100-kD Proteins of Golgi- and *Trans*-Golgi Network-Associated Coated Vesicles Have Related But Distinct Membrane Binding Properties

Darren H. Wong* and Frances M. Brodsky*‡

* Departments of Pharmacy and Pharmaceutical Chemistry, School of Pharmacy; and ‡ Department of Microbiology and Immunology, School of Medicine, University of California San Francisco, San Francisco, California 94143-0446

Abstract. The 100–110-kD proteins (α-, β-, β′-, and γ-adaptins) of clathrin-coated vesicles and the 110-kD protein (β-COP) of the nonclathrin-coated vesicles that mediate constitutive transport through the Golgi have homologous protein sequences. To determine whether homologous processes are involved in assembly of the two types of coated vesicles, the membrane binding properties of their coat proteins were compared. After treatment of MDBK cells with the fungal metabolite Brefeldin A (BFA), β-COP was redistributed to the cytoplasm within 15 s, γ-adaptin and clathrin in the *trans*-Golgi network (TGN) dispersed within 30 s, but the α-adaptin and clathrin present on coated pits and vesicles derived from the plasma membrane remained membrane associated even after a 15-min exposure to BFA. In PtK_1 cells and MDCK cells, BFA did not affect β-COP binding or Golgi morphology but still induced redistribution of γ-adaptin and clathrin from TGN membranes to the cytoplasm. Thus BFA affects the binding of coat proteins to membranes in the Golgi region (Golgi apparatus and TGN) but not plasma membranes. However, the Golgi binding interactions of β-COP and γ-adaptin are distinct and differentially sensitive to BFA. BFA treatment did not release γ-adaptin or clathrin from purified clathrin-coated vesicles, suggesting that their distribution to the cytoplasm after BFA treatment of cells was due to interference with their rebinding to TGN membranes after a normal cycle of disassembly. This was confirmed using an in vitro assay in which γ-adaptin binding to TGN membranes was blocked by BFA and enhanced by GTPγS, similar to the binding of β-COP to Golgi membranes. These results suggest the involvement of GTP-dependent proteins in the association of the 100-kD coat proteins with membranes in the Golgi region of the cell.

PROTEIN transport between membrane-bound cellular compartments is mediated by protein-coated vesicular carriers. Clathrin-coated vesicles are responsible for selective budding of proteins during receptor-mediated endocytosis and the sorting of proteins in the *trans*-Golgi network (TGN)[1] (6, 29). The coat of the vesicles that mediate nonselective, constitutive export of proteins through the Golgi apparatus to the plasma membrane is composed of COP (coat protein) subunits of 160-, 110-, 98- and 61-kD (23, 35). The 100-kD coat components of clathrin-coated and COP-coated vesicles have homologous protein sequences and domain structures, suggesting that assembly of these vesicular coats may share some biochemical properties.

In clathrin-coated vesicles, the 100-kD proteins are subunits of multimeric protein complexes called adaptors (29) or assembly polypeptides (16). Adaptors bind clathrin and recognize a specific motif in the cytoplasmic tails of receptors (28, 37), thereby linking the clathrin coat to the vesicle membrane and allowing receptors to be sequestered and concentrated by the clathrin lattice. Adaptors localized to endocytic-coated vesicles at the plasma membrane are composed of two ~100-kD proteins, α- and β-adaptin, and one each of two smaller subunits of 50 and 17 kD. Adaptors in clathrin-coated vesicles of the TGN consist of the ~100-kD γ- and β′-adaptins plus two smaller subunits of 47 and 19 kD. The protein sequences of all four 100-kD adaptins are clearly related and suggest that these subunits have similar structures (32).

The 110-kD β-COP subunit of COP-coated vesicles in the Golgi apparatus shows significant protein sequence homology with β-adaptin (13). The molecular weights of other COP coat proteins appear to correspond approximately to the heavy and light chain subunits of clathrin and to the low molecular weight proteins in adaptors, which has led to the suggestion that there are equivalent structural components in the COP coat (35). However, all the COP coat proteins can be found associated together in a multimeric complex in the cytoplasm, while clathrin and adaptors are separate in their cytoplasmic form and apparently bind to membranes in distinct steps (16, 38).

Several factors that influence the binding of COP proteins to Golgi membranes have been identified. GTP is required

1. *Abbreviations used in this paper*: ARF, ADP-ribosylation factor; BFA, brefeldin A; MDBK, Madin-Darby bovine kidney; TGN, *trans*-Golgi network.

© The Rockefeller University Press, 0021-9525/92/06/1171/9 $2.00

for COP proteins to bind Golgi membranes and they are released by GTP hydrolysis (27). Using a mAb to follow the presence of β-COP on membranes, it has been observed that the fungal metabolite Brefeldin A (BFA) inhibits membrane binding of β-COP and causes a cytoplasmic distribution of β-COP when exposed to cells, while GTPγS and aluminum fluoride enhance β-COP binding to membranes (11). Since the cytoplasmic form of β-COP appears to be in association with the other COP proteins, it is assumed that these proteins accompany β-COP during association with and dissociation from membranes. Although it is not clear whether the β-COP protein is the direct target of the GTP and BFA effects, it was of interest to determine whether the ~100-kD adaptin molecules of clathrin-coated vesicles respond in a similar fashion to those agents that influence membrane binding of β-COP. Here we demonstrate, based on the effects of BFA and GTPγS, that γ-adaptin and β-COP have related properties influencing their association with the membranes in the Golgi region (Golgi apparatus and TGN) that are distinct from the binding properties of the α-adaptin present in endocytic clathrin-coated vesicles.

Materials and Methods

Reagents

Brefeldin A (BFA), poly-D-lysine, ATP, GTP, GTPγS, and *p*-phenylene diamine were purchased from Sigma Chemical Co. (St. Louis, MO). BFA was stored at −20°C as a 5-mg/ml stock solution in methanol. FITC-coupled goat anti-mouse Ig was purchased from Organon Technika (Durham, NC). Biotinylated horse anti-mouse Ig was purchased from Vector Laboratories, Burlingame, CA). Fluoromount G was purchased from Fisher Scientific Co. (Pittsburgh, PA). 0.2-μm pore nitrocellulose was purchased from Schleicher & Schuell (Keene, NH). ^{125}I-streptavidin was prepared by iodinating 25 μg purified streptavidin (Pierce Chemical Co., Rockford, IL) with 1mCi ^{125}I using Iodobeads™ (Pierce Chemical Co.). High purity digitonin (Calbiochem-Behring Corp., La Jolla, CA) was stored as a 20-mg/ml stock solution in DMSO. The following mAbs were used in this study: TD.1 (anticlathrin heavy chain terminal domain [26]), X22 (anticlathrin heavy chain [5]), AP.6 (anti-α-adaptin [7]), 100/3 and 100/1 (anti-γ-adaptin and anti-β/β′-adaptin, gift from E. Ungewickell [1], Max-Planck-Institut für Biochemie, Martinsried bei München, Germany), M3A5 (anti-β-COP, gift from V. Allan [2], EMBL, Heidelberg, Germany), and AC1-M11 (anti-α-adaptin, gift from M. Robinson [31], University of Cambridge, Cambridge, England).

