*E* and *F*) Confocal micrographs of a mitotic cell double labeled for newly recruited β-NAP (*E*) and γ-adaptin (*F*). When the organelles are dispersed during cell division, the two proteins are generally not colocalized. Bars, 10 μm.

ity, we made use of an in vitro system originally designed to study the recruitment of adaptors onto membranes (Robinson and Kreis, 1992; Seaman et al., 1993). Permeabilized NRK cells are incubated with pig brain cytosol under various conditions. Because p47 is much more strongly expressed in brain than in NRK cells (see Fig. 1 *B*), recruitment of the exogenous complex from the cytosol can be assayed by centrifuging the cells at low speed and probing Western blots with anti-p47 under conditions where there is no detectable labeling of endogenous p47. Experiments were carried out in triplicate, and the upper half of the blots was probed with a mAb against γ-adaptin, which also only detects the newly recruited protein because it recognizes pig but not rat, while the bottom half was probed with anti-p47 (Fig. 3, *A* and *B*).

The signal from γ-adaptin associated with the cell pellet increased about 10-fold when GTPγS was added to the in-

cubation mixture, consistent with previous reports (Robinson and Kreis, 1992). The signal from p47 was also found to increase in the presence of GTPγS, by about threefold. In both cases the GTPγS effect was strongly reduced by prior incubation with BFA, indicating that an ARF family member regulates the recruitment of the p47 complex as well as TGN adaptors. One important difference between the two complexes, however, was that GTPγS stimulated γ-adaptin recruitment whether or not ATP was present, while recruitment of the p47 complex absolutely required ATP and a regenerating system. When an ATP-depleting system was added instead, only basal recruitment was observed even in the presence of GTPγS. One possible explanation for the ATP requirement of the p47 complex may be that a substrate needs to be phosphorylated before binding can occur.

