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Retrograde Transport from the Pre-Golgi Intermediate Compartment and the Golgi Complex Is Affected by the Vacuolar H^+-ATPase Inhibitor Bafilomycin A1

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The effect of the vacuolar H^+-ATPase inhibitor bafilomycin A1 (Baf A1) on the localization of pre-Golgi intermediate compartment (IC) and Golgi marker proteins was used to study the role of acidification in the function of early secretory compartments. Baf A1 inhibited both brefeldin A- and nocodazole-induced retrograde transport of Golgi proteins to the endoplasmic reticulum (ER), whereas anterograde ER-to-Golgi transport remained largely unaffected. Furthermore, p58/ERGIC-53, which normally cycles between the ER, IC, and *cis*-Golgi, was arrested in pre-Golgi tubules and vacuoles, and the number of p58-positive ~80-nm Golgi (coatomer protein I) vesicles was reduced, suggesting that the drug inhibits the retrieval of the protein from post-ER compartments. In parallel, redistribution of β-coatomer protein from the Golgi to peripheral pre-Golgi structures took place. The small GTPase rab1p was detected in short pre-Golgi tubules in control cells and was efficiently recruited to the tubules accumulating in the presence of Baf A1. In contrast, these tubules showed no enrichment of newly synthesized, anterogradely transported proteins, indicating that they participate in retrograde transport. These results suggest that the pre-Golgi structures contain an active H^+-ATPase that regulates retrograde transport at the ER–Golgi boundary. Interestingly, although Baf A1 had distinct effects on peripheral pre-Golgi structures, only more central, p58-containing elements accumulated detectable amounts of 3-(2,4-dinitroanilino)-3′-amino-*N*-methyldipropylamine (DAMP), a marker for acidic compartments, raising the possibility that the lumenal pH of the pre-Golgi structures gradually changes in parallel with their translocation to the Golgi region.

INTRODUCTION

The function of vesicular intermediates in intracellular transport (Palade, 1975) has been firmly established, and a number of proteins involved in the formation and fusion of different vesicular carriers have been identified using biochemical and genetic approaches (Rothman and Wieland, 1996; Schekman and Orci, 1996; Robinson, 1997). In the early secretory pathway, two types of cytoplasmic vesicle coats, coatomer protein I (COPI)[1] and COPII, whose assembly is regulated by the small GTPases Arf1 and Sar1, respectively, operate sequentially in protein transport (Kreis *et al.*, 1995; Aridor and Balch, 1996; Schekman and Orci, 1996). The COPII coats are recruited to vesicles budding from the endoplasmic reticulum (ER) (Barlowe *et al.*, 1994; Bannykh *et al.*, 1996; Scales *et al.*, 1997), whereas the COPI coats preferentially associate with the pre-Golgi intermediate compartment (IC) and *cis*-

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[1] Abbreviations used: Baf A1, bafilomycin A1; BFA, brefeldin A; COP, coatomer protein; DAMP, 3-(2,4-dinitroanilino)-3′-amino-*N*-methyldipropylamine; IC, pre-Golgi intermediate compartment; SFV, Semliki Forest virus; TfR, transferrin receptor; VSV, vesicular stomatitis virus.

Golgi membranes (Oprins *et al.*, 1993; Griffiths *et al.*, 1995). Although it has been suggested that the COPI vesicles also participate in anterograde transport (Orci *et al.*, 1997, and references therein), increasing evidence points to their primary role in retrograde transport (Letourneur *et al.*, 1994; Sönnichsen *et al.*, 1996; Gaynor and Emr, 1997; Scales *et al.*, 1997; for reviews, see Pelham, 1994; Schekman and Mellman, 1997), e.g. the retrieval of proteins such as the KDEL-receptor and the mannose-binding lectin p58/ERGIC-53 from the IC and *cis*-Golgi back to the ER (Lewis and Pelham, 1996; Tisdale *et al.*, 1997).