Cell Culture

All cell lines, tissue culture media, and supplements were supplied by the University of California, San Francisco Tissue Culture Facility except where noted. Madin-Darby bovine kidney (MDBK) cells used for indirect immunofluorescence were grown in RPMI 1640 supplemented with 10% horse serum (Gemini Bioproducts, Inc., Calabasas, CA). MDBK cells used for making cytosol were grown in DME-H21 supplemented with 10% horse serum. The rat kangaroo kidney cell line PtK_1 (ATCC) was grown in MEM-EBSS supplemented with nonessential amino acids, 1 mM sodium pyruvate, and 10% FCS. NRK cells were grown in DME-H21 supplemented with 10% FCS. MDCK cells (from M. Bomsel and K. Mostov, University of California, San Francisco) were grown in MEM-EBSS supplemented with 5% FCS. All cells were grown at 37°C/5% CO_2 and passaged every 3–4 d.

Indirect Immunofluorescence Microscopy

Cells growing for 1–3 d on poly-D-lysine coverslips were treated with BFA (diluted from a 1,000× stock to 5 μg/ml) at 37°C for indicated times and immediately fixed with 3.7% paraformaldehyde in PBS, pH 7.4, for 10 min and permeabilized for 10 min in 0.04% saponin in PBS. Samples were processed for indirect immunofluorescence microscopy as described in Wong et al. (39) and mounted in 1 mg/ml *p*-phenylene diamine in Fluoromount G. Antibodies were used at the following concentrations: purified mAb, 10 μg/ml; ascites fluid, 1:40; FITC-coupled secondary antibody, 1:500. Samples were visualized on a BioRad MRC600 confocal microscope system, equipped with a 25 mW argon laser and mounted on a Nikon Optiphot with a Nikon CFN Plan Apo 60/1.40 oil lens, WD 0.17 mm. Each image was recorded from the central plane of the cell (except for AP.6 labeling of α-adaptin which is imaged closer to the plasma membrane) and is the arithmetic average of 15 frames.

Immunoblotting of Clathrin-coated Vesicles

Clathrin-coated vesicles were purified from bovine brain as previously described (24). 100 μg of purified clathrin-coated vesicles were spun at 100,000 *g* for 10 min in TL100 tabletop ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA), and resuspended in 500 μl buffer D+ (50 mM NaHepes, pH 7.0, 100 mM sucrose, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.02% sodium azide). 5 μg/ml BFA was added to appropriate samples and incubated for 30 min at room temperature. Samples were then spun at 100,000 *g* for 10 min to separate assembled from unassembled clathrin-coated vesicles. High speed supernatant (unassembled clathrin) was collected and high speed pellet (assembled clathrin) was resuspended in an equivalent volume of buffer D+. Equal amounts (10 μl) of assembled and unassembled fractions were analyzed by SDS-PAGE (18) and transferred to nitrocellulose. Blots were probed with mAbs TD.1 (5 μg/ml), 100/3 (5 μg/ml), and AC1-M11 (1:100) followed by biotinylated horse-anti-mouse Ig (1.5 μg/ml) and ^{125}I-streptavidin (800,000 cpm/ml). Immunoblot was exposed to Kodak X-OMAT x-ray film and developed.

Permeabilized NRK Cell Assay

MDBK cells grown in DME-H21 + 10% horse serum were trypsinized from culture flask and rinsed twice with cold transport buffer (25 mM Hepes-KOH, pH 7.0, 125 mM KOAc, 2.5 mM $Mg(OAc)_2$, 1 mM DTT) and resuspended in double strength transport buffer with protease inhibitors (0.05% PMSF, 2.5 μg/ml aprotinin, and leupeptin). Cells were then pelleted and homogenized with 25 strokes in Potter-Elvehjem homogenizer (Fisher Scientific Co.). Resulting cell lysate was spun for 15 min at 12,000 *g* followed by 30 min spin at 100,000 *g* in Beckman TL-100 tabletop ultracentrifuge. Resulting supernatant was used as the cytosol fraction in these assays. Protein concentration of the cytosol fraction was 3–4 mg/ml as determined by a Bradford protein assay (US Biochemicals, Cleveland, OH) using BSA as a standard.

NRK cells were grown 4–5 d to confluency on poly-D-lysine–coated coverslips. Cells were permeabilized with 40 μg/ml digitonin in ice-cold transport buffer for 5 min on ice and transferred to transport buffer without digitonin. Cells were incubated at room temperature for 20 min before adding cytosol, digitonin, and other components as noted. The energy regenerating system consisted of 1 mM ATP, 1 mM GTP, 1.5 mM phosphocreatine, 15.1 U/ml creatine kinase, and 1 mg/ml glucose stored at −20°C as 10× stock solutions in transport buffer. Final reaction mixture consisted of 100 μl cytosol and 100 μl energy regenerating system. Coverslips were incubated for 30 min in 37°C water bath. After incubation, cells were gently rinsed once with transport buffer and fixed with 3.7% paraformaldehyde in PBS for 10 min. Samples were then processed for indirect immunofluorescence microscopy as described above, omitting saponin permeabilization step. Cells were visualized using a Nikon Plan Apo 100/1.40 oil lens DM Ph4 by confocal microscopy, as above.

Table I. mAbs Used in Study

mAb	Specificity	Binding location	Reference
AC1-M11	α-adaptin	Plasma membrane	31
AP.6	α-adaptin	Plasma membrane	7
M3A5	β-COP	Golgi region	2
TD.1	Clathrin heavy chain terminal domain	Unreactive in immunofluorescence	26
X22	Clathrin heavy chain	TGN and plasma membrane	5
100/1	β and β′-adaptin	Same as X22	1
100/3	γ-adaptin	TGN	1