Recruitment was also investigated using membrane

Figure 5. Immunogold EM labeling of newly recruited coat proteins. Frozen thin sections were prepared of permeabilized NRK cells that had been allowed to recruit proteins from pig brain cytosol in the presence of GTPγS. The sections were labeled with antibodies against newly recruited β-NAP (15 nm gold) (*A*), or double labeled for β-NAP (10 nm gold) and γ-adaptin (15 nm gold) (*B*), β-NAP (10 nm gold) and clathrin (15 nm gold) (*C*), or γ-adaptin (10 nm gold) and clathrin (15 nm gold) (*D*). The arrowheads in *B* and *C* indicate β-NAP–positive membranes. All three proteins are associated with tubulovesicluar clusters of membrane near the Golgi stack (*G*). β-NAP is often present on the same clusters as γ-adaptin and clathrin but generally not on the same buds. The degree of coincidence
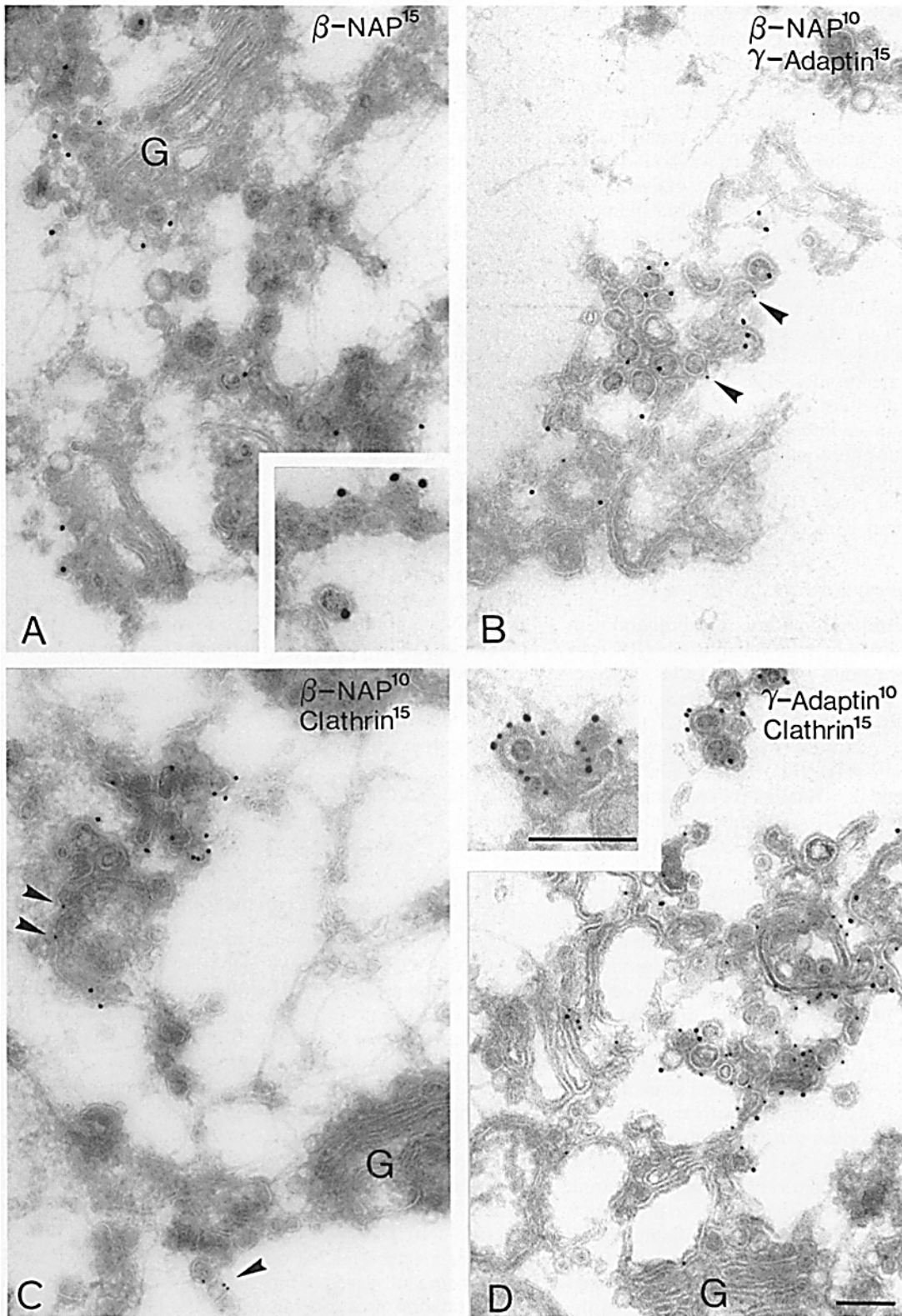
was quantified by counting vesicular profiles labeled for β-NAP and determining how many of these were also positive for γ-adaptin or clathrin. Out of 89 such profiles from cells such as the one in *B*, only four (4%) also had γ-adaptin associated with them. Similarly, out of 145 β-NAP–positive profiles from cells such as the one in *C*, only six (4%) were also labeled with anticlathrin. Even these numbers may be an overestimate of the degree of colocalization, since when the vesicles appeared in clusters, it was often difficult to assign a gold particle to a particular vesicle. However, when cells such as the one in *D* were scored for colocalization of γ-adaptin and clathrin, a much higher degree of coincidence was seen: out of 71 γ-adaptin–positive vesicular profiles, 30 (42%) were also positive for clathrin. The insets show coated buds labeled either for β-NAP (*A*) or γ-adaptin and clathrin (*D*) and reveal that the β-NAP–containing coats are thinner than the clathrin coats. Bars, 200 nm.

fractions prepared from rat liver (Fig. 3, *C* and *D*). A post-mitochondrial supernatant was fractionated by Nycodenz gradient centrifugation, and the fractions were incubated with pig brain cytosol plus ATP and a regenerating system, with or without GTPγS, pelleted, blotted, and probed for the presence of newly recruited α-adaptin, γ-adaptin, or p47. The recruitment of all three proteins was found to be enhanced by GTPγS. In addition, in the case of α-adaptin, a different fractionation profile was observed in the presence and in the absence of GTPγS, presumably because GTPγS causes plasma membrane adaptors to be mistargeted to an endosomal compartment instead of binding to the plasma membrane. The fractionation profile for p47-recruiting membranes in the presence of GTPγS was different from both of these but similar to the profile for γ-adaptin–recruiting membranes. These results indicate that the p47 complex does not bind to either of the membranes that recruit plasma membrane adaptors (the plasma membrane itself and the endosomal mistargeting compartment) but to a denser membrane compartment: either the same compartment that binds TGN adaptors or another compartment with a similar fractionation profile.