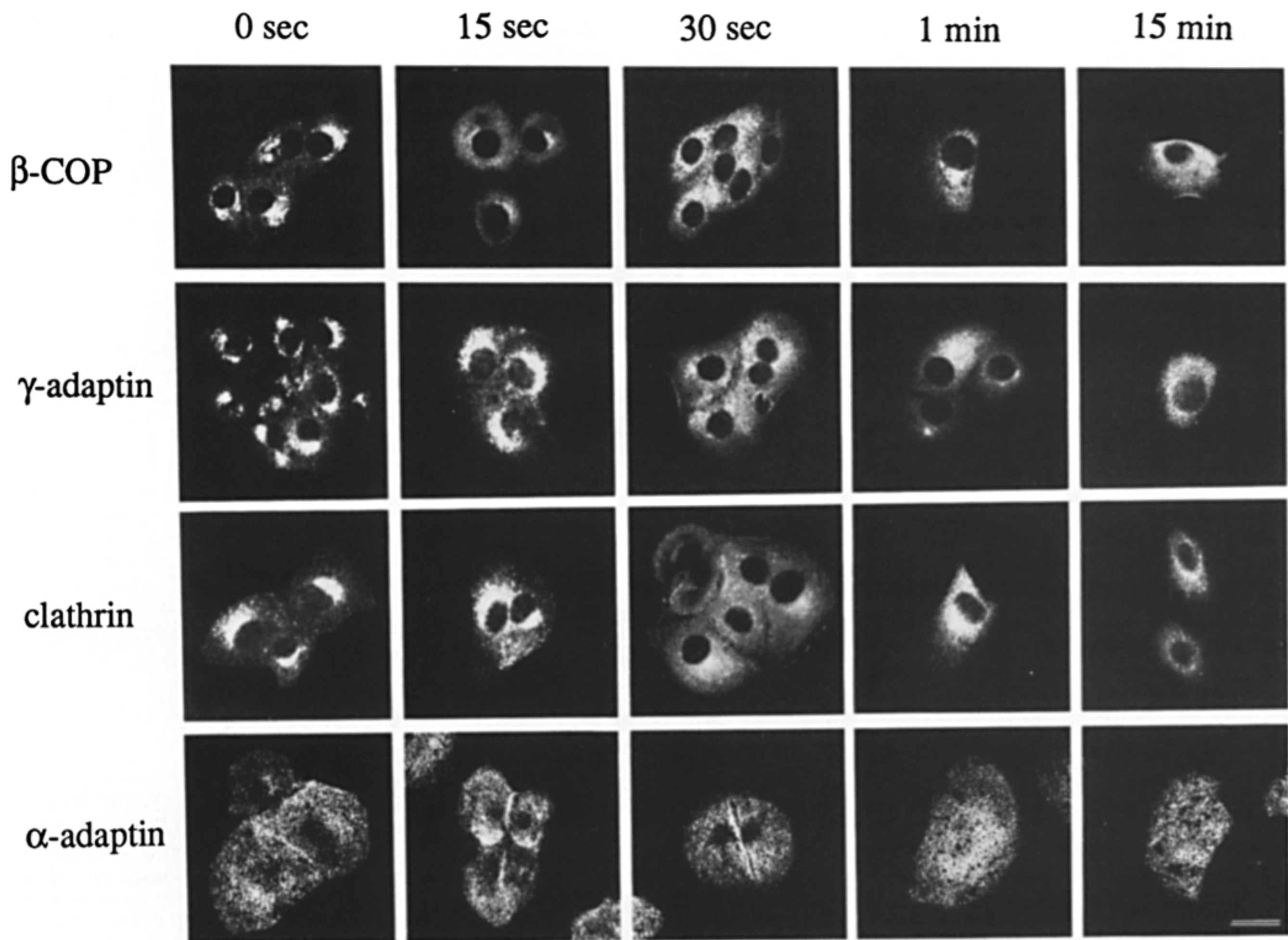

Figure 1. Time course of BFA treatment and effects on clathrin-coated and COP-coated vesicle proteins. MDBK cells grown 2–3 d on poly-D-lysine–coated coverslips were treated with 5 μg/ml BFA at 37°C for the indicated times. BFA treatment was terminated by fixing cells in 3.7% paraformaldehyde. Cells were processed for indirect immunofluorescence with mAbs recognizing the following proteins: β-COP (M3A5), γ-adaptin (100/3), clathrin heavy chain (X22), and α-adaptin (AP.6). Bar, 10 μm.

Results

BFA Causes γ-Adaptin to Redistribute to the Cytoplasm

The fungal metabolite BFA blocks protein transport into the Golgi apparatus and results in the fusion of the Golgi apparatus with the ER (20, 21, 25). One of the earliest events in cells treated with BFA is the redistribution of β-COP from Golgi membranes to the cytoplasm (9). The time course of β-COP redistribution from Golgi membranes was compared to the effects of BFA on the membrane association of the homologous 100-kD adaptins of clathrin-coated vesicles. MDBK cells were exposed to 5 μg/ml BFA for increasing periods of time and then processed for indirect immunofluorescence microscopy using mAbs recognizing β-COP, α-, β-, β′-, and γ-adaptin, and clathrin heavy chain (Table I). In untreated control cells, anti-β-COP (mAb M3A5) and anti-γ-adaptin (mAb 100/3) primarily labeled vesicles in the perinuclear region, representing coated vesicles of the Golgi and TGN, respectively. Anticlathrin heavy chain (mAb X22) labeled vesicles in the Golgi region and vesicles in the cytoplasm and at the plasma membrane, while anti-α-adaptin (mAb AP.6) only labeled the vesicles near the plasma membrane, being restricted to endocytic clathrin-coated vesicles (Fig. 1). After 15 s of exposure to BFA, a substantial proportion of β-COP labeling was no longer tightly associated with the Golgi region but was more cytoplasmically distributed. With increasing time of BFA treatment, staining of β-COP became less Golgi associated and more dispersed. Staining of γ-adaptin and clathrin heavy chain within the TGN also became more diffuse over time, and occurred at a slightly slower rate than the redistribution of β-COP, becoming obvious 30 s to 1 min after exposure of the cells to BFA. The difference in kinetics of the change in staining with antibody to γ-adaptin (100/3) compared to the changes in staining with anti-β-COP (M3A5) rules out the possibility that 100/3 crossreacts with β-COP. mAb crossreactivity is also ruled out in Figs. 2 and 3 (see below). Furthermore, clathrin dissociation from the TGN followed the same slower time course as γ-adaptin dissociation. Staining with antibody to α-adaptin (AP.6) remained unchanged over the time course of BFA treatment, indicating that the factors influencing binding of α-adaptin to membranes of endocytic-coated vesicles differ from those affecting association of γ-adaptin and β-COP with TGN and Golgi membranes. Both endocytic and TGN adaptors and COP proteins behave biochemically as protein complexes (16, 38), suggesting that the staining patterns observed using mAbs to the individual subunits most

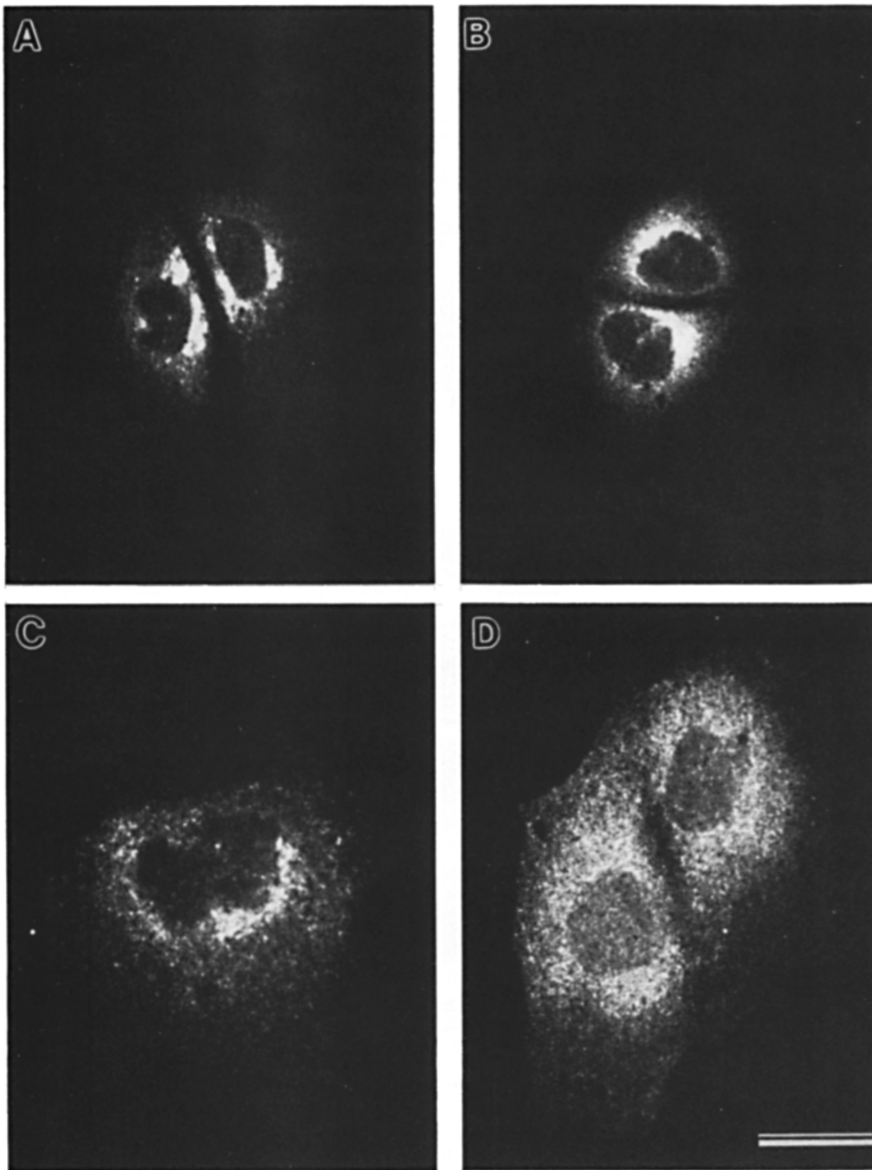

Figure 2. BFA induces a cytoplasmic distribution of γ-adaptin in PtK_1 cells. PtK_1 cells grown for 3 d on poly-D-lysine-coated coverslips were mock-treated (*A* and *C*) or treated with 5 μg/ml BFA (*B* and *D*) for 30 min at 37°C, immediately fixed in 3.7% paraformaldehyde and processed for indirect immunofluorescence microscopy with mAbs recognizing β-COP (M3A5) (*A* and *B*) and γ-adaptin (100/3) (*C* and *D*). *B* demonstrates that β-COP is not affected by BFA in this cell line as previously reported (17). Bar, 10 μm.

likely represent association and dissociation of whole adaptor and COP complexes. For the adaptors in clathrin-coated vesicles, this was tested by staining with a mAb to the β and β′ subunits of the plasma membrane and Golgi adaptors, respectively (100/1). The β′ subunits in the Golgi region dispersed within 30 s to 1 min of BFA treatment, while the disruption of the β subunits remained unchanged (data not shown), following the behavior of their associated γ- and α-adaptin subunits.

BFA Differentially Affects β-COP and γ-Adaptin

To determine whether the similar effects of BFA on the association of β-COP and γ- and β′-adaptin with TGN membranes reflect shared mechanisms for Golgi-coated vesicle assembly, the effects of BFA on TGN adaptors were studied in PtK_1 and MDCK cells. Ktistakis et al. (17) have reported that after treating PtK_1 cells with BFA, β-COP remains associated with Golgi membranes and the Golgi apparatus does not fuse with the ER. Similar properties of "BFA resistance" were observed by Hunziker et al. (15) for MDCK cells. In MDCK cells, treatment with BFA for an extended period of time did not change the distribution of β-COP in the Golgi apparatus or the structural integrity of the TGN. We also observed that β-COP association with the Golgi apparatus was unaffected after lengthy treatment of either PtK_1 (Fig. 2) or MDCK (Fig. 3) with 5 μg/ml BFA, while γ-adaptin redistributed to the cytoplasm under the same conditions. In PtK_1 cells the cytoplasmic distribution of γ-adaptin was complete after 30 min of exposure to BFA (Fig. 2 *D*), while even after 2 h of exposure of MDCK cells to BFA, a small amount of γ-adaptin remained concentrated in the perinuclear region, with most redistributed to the cytoplasm (Fig. 3 *D*). The same behaviors of γ-adaptin and β-COP were observed after BFA treatment of MDCK cells grown at a density that promotes tight junction formation (data not shown). As with MDBK cells, α-adaptin and plasma membrane–associated clathrin was unaffected by BFA treatment of either PtK_1 or MDCK cells (data not shown). The differential effects of BFA on the membrane association of β-COP and γ-adaptin in PtK_1 and MDCK cells indicate that membrane binding mechanisms of these coat proteins are not identical, although they are similarly affected by BFA in MDBK cells.

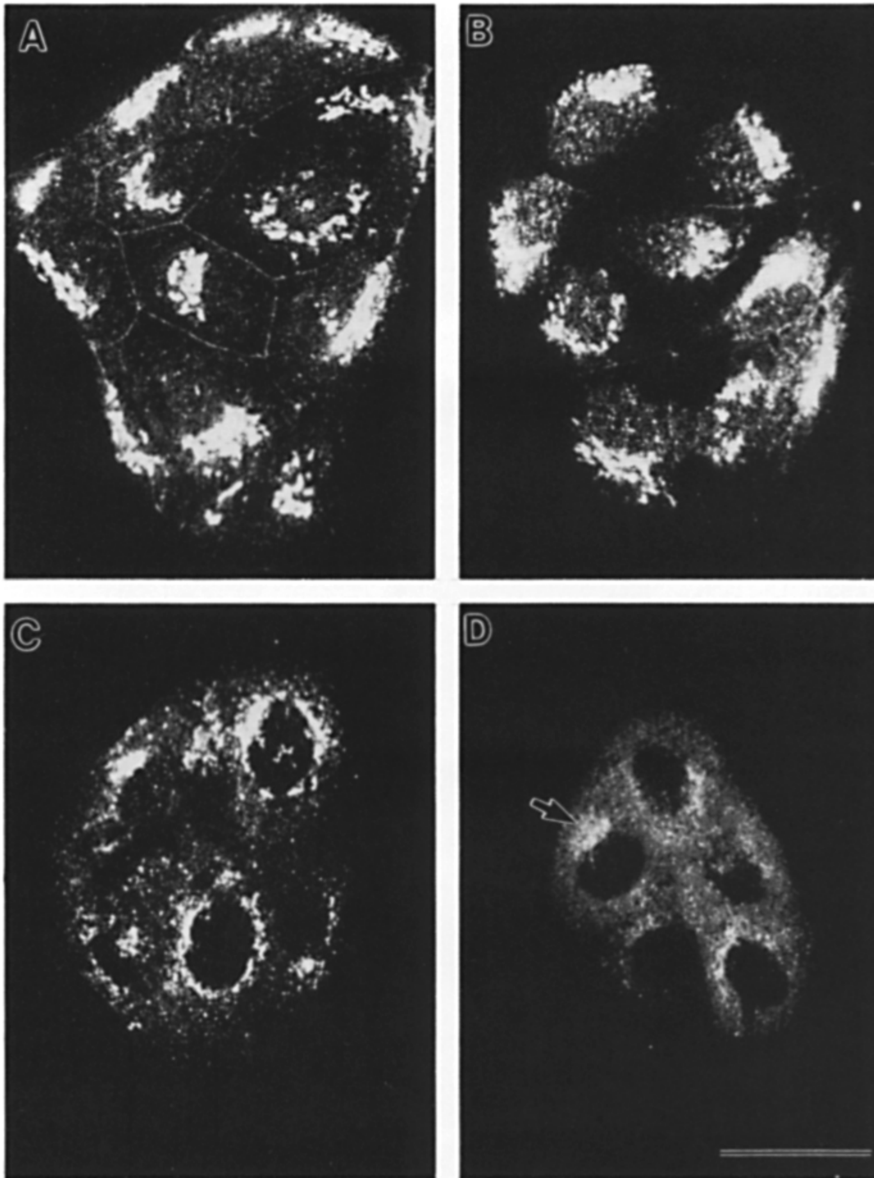

Figure 3. BFA induces a cytoplasmic distribution of γ-adaptin in MDCK cells. MDCK cells grown overnight on glass coverslips were mock-treated (*A* and *C*) or treated with 5 μg/ml BFA (*B* and *D*) for 2 h at 37°C, fixed in 3.7% paraformaldehyde, and processed for indirect immunofluorescence microscopy with mAbs recognizing β-COP (M3A5) (*A* and *B*) and γ-adaptin (100/3) (*C* and *D*). *B* demonstrates that β-COP is not affected by BFA in this cell line, as previously reported (15). *D* demonstrates that BFA treatment causes the majority of γ-adaptin to redistribute to the cytoplasm with a small population of γ-adaptin remaining localized to the perinuclear region (*arrow*). Bar, 25 μm.