Localization of the Newly Recruited Complex

For a more precise identification of the membrane(s) with which the complex is associated, permeabilized NRK cells were incubated with pig brain cytosol plus ATP and a regenerating system in the presence of GTPγS, and then double labeled with anti-β-NAP (Fig. 4, *A* and *C*) and antibodies against newly recruited (mistargeted) α-adaptin (Fig. 4 *B*) or γ-adaptin (Fig. 4 *D*). By immunofluorescence, the compartment that recruits β-NAP is primarily perinuclear, with more punctate labeling extending out towards the cell periphery. Omission of ATP from the incubation mixture blocked the β-NAP recruitment, while omission of GTPγS reduced the amount of recruitment (data not shown), consistent with the results shown in Fig. 3. Superficially at least, the structures labeled with anti-β-NAP look similar to those labeled with anti-α-adaptin and with anti-γ-adaptin. However, closer examination reveals that the fine details of the labeling are different. The β-NAP perinuclear pattern is more fine grained than either of the other two, and the peripheral labeling is more pronounced. Moreover, when the peripheral structures labeled with anti-β-NAP are compared with those labeled with the other two antibodies, for the most part they do not coincide.

Differences in the labeling patterns are more apparent in mitotic cells, where organelles that cluster in the perinuclear region around the microtubule organizing center become more dispersed. Fig. 4, *E* and *F*, shows confocal micrographs comparing newly recruited β-NAP (*E*) and γ-adaptin (*F*) in a mitotic cell. It is clear that most of the structures labeled with the two antibodies are different. Thus, although the membranes that bind these two proteins fractionate in a similar way and have a similar perinuclear distribution in interphase cells, they become segregated from each other during cell division.

To examine the β-NAP–binding compartment at higher resolution, we used immunogold EM. Fig. 5 *A* reveals that newly recruited β-NAP is associated with tubulovesicular

clusters of membrane in close proximity to the Golgi stack but not with the Golgi stack itself. Often the β-NAP–positive membranes appear to be coated on the cytoplasmic side (*inset*). The appearance of these membranes suggests that they may correspond to the TGN; however, the intermediate compartment between the Golgi and the ER also has a tubulovesicular appearance in permeabilized cells (Griffiths et al., 1995). To determine whether the compartment is *cis* or *trans* to the Golgi stack, cells were double labeled with anti-β-NAP and with either anti-γ-adaptin (Fig. 5 *B*) or anti-clathrin (Fig. 5 *C*). In both cases, the two labels are frequently associated with the same clusters, although not with the same buds and vesicles. In other regions of the cell, membranes can be seen that are labeled with only one of the two antibodies (e.g., in Fig. 5 *C*). In contrast, when cells are double labeled with anti-clathrin and anti-γ-adaptin (Fig. 5 *D*), the labeling is much more coincident, and both labels are often seen on the same coated buds. The clathrin coat can be distinguished from the β-NAP–containing coat because it generally appears to be thicker (Fig. 5 *D*, *inset*; compare with Fig. 5 *A*, *inset*).

Thus, these observations indicate that the β-NAP/p47 complex binds to a compartment that is *trans* to the Golgi stack and that is most likely a subcompartment of the TGN, a possibility supported by partial colocalization with the TGN membrane protein TGN38 (not shown). The less perinuclear, more peripheral labeling that can be seen by immunofluorescence may correspond to an endosomal subcompartment. Indeed, recent studies have shown that the TGN and endosomes are closely connected compartments with extensive trafficking between them, and "classical" TGN markers, such as γ-adaptin and TGN38, are found in endosomes as well as in the TGN (Luzio and Banting, 1993; Seaman, M.N.J., and M.S. Robinson, unpublished observations).

Localization of the Endogenous Complex

Because studies on plasma membrane adaptors have shown that GTPγS may cause coat proteins to be mistargeted (Seaman et al., 1993), we also examined the distribution of endogenous β-NAP in primary cultures of cerebellar granule neurons. The confocal micrographs in Fig. 6 show that both γ-adaptin (*A*) and β-NAP (*B*) are concentrated in the cell body, next to or surrounding the nucleus. In addition, β-NAP labeling can also be seen in neurites (Fig. 6 *C*, *small arrowheads*), consistent with previous observations on brain sections in which the protein was found to be present not only in the perikaryon but also in cellular processes and in the nerve terminal (Newman et al., 1995).