BFA Inhibits γ-Adaptin from Binding to Membranes

BFA could induce the cytoplasmic distribution of coat proteins in the Golgi region by two possible mechanisms. BFA might cause dissociation of bound proteins from membranes (stripping) or, if coat proteins cycle between a membrane-bound and cytoplasmic state, BFA might inhibit their rebinding to membranes (blocking). It has already been demonstrated that BFA inhibits β-COP rebinding to membranes (11), favoring the latter mechanism for its effect on COP proteins. To distinguish between a stripping or blocking effect on γ-adaptin binding, BFA was tested for its ability to remove bound adaptors from purified clathrin-coated vesicles and for its ability to block the rebinding of cytoplasmic adaptors to TGN membranes.

Clathrin-coated vesicles purified from bovine brain (24), comprising both endocytic and TGN-derived coated vesicles were exposed to 5 μg/ml BFA, for 30 min, then centrifuged to separate membrane-associated (pellet) and released (supernatant) clathrin and adaptor proteins. BFA treatment had no effect on the amount of γ-adaptin or α-adaptin detected in the supernatant or associated with the coated vesicle pellet, indicating that BFA does not strip clathrin and γ-adaptin from membranes (Fig. 4 *A*). The clathrin heavy chain that was detected in the supernatant harvested from both control and BFA-treated clathrin-coated vesicles was most likely released during the resuspension procedure, before centrifugation. This minor mechanical effect on the clathrin coat has been observed in other experiments in the laboratory.

To test whether BFA could block the binding of γ-adaptin to TGN membranes, an in vitro assay monitoring the recruitment of cytoplasmic adaptors onto TGN membranes was developed. The mAb to γ-adaptin (100/3) recognizes bovine but not rodent γ-adaptin (1). Therefore, the adherent rat kidney cell line NRK was used as the target membrane source and cytosol generated from the bovine kidney cell line MDBK was chosen as the source of adaptor proteins. This strategy was chosen so that the binding of adaptors recruited from the cytosol could be distinguished from the adaptors already present on the TGN membranes. Cultured NRK

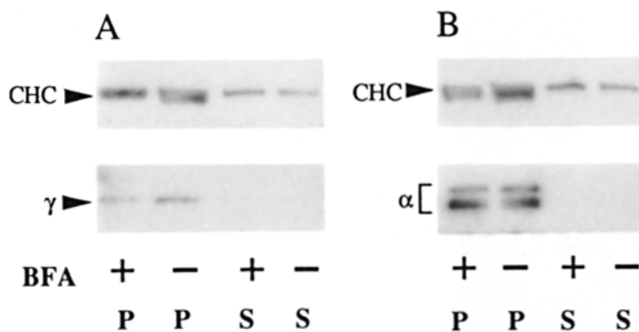

Figure 4. BFA does not strip adaptor molecules from purified clathrin-coated vesicles. Purified clathrin-coated vesicles were treated with 5 μg/ml BFA or mock-treated for 30 min at room temperature and spun at 100,000 *g* to separate assembled clathrin-coated vesicles (*P*) from unassembled coat proteins (*S*). Pellets were resuspended to original volume. Equal volumes of pellet and supernatant samples were applied to a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. Nitrocellulose was cut between the clathrin heavy chain and 100-kD adaptor polypeptides, visualized by staining for protein. The clathrin portions of the blots were probed with anticlathrin heavy chain (mAb TD.1) and the adaptor portions probed with anti-γ-adaptin (mAb 100/3) (*A*) or anti-α-adaptin (mAb AC1-M11) (*B*).

cells were permeabilized with digitonin and rinsed to dilute out the endogenous cytosol. Then exogenous MDBK cytosol was added to the adherent membranes, incubated at 37°C for 30 min and γ-adaptin binding to TGN membranes was assessed by indirect immunofluorescence microscopy (Fig. 5). When cytosol alone was added to permeabilized cells, weak labeling of the TGN with antibody to bovine γ-adaptin (100/3) was observed in the Golgi region (presumably the TGN) (Fig. 5 *C*). When MDBK cytosol and a creatine kinase–based energy regenerating system with ATP and GTP were added to NRK membranes, binding of bovine γ-adaptin to rat membranes in the Golgi region was clearly observed (Fig. 5 *E*).

To identify the nucleotide components produced by the energy regenerating system that were influencing the binding of γ-adaptin to Golgi membranes, individual nucleotides and nonhydrolyzable analogues were tested for their effects on reconstitution of γ-adaptin binding. In the presence of an energy regenerating system, γ-adaptin binding to TGN membranes was barely enhanced when either GTP or ATP were added individually to MDBK cytosol (data not shown) as compared to binding from cytosol alone (Fig. 5 *C*), whereas binding was increased when ATP and GTP were added in combination (Fig. 5 *E*). The weak γ-adaptin binding observed when no energy generating system was added may be influenced by low levels of endogenous ATP and GTP. In the presence of an energy regenerating system, addition of 1 mM GTPγS strongly increased γ-adaptin binding to TGN membranes (Fig. 5 *G*), while the nonhydrolyzable ATP analogue AMP-PNP did not significantly affect γ-adaptin binding (data not shown). The enhanced binding observed with GTPγS implies that a GTP-dependent protein may be involved in regulating γ-adaptin association with TGN membranes.

Having reconstituted γ-adaptin binding to TGN membranes, BFA was tested for inhibition of this process by addition of BFA (5 μg/ml) to MDBK cytosol before and after addition of GTPγS (1 mM) (Fig. 6). Addition of BFA to cytosol and membranes prevented the binding of γ-adaptin to TGN

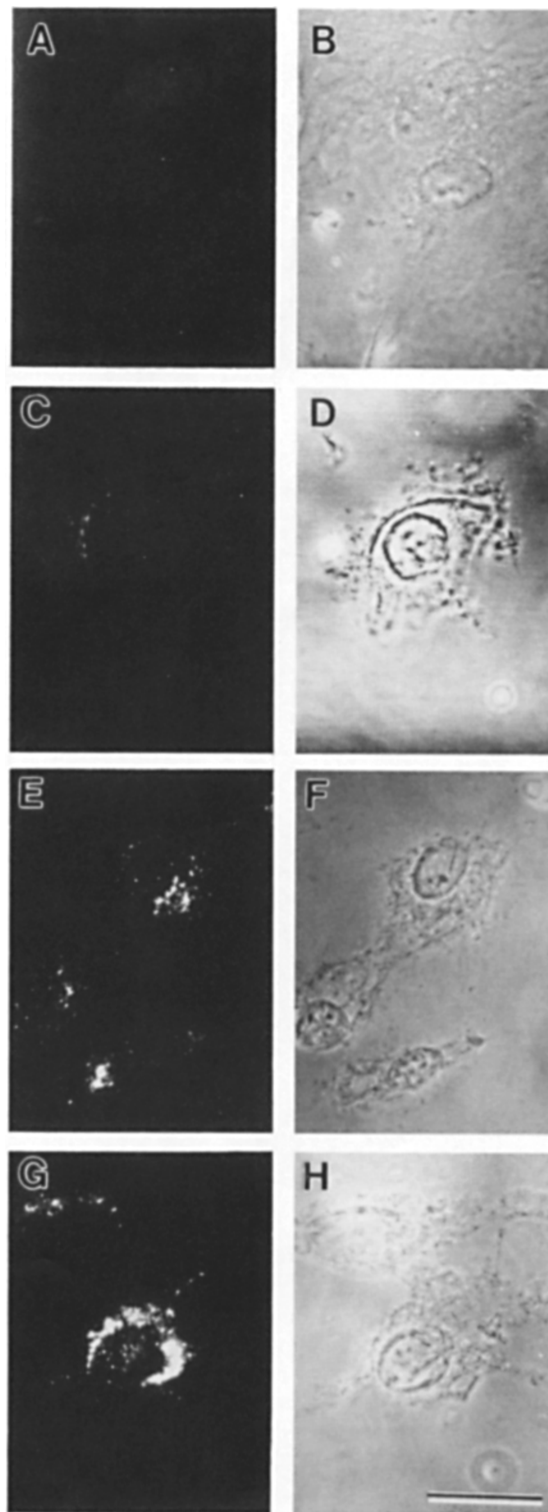

Figure 5. Reconstitution of γ-adaptin binding to TGN membranes. NRK cells grown 4 d on poly-D-lysine-coated coverslips were permeabilized for 5 min on ice with 40 μg/ml digitonin in ice cold transport buffer followed by 20 min incubation with transport buffer at room temperature. After addition of cytosol, an energy regenerating system and GTPγS as indicated below, samples were incubated 30 min at 37°C, rinsed with cold transport buffer, fixed and processed for immunofluorescence. *A*, *C*, *E*, and *G* are cells labeled with anti-γ-adaptin (mAb 100/3). *B*, *D*, *F*, and *H* are phase-contrast images of *A*, *C*, *E*, and *G*. (*A* and *B*) −Cytosol, −energy regenerating system; (*C* and *D*) +cytosol, −energy regenerating system; (*E* and *F*) +cytosol, +energy regenerating system; and (*G* and *H*) +cytosol, +energy regenerating system, +GTPγS. Bar, 25 μm.

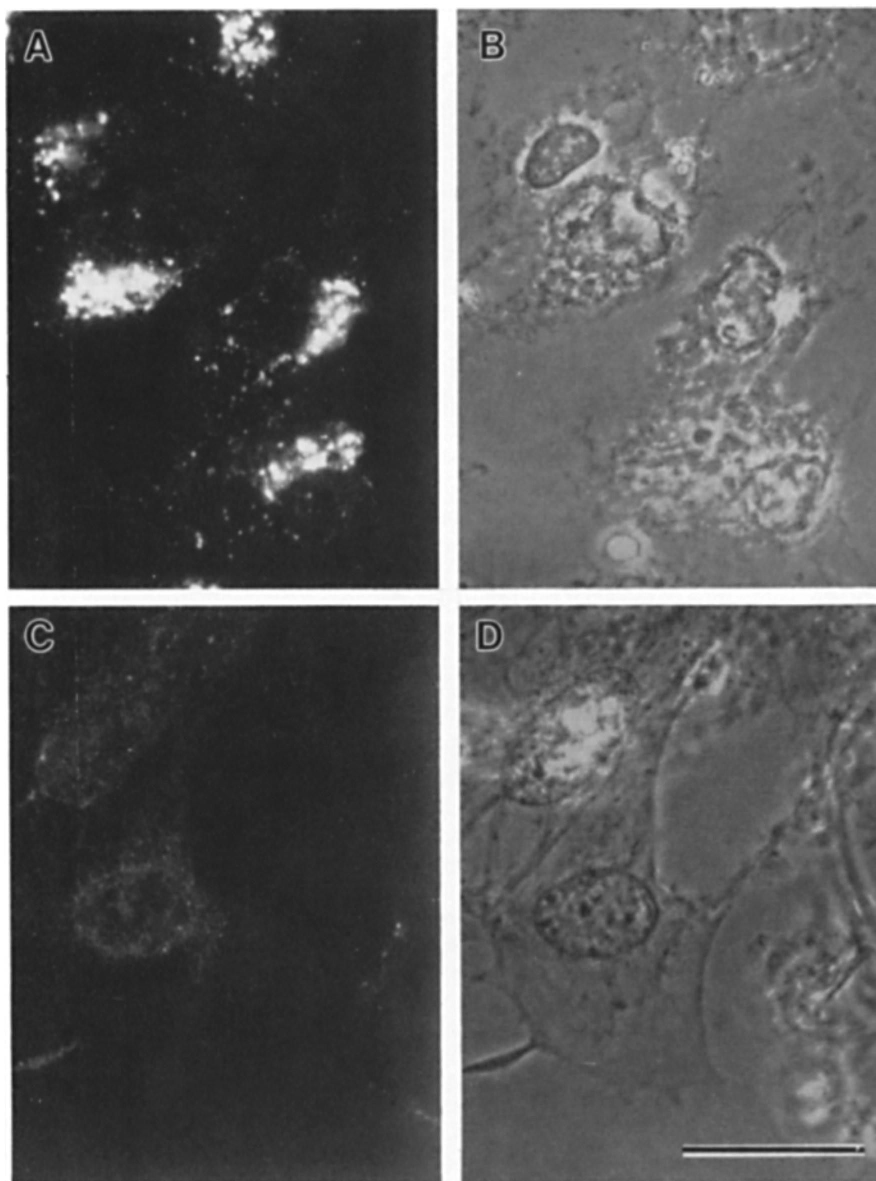

Figure 6. BFA inhibits γ-adaptin rebinding to TGN membranes. NRK cells grown 4 d on poly-D-lysine-coated coverslips were permeabilized for 5 min on ice with 40 μg/ml digitonin in ice-cold transport buffer followed by 20 min incubation with transport buffer at room temperature. Cytosol and an energy regenerating system were added to each coverslip, and incubated at 37°C as follows: (*A* and *B*) 15 min with 1 mM GTPγS followed by 15 min with 5 μg/ml BFA; (*C* and *D*) 15 min with 5 μg/ml BFA followed by 15 min with 1 mM GTPγS. *A* and *C* are cells labeled with anti-γ-adaptin (mAb 100/3). *B* and *D* are phase-contrast images of *A* and *C*. Bar, 25 μm.

membranes in the presence of an energy regenerating system. Subsequent addition of GTPγS had no further influence on γ-adaptin binding (Fig. 6 *C*). Using a similar assay, BFA did not appear to inhibit α-adaptin binding to the plasma membrane (Mallet, W. G., and F. M. Brodsky, unpublished observation). Conversely, if γ-adaptin from MDBK cytosol was stably bound to NRK membranes in the presence of GTPγS and energy, subsequent addition of BFA did not remove the bound molecules (Fig. 6 *A*). These results confirm that BFA does not strip γ-adaptin from membranes and indicate that BFA blocks the recruitment of γ-adaptin from the cytosol to the TGN membrane. Furthermore, they suggest that GTPγS prevents adaptor dissociation from membranes, rather than promoting their binding.