At the electron microscope level (Fig. 7), β-NAP labeling is mainly observed to one side of the Golgi stack. Some of the label is also associated with the plasma membrane (Fig. 7 *A*, *arrowheads*). In the Golgi region of the cell, vesicles with a thick, clathrin-like coat frequently appear interspersed with the β-NAP–positive membranes (Fig. 7 *B*, *arrow*). To confirm that these vesicles are clathrin coated and thus can be used as markers for the TGN, cells were double labeled with antibodies against β-NAP and γ-adaptin (Fig. 7 *C*) or with antibodies against β-NAP and clathrin (Fig. 7 *D*). Both sets of labels are localized to the same tu-
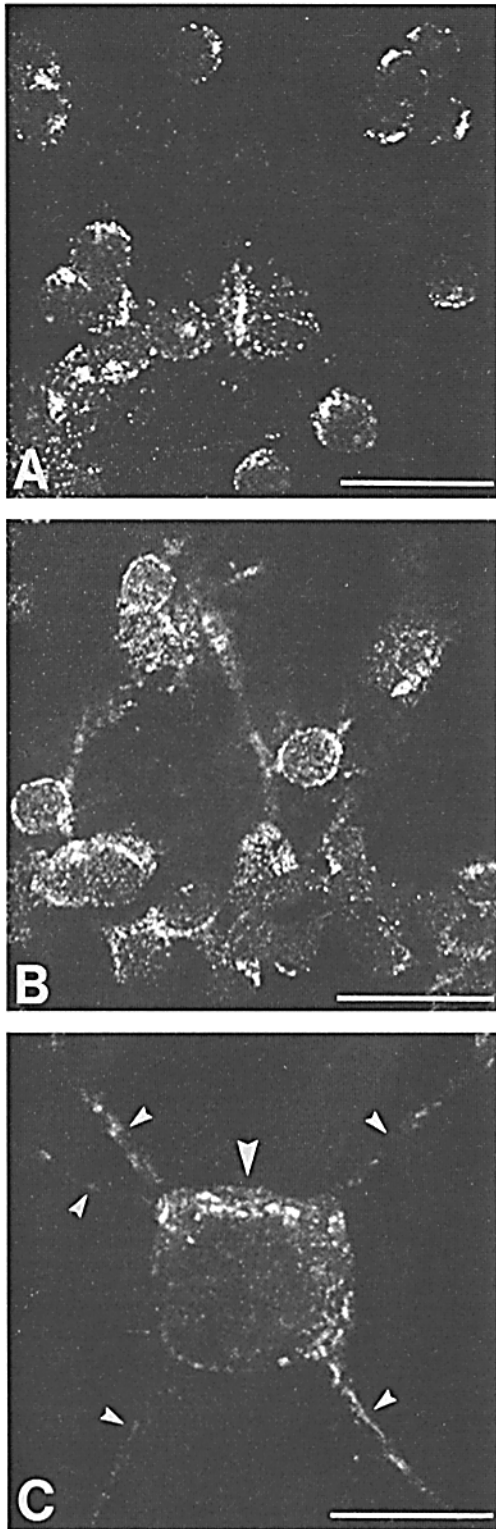
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Figure 6. Confocal micrographs of endogenous coat proteins in neuronal cells. Cultured cerebellar