Discussion

The sequence homology between the ∼100-kD components of coated vesicles that mediate intracellular membrane traffic prompted an investigation into whether vesicle coat proteins might have common mechanisms of binding to intracellular membranes. BFA and GTPγS are agents that respectively inhibit and enhance the binding of the COP-coated vesicle coat protein β-COP to Golgi membranes. Both compounds were found to affect the binding of the TGN-associated clathrin-coat protein, γ-adaptin, in a similar fashion. However, in PtK_1 and MDCK cells, BFA did not affect the association of β-COP with Golgi membranes but still caused a cytoplasmic distribution of γ-adaptin. This demonstrates that γ-adaptin and β-COP have a related but distinct mechanism for binding to membranes. This has been supported by analysis of cells recovering from BFA treatment, indicating that γ-adaptin reassociates more rapidly with membranes than β-COP after withdrawal of BFA (Wong, D. H., and F. M. Brodsky, data not shown; [33]). Although the α-adaptin subunit of adaptors in endocytic clathrin-coated vesicles has homology to β-COP and γ-adaptin, the membrane binding of α-adaptin and presumably its associated subunits was unaffected by BFA in all cell types tested. This suggests that the membrane binding of the adaptors in plasma membrane clathrin coats is regulated differently from adaptor and COP protein binding in the TGN and Golgi apparatus respectively. This correlates with the observation that endocytosis of receptors that

are internalized in clathrin-coated pits continues in the presence of BFA (8, 25), but that constitutive secretion is completely inhibited (30).

The sensitivity of both β-COP and γ-adaptin to BFA and GTPγS indicates some common features in their interactions with membranes in the Golgi region. The binding of β-COP to Golgi membranes may, in turn, be similar to the Golgi association of the small GTP-binding protein ADP-ribosylation factor (ARF) (34). ARF is also a component of COP-coated vesicles and its binding to Golgi membranes is inhibited by BFA and enhanced by GTPγS. Enhanced binding of ARF by GTPγS treatment can be inhibited by exogenously added $G_{\beta\gamma}$ subunits of heterotrimeric G proteins (10). This would suggest that ARF binding is dependent on activation of a heterotrimeric G protein. In apparent contradiction to this conclusion, ARF binding is not enhanced by aluminum fluoride, but it has been postulated that aluminum fluoride may activate the appropriate G protein but not activate ARF itself, only partially fulfilling the requirement for ARF binding (10). β-COP binding to the Golgi apparatus is both enhanced by aluminum fluoride and inhibited by $G_{\beta\gamma}$ subunits, suggesting β-COP does not require additional activation for membrane binding but depends on the activation of a heterotrimeric G protein, similar to ARF binding. The $G\alpha_{i-3}$ subunit of a heterotrimeric G protein has been localized to Golgi membranes and appears to modulate constitutive secretion of heparan sulfate proteoglycan in $LLC\text{-}PK_1$ cells (36). Thus, $G\alpha_{i-3}$ may be the G protein that regulates COP-coated vesicle assembly, and influences binding of ARF and β-COP subunits.

There is no direct evidence for the presence of ARF or heterotrimeric G proteins in clathrin-coated vesicles in the TGN, although small GTP binding proteins have been found in purified clathrin-coated vesicles (19). However, BFA sensitivity of clathrin coat formation in the TGN suggests a possible involvement of the ARF family of proteins. Furthermore, Robinson and Kreis have reported that γ-adaptin binding to TGN membranes is enhanced by aluminum fluoride, implicating a heterotrimeric G protein in this process (33) and correlating with enhanced binding in the presence of GTPγS. γ-adaptin and β-COP are differentially sensitive to BFA in different cell lines, indicating that different BFA-sensitive elements are required for their binding. This difference in sensitivity could reflect the sequence differences in the 100-kD coat proteins themselves or in associated proteins, as the direct target for BFA is not known. The heterotrimeric G proteins and ARF proteins that may be involved in clathrin-coated vesicle formation in the TGN could certainly be different from those involved in COP-coated vesicle nucleation in the Golgi apparatus, since the G protein and ARF protein families have numerous related members (3, 4).

The adaptor molecules are believed to recognize protein motifs in the cytoplasmic domains of receptors (29, 37), both at the plasma membrane and in the TGN (29). The BFA-sensitive component affecting adaptor binding to the TGN may represent an additional membrane interaction site, perhaps influencing intracellular localization. Analysis of adaptor interaction with receptor tail peptides has revealed that the affinity of adaptors for the cytoplasmic domain of receptors is quite low. However, membrane binding assays indicate that adaptors associate with plasma membranes with high affinity (Chang, M. P., and F. M. Brodsky, unpublished results, [14, 22]). This would suggest that adaptors may bind to membranes by a high affinity "localization" binding site and then trap receptor tails through a weaker interaction. Identifying the target for BFA action could provide insight into the differential localization of TGN and plasma membrane adaptors.

COP proteins and TGN adaptors may have an additional role in the cell besides vesicular trafficking. It has been proposed that COP proteins provide a dynamic scaffolding that maintains the Golgi apparatus as a distinct organelle (12, 13). Disruption of the COP-membrane association, as seen with BFA, precedes the disruption of the Golgi apparatus. By analogy to COP proteins, TGN adaptors may also serve as a scaffolding molecule maintaining the TGN structure. In some cells BFA induces formation of tubular processes that fuse with the endosomal network (40), which could be a result of complete TGN adaptor dispersal that we observed after BFA treatment of MDBK cells. In MDCK cells, however, the TGN remains intact after extended BFA treatment (15). Although we observed that most γ-adaptin was localized to the MDCK cytoplasm under these conditions, a small population of γ-adaptin remained in the TGN area, which could be sufficient to maintain the TGN as a stable entity. A role for adaptors in maintaining organelle stability would indicate further common properties shared by the components of clathrin and COP-coated vesicles, which may even extend to similar interactions with cytoskeletal elements. Certainly the complexity of the adaptors and their COP homologues suggests that they will be able to mediate multiple functions in the control of intracellular membrane traffic.

M. Robinson and T. Kreis independently carried out similar experiments on the effects of BFA on clathrin-coated vesicles at the same time that we did. We thank them for communicating unpublished results. The authors would like to thank V. Allan, M. Robinson, and E. Ungewickell for their generous gifts of mAbs; S. Acton, M. Chang, B. Koppelman, W. Mallet, and I. Näthke for valuable discussion; and D. Crumrine, C. Cullander, and W. Theurkauf for technical assistance.

This work is supported by National Institutes of Health (NIH) Biotechnology Training Grant GM08388-02 (D. H. Wong) and NIH Grants GM38093, GM26691 and the Pew Charitable Trusts (F. M. Brodsky) and the Markey Charitable Trust (confocal microscope).

Received for publication 15 January 1992 and in revised form 24 March 1992.

References

1. Ahle, S., A. Mann, U. Eichelsbacher, and E. Ungewickell. 1988. Structural relationships between clathrin assembly proteins from the Golgi and the plasma membrane. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:919–929.
2. Allan, V. J., and T. E. Kreis. 1986. A microtubule-binding protein associated with membranes of the Golgi apparatus. *J. Cell Biol.* 103:2229–2239.
3. Balch, W. E. 1990. Small GTP-binding proteins in vesicular transport. *Trends Biol. Sci.* 15:473–477.
4. Bourne, H. R., D. A. Sanders, and F. McCormick. 1991. The GTPase superfamily: conserved structure and molecular mechanism. *Nature (Lond.).* 349:117–127.
5. Brodsky, F. M. 1985. Clathrin structure characterized with monoclonal antibodies. I. Analysis of multiple antigenic sites. *J. Cell Biol.* 101:2047–2054.
6. Brodsky, F. M. 1988. Living with clathrin: its role in intracellular membrane traffic. *Science (Wash. DC).* 242:1396–1402.
7. Chin, D. J., R. M. Straubinger, S. L. Acton, I. Näthke, and F. M. Brodsky. 1989. Adaptor proteins in peripheral clathrin-coated vesicles are required for receptor-mediated endocytosis. *Proc. Natl. Acad. Sci. USA.* 86: 9289–9293.
8. Damke, H., J. Klumperman, K. von Figura, and T. Braulke. 1991. Effects of Brefeldin A on the endocytic route. *J. Biol. Chem.* 266:24829–24833.