granule cells were labeled with antibodies against either γ-adaptin (*A*) or β-NAP (*B* and *C*). Both γ-adaptin and β-NAP have a perinuclear or juxtanuclear distribution, consistent with their being associated with the TGN; in addition, β-NAP is also present in neurites. In the extended focus projection of a Z series of images shown in *C*, both the perinuclear region of the cell body (*large arrowhead*) and the neurites (*small arrowheads*) are labeled. Bars: (*A* and *B*) 25 μm; (*C*) 10 μm.
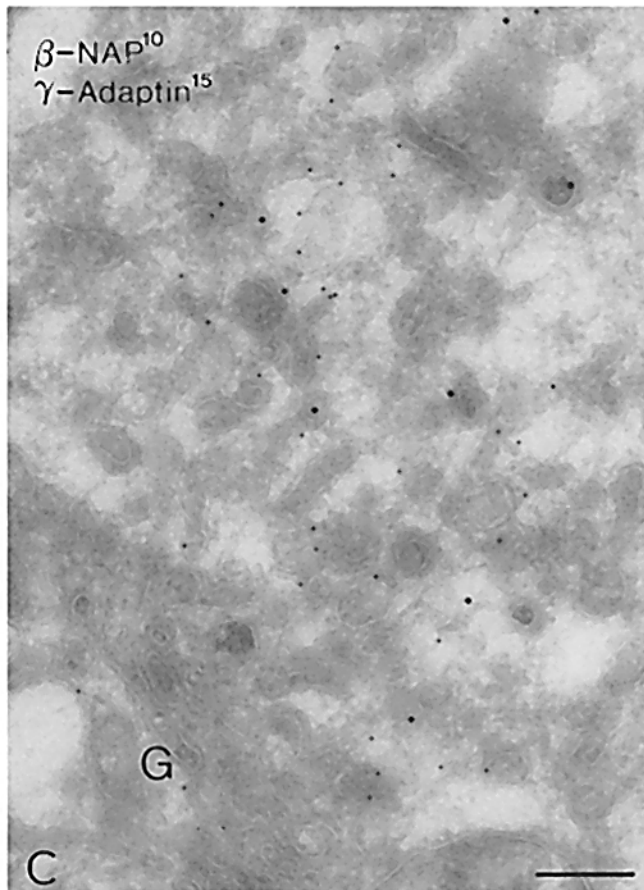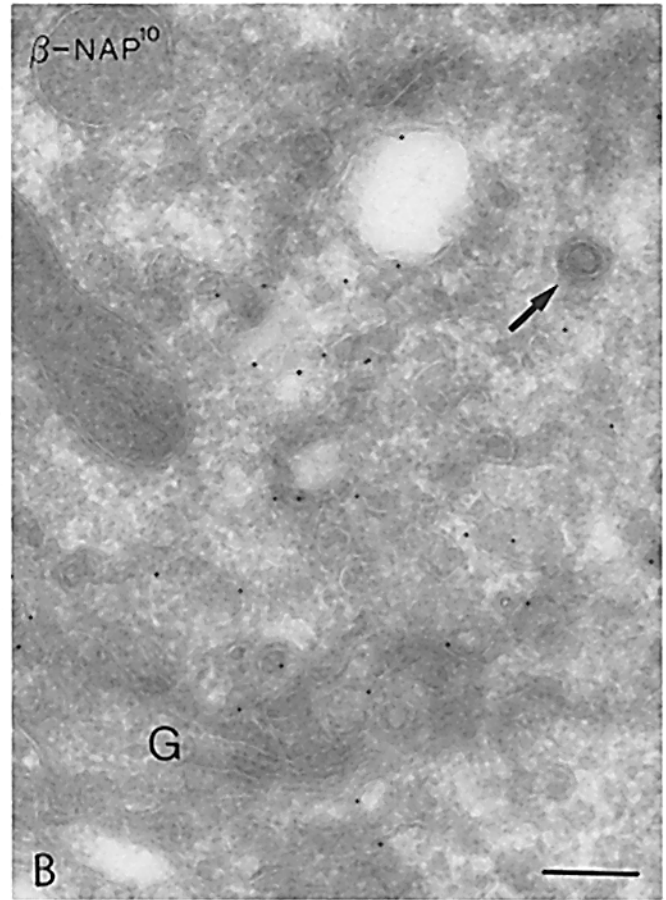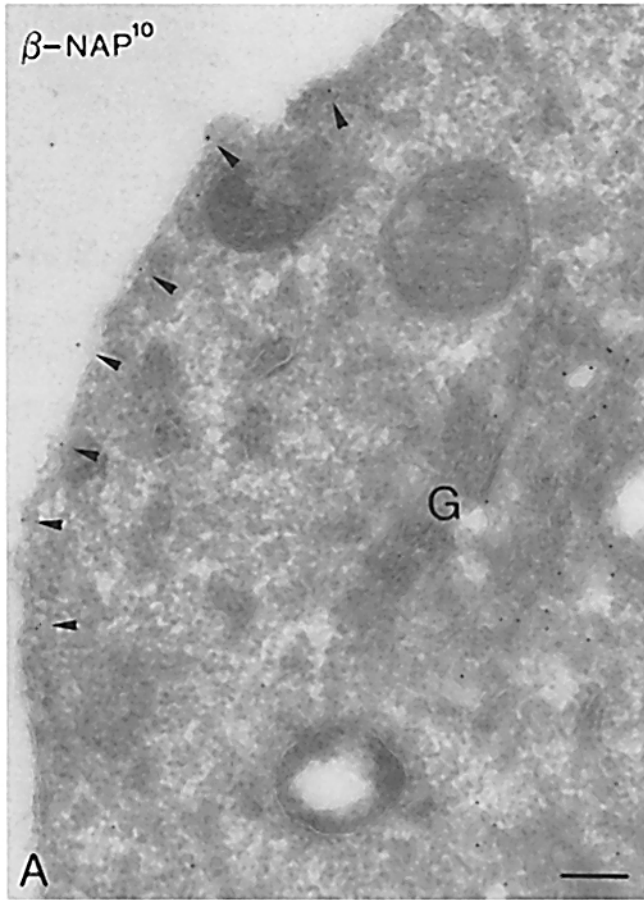
bulovesicular network. Thus, these observations indicate that the β-NAP/p47 complex associates with the TGN not only when added to heterologous membranes in vitro, but also under physiological conditions in vivo.

Discussion

We have shown that p47 and β-NAP are subunits of a novel adaptor-related protein complex and that this complex is a component of a new type of non-clathrin coat. The existence of such coats has long been suspected, since there are a number of budding events for which coat proteins have not yet been identified, including some that make use of tyrosine-containing sorting signals related to the internalization signals, which have been shown to bind to adaptor complexes (Matter and Mellman, 1994; Luzio and Banting, 1993). Our evidence that the complex actually functions as a coat component is twofold. First, recruitment of the complex onto target membranes is enhanced by GTPγS and blocked by brefeldin A, indicating that, like other coats, its membrane association is regulated by a GTP-binding protein, probably an ARF. Second, immunogold EM reveals that the complex is associated with membrane buds that display a distinctive ~10-nm coat on their cytoplasmic side. This coat is thinner than a clathrin coat; and this observation, combined with the lack of colocalization of β-NAP with clathrin and adaptors at both the light and the electron microscope level, and the finding by Western blotting that both p47 and β-NAP are depleted rather than enriched in preparations of clathrin-coated vesicles (see Newman et al., 1995), indicates that the complex is not simply another clathrin-associated adaptor but is part of a different type of coat. Presumably this coat must also contain a structural protein, analogous although not necessarily homologous to clathrin, which would act as a scaffold to facilitate vesicle budding. The lace-like coats that have been observed on vesicles budding from the TGN by high voltage EM tomography (Ladinsky et al., 1994) may be the same coats that we see by immunogold EM, viewed with a different technique.

Under conditions where p47 and β-NAP coimmunoprecipitate, two other proteins come down as well, p160 and p25. We predict that p160 will be found to share homology with the α- and γ-adaptins, and p25 with the adaptor small chains, but that in both cases the homology will be less than that between α- and γ-adaptin and between AP17 and AP19. Interestingly, cDNAs encoding proteins that have these properties have recently been cloned as expressed sequence tags and can be found in the public database. We also predict that there must be a β-NAP homologue expressed in nonneuronal cells to form complexes with the universally expressed p47A. Again, we believe that we have identified such a protein in the database, and we are currently raising antibodies against all of these proteins to determine whether they coimmunoprecipitate with p47. Even if they are not subunits of the p47-containing complex, they are still likely to be of interest as components of yet another type of adaptor-related complex.

What pathway or pathways might the p47/β-NAP complex mediate? In their recent report on the cloning and characterization of β-NAP, Newman et al. suggested that it might play a role in the trafficking of endosomally de-

rived vesicles between the cell body and the nerve terminal. This hypothesis was based on the neuronal-specific expression of the protein and its presence in synaptosome fractions as well as in the cell body (Newman et al., 1995). A neuronal-specific role was also proposed by Pevsner et al. (1994) in their study of p47. They suggested that the protein might be a component of a coat involved in the biogenesis of synaptic vesicles from endosomes. We would like to propose an additional or alternative model that is supported by our EM localization studies, both of newly recruited β-NAP and of endogenous β-NAP in neurons. Both studies suggest that the complex is required for a non–clathrin-mediated budding event from the TGN. Our results are not inconsistent with the possibility that the complex might also be involved in trafficking from endosomes, especially since more peripheral structures were also labeled with anti-β-NAP; however, the endosomes in the nerve terminal are an early endosomal compartment, and in the NRK cells we failed to see significant colocalization with the transferrin receptor, which is concentrated in early endosomes, or with fluorescent wheat germ agglutinin, which the cells had been allowed to internalize for 2, 5, or 12 min before permeabilization (data not shown).