9. Donaldson, J. G., J. Lippincott-Schwartz, G. S. Bloom, T. E. Kreis, and R. D. Klausner. 1990. Dissociation of a 110-kD peripheral membrane protein from the Golgi apparatus is an early event in Brefeldin A action. *J. Cell Biol.* 111:2295–2306.
10. Donaldson, J. G., R. A. Kahn, J. Lippincott-Schwartz, and R. D. Klausner. 1991. Binding of ARF and β-COP to Golgi membranes: possible regulation by a trimeric G protein. *Science (Wash. DC).* 254:1197–1199.
11. Donaldson, J. G., J. Lippincott-Schwartz, and R. D. Klausner. 1991. Guanine nucleotides modulate the effects of Brefeldin A in semipermeable cells: regulation of the association of a 110-kD peripheral membrane protein with the Golgi apparatus. *J. Cell Biol.* 112:579–588.
12. Duden, R., V. Allan, and T. Kreis. 1991. Involvement of β-COP in membrane traffic through the Golgi complex. *Trends Cell Biol.* 1:14–19.
13. Duden, R., G. Griffiths, R. Frank, P. Argos, and T. E. Kreis. 1991. β-COP, a 110 kd protein associated with non-clathrin-coated vesicles and Golgi complex, shows homology to β-adaptin. *Cell.* 64:649–665.
14. Glickman, J. N., E. Conibear, and B. M. F. Pearse. 1989. Specificity of binding of clathrin adaptors to signals on the mannose-6-phosphate/insulin-like growth factor II receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1041–1047.
15. Hunziker, W., J. A. Whitney, and I. Mellman. 1991. Selective inhibition of transcytosis by Brefeldin A in MDCK cells. *Cell.* 67:617–627.
16. Keen, J. H. 1990. Clathrin and associated assembly and disassembly proteins. *Annu. Rev. Biochem.* 59:115–138.
17. Ktistakis, N. T., M. G. Roth, and G. S. Bloom. 1991. PtK_1 cells contain a nondiffusible, dominant factor that makes the Golgi apparatus resistant to Brefeldin A. *J. Cell Biol.* 113:1001–1023.
18. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
19. Lenhard, J. M., M. A. Levy, and P. D. Stahl. 1991. Clathrin-coated vesicles from human placenta contain GTP-binding proteins. *Biochem. Biophys. Res. Commun.* 174:197–203.
20. Lippincott-Schwartz, J., L. C. Yuan, J. S. Bonifacino, and R. D. Klausner. 1989. Rapid redistribution of Golgi proteins into the ER in cells treated with Brefeldin A: evidence for membrane cycling from Golgi to ER. *Cell.* 56:801–813.
21. Lippincott-Schwartz, J., J. G. Donaldson, A. Schweizer, E. G. Berger, H.-P. Hauri, L. C. Yuan, and R. D. Klausner. 1990. Microtubule-dependent retrograde transport of proteins into the ER in the presence of Brefeldin A suggests an ER recycling pathway. *Cell.* 60:821–836.
22. Mahaffey, D. T., J. S. Peeler, F. M. Brodsky, and R. G. W. Anderson. 1990. Clathrin-coated pits contain an integral membrane protein that binds the AP-2 subunit with high affinity. *J. Biol. Chem.* 265:16514–16520.
23. Malhotra, V., T. Serafini, L. Orci, J. C. Shepherd, and J. E. Rothman. 1989. Purification of a novel class of coated vesicles mediating biosynthetic protein transport through the Golgi stack. *Cell.* 58:329–336.
24. Manfredi, J. J., and W. L. Bazari. 1987. Purification and characterization of two distinct complexes of assembly polypeptides from calf brain coated vesicles that differ in their polypeptide composition and kinase activities. *J. Biol. Chem.* 262:12182–12188.
25. Misumi, Y., Y. Misumi, K. Miki, A. Takatsuki, G. Tamura, and Y. Ikehara. 1986. Novel blockade by Brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. *J. Biol. Chem.* 261:11398–11403.
26. Näthke, I. S., J. Heuser, A. Lupas, J. Stock, C. W. Turck, and F. M. Brodsky. 1992. Folding and trimerization of clathrin subunits at the triskelion hub. *Cell.* 68:899–919.
27. Orci, L., V. Malhotra, M. Amherdt, T. Serafini, and J. E. Rothman. 1989. Dissection of a single round of vesicular transport: sequential coated and uncoated intermediates mediate intercisternal movement in the Golgi stack. *Cell.* 56:357–368.
28. Pearse, B. M. F. 1988. Receptors compete for adaptors found in plasma membrane coated pits. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3331–3336.
29. Pearse, B. M. F., and M. S. Robinson. 1990. Clathrin, adaptors, and sorting. *Annu. Rev. Cell Biol.* 6:151–171.
30. Pelham, H. R. B. 1991. Multiple targets for Brefeldin A. *Cell.* 67:449–451.
31. Robinson, M. S. 1987. 100-kD coated vesicle proteins: molecular heterogeneity and intracellular distribution studied with monoclonal antibodies. *J. Cell Biol.* 104:887–895.
32. Robinson, M. S. 1990. Cloning and expression of the γ adaptin, a component of clathrin-coated vesicles associated with the Golgi apparatus. *J. Cell Biol.* 111:2319–2326.
33. Robinson, M. W., and T. E. Kreis. 1992. Recruitment of coat proteins onto Golgi membranes in intact and permeabilized cells: effects of Brefeldin A and G-protein activators. *Cell.* 69:129–138.
34. Serafini, T., L. Orci, M. Amherdt, M. Brunner, R. A. Kahn, and J. E. Rothman. 1991. ADP-ribosylation factor is a subunit of the coat of Golgi-derived COP-coated vesicles: a novel role for a GTP-binding protein. *Cell.* 67:239–253.
35. Serafini, T., G. Stenbech, A. Brecht, F. Lottspeich, L. Orci, J. E. Rothman, and F. T. Wieland. 1991. A coat subunit of Golgi-derived non-clathrin-coated vesicles with homology to the clathrin-coated vesicle coat protein, β-adaptin. *Nature (Lond.).* 349:215–220.
36. Stow, J. L., J. B. de Almeida, N. Narula, E. J. Holtzman, L. Ercolani, and D. A. Ausiello. 1991. A heterotrimeric G protein, $G\alpha_{i-3}$, on Golgi membranes regulates the secretion of a heparan sulfate proteoglycan in $LLC\text{-}PK_1$ epithelial cells. *J. Cell Biol.* 114:1113–1124.
37. Trowbridge, I. S. 1991. Endocytosis and signals for internalization. *Curr. Opin. Cell. Biol.* 3:634–641.
38. Waters, M. G., T. Serafini, and J. E. Rothman. 1991. "Coatomer": a cytosolic protein complex containing subunits of non-clathrin-coated Golgi transport vesicles. *Nature (Lond.).* 349:248–251.
39. Wong, D. H., M. J. Ignatius, G. Parosky, P. Parham, J. Q. Trojanowski, and F. M. Brodsky. 1990. Neuron-specific expression of high molecular weight clathrin light chain. *J. Neurosci.* 10:3025–3031.
40. Wood, S. A., J. E. Park, and W. J. Brown. 1991. Brefeldin A causes a microtubule-mediated fusion of the trans-Golgi network and early endosomes. *Cell.* 67:591–600.