A number of different types of vesicles are formed from the TGN: clathrin-coated vesicles, which sequester newly synthesized lysosomal enzymes; specialized secretory granules in cells with a regulated secretory pathway; and constitutive secretory vesicles, which exist as two distinct populations in polarized epithelial cells and possibly in other cells as well (Matter and Mellman, 1994). There is some evidence that the same sorting signals that are used to send different membrane proteins to the apical and basolateral domains in epithelial cells are used for trafficking to the axon and dendrites in neuronal cells (Dotti and Simons, 1990), and Newman et al. have proposed that the β-NAP–containing complex might play a role in the generation of axonally directed vesicles, which in epithelial cells would correspond to apically directed vesicles (Newman et al., 1995). On the other hand, it is also possible that an adaptor-related complex might function in the basolateral pathway, since the signals that target proteins into this pathway are closely related to the internalization signals, which bind to adaptors, while the apical pathway is thought to make use of a different type of machinery (Matter and Mellman, 1994). The localization of the endogenous complex in neuronal cells not only to the cell body but also to neurites, most of which are likely to be dendrites rather than axons, is consistent with this latter possibility.

The association of label with the plasma membrane as well as with internal membranes is striking, and its significance is not yet clear. One possibility is that the complex might be required for a budding event at the plasma mem-

brane as well as at the TGN, just as clathrin and adaptors are used at both locations. However, the labeled regions of plasma membrane are flat, which is inconsistent with a role in budding. An alternative possibility is that the complex may stay membrane associated long after the vesicles bud from their donor compartment (the TGN) and remain associated even after the vesicles fuse with their acceptor compartment (the plasma membrane).

It is clear that further studies will need to be carried out before the function of the complex can be established definitively. New antibodies will need to be generated to find out whether, for instance, p47B can only coassemble with β-NAP and p47A with a nonneuronal β-NAP homologue or whether the subunits are interchangeable. Our immunolocalization studies on NRK cells will need to be confirmed with antibodies that recognize the endogenous p47A-containing complex in these cells, and the distribution of the two types of complexes will need to be compared in neuronal and nonneuronal cells. Although it is tempting to speculate that the neuronal specificity of p47B and β-NAP means that these proteins are required for a neuronal-specific pathway, it is equally possible that they are functionally identical to their nonneuronal isoforms but that their expression is regulated differently. Thus, the proteins could be required for a constitutive pathway that exists in all cells but that is used much more extensively in neurons. One such pathway is the route from the TGN to the plasma membrane, which must be upregulated in neurons as the cells send out axons and dendrites.

Immunolocalization will only provide clues about what such a pathway might be. Functional studies will also be required, using antibodies or mutagenesis to try to inactivate the complex. Interestingly, a number of homologues of adaptor subunits have been identified in the budding yeast *Saccharomyces cerevisiae*, including some that appear, by genetic and biochemical criteria, not to be associated with clathrin (Phan et al., 1994; Stepp et al., 1995), and these proteins are candidates for components of a different type of coat, which may be more like the p47- and β-NAP–containing coat. The nematode worm *Caenorhabditis elegans* may prove to be an even better model system, especially for studying the role of the coat in neurons. We have recently identified a close homologue of β-NAP cloned from *C. elegans* as an expressed sequence tag, which is 78% identical to the mammalian protein. Experiments are currently underway to investigate the function of this protein by taking advantage of *C. elegans* genetics. But even though the role of the complex remains to be established, the fact that it exists, and that its recruitment onto the membrane is regulated by GTP, supports the view that all membrane traffic pathways make use of similar underlying mechanisms.

Figure 7. Immunogold EM labeling of endogenous coat proteins in neuronal cells. Frozen thin sections were prepared of cultured cerebellar granule cells and labeled with antibodies against β-NAP (10 nm gold), either alone (*A* and *B*) or together with 15-nm gold-conjugated antibodies against γ-adaptin (*C*) or clathrin (*D*). Most of the β-NAP label is associated with tubulovesicular membranes near the Golgi stack (*G*), although some also appears at the plasma membrane (*arrowheads* in *A*). Clathrin-coated vesicles (*arrow* in *B*) are found in the same region near the Golgi stack, and their identity can be confirmed by double labeling (*C* and *D*). Again, quantification revealed little colocalization of β-NAP with γ-adaptin or clathrin. Out of 176 β-NAP–positive vesicular profiles from cells such as the one in *C*, three (2%) were also labeled with anti-γ-adaptin, while out of 183 β-NAP–positive vesicular profiles from cells such as the one in *D*, six (3%) were also labeled with anticlathrin. Bars, 200 nm.

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