Electron microscopic studies have demonstrated the regular presence of a network of narrow tubules at the *cis*-face of the Golgi complex (Rambourg and Clermont, 1997) and the localization of recycling proteins in such tubular extensions (Saraste *et al.*, 1987; Stinchcombe *et al.*, 1995). In addition, the pleiomorphic pre-Golgi structures have been reported to develop tubular domains (Saraste and Kuismanen, 1984; Pepperkok *et al.*, 1993; Stinchcombe *et al.*, 1995; Tang *et al.*, 1995; Bannykh *et al.*; 1996; Jäntti *et al.*, 1997; Presley *et al.*, 1997). When the formation of COPI vesicles (caused by lack of coatomer binding) is inhibited by brefeldin A (BFA), long tubules extend from the Golgi and fuse with the ER (Lippincott-Schwartz *et al.*, 1990). Similar Golgi tubules also form in vitro in the absence of BFA (Cluett *et al.*, 1993; Weidman *et al.*, 1993), and recent studies of Golgi dynamics in living cells support the idea that they function as intermediates in retrograde Golgi-to-ER transport (Sciaky *et al.*, 1997). In unperturbed cells, the formation of long tubules could be restricted by an efficient vesiculation process (Klausner *et al.*, 1992).

Bafilomycins, a group of macrolide antibiotics that at low concentrations specifically inhibit vacuolar (V-type) proton ATPases (Bowman *et al.*, 1988), have been commonly used to neutralize acidic compartments, such as endosomes, where the lumenal low pH is known to affect receptor–ligand interactions and protein sorting (for reviews, see Mellman *et al.*, 1986; Mukherjee *et al.*, 1997; Stevens and Forgac, 1997). Recent studies have shown that bafilomycin A1 (Baf A1) inhibits the delivery of endocytosed material from endosomes to lysosomes (Clague *et al.*, 1994; Van Weert *et al.*, 1995; Van Deurs *et al.*, 1996) and also causes tubulation of endosomes (Clague *et al.*, 1994; D'Arrigo *et al.*, 1997). In an in vitro system, the drug was shown to inhibit the formation of carrier vesicles operating between early and late endosomes (Clague *et al.*, 1994) and the association of a subset of coatomer subunits with early endosomal membranes (Aniento *et al.*, 1996). These studies, together with results showing that the binding of Arf to membranes requires intravesicular acidification (Zeuzem *et al.*, 1992), suggest that a functional H^+-ATPase is required not only for lumenal processes but also for coat-mediated, cytoplasmic transport events.

Immunolocalization of the weak bases 3-(2,4-dinitroanilino)-3′-amino-*N*-methyldipropylamine (DAMP) and primaquine first demonstrated that the lumenal pH of Golgi-associated vacuoles and *trans*-Golgi elements is acidic (Anderson and Pathak, 1985; Schwartz *et al.*, 1985). Recently, FITC-conjugated verotoxin, delivered to the Golgi complex via endocytosis, provided a novel means to more precisely determine the mildly acidic lumenal pH of Golgi elements (Kim *et al.*, 1996). Regarding the early secretory pathway, it has been shown previously that the interaction between the KDEL-receptor and KDEL-containing peptides in vitro prefers acidic pH (Wilson *et al.*, 1993; Scheel and Pelham, 1996) and that the pre-Golgi dissociation of T cell receptor-associated protein (TRAP) from receptor subunits requires organelle acidification (Bonifacino *et al.*, 1988). In addition, the emerging similarities of pre-Golgi structures and early (sorting) endosomes (Saraste and Kuismanen, 1984; Presley *et al.*, 1997; Scales *et al.*, 1997; for reviews, see Saraste and Kuismanen, 1992; Lippincott-Schwartz, 1993; Aridor and Balch, 1996), and previous studies on the effect of Baf A1 on the exocytic pathway (Palokangas *et al.*, 1994), prompted us to examine further the role of acidification in the functional organization of the early secretory compartments. Our present results indicate that the pre-Golgi and *cis*-Golgi membranes contain an active vacuolar H^+-ATPase that regulates retrograde membrane traffic at the ER–Golgi boundary.

MATERIALS AND METHODS

Antibodies

Rabbit polyclonal antibodies against rat p58 were prepared and affinity-purified as described previously (Saraste and Svensson, 1991). The monoclonal G1/93 antibody against ERGIC-53 was a gift from Dr. Hans-Peter Hauri (Biozentrum, Basel, Switzerland). Affinity-purified antibodies against rab1p, recognizing the A and B isoforms of the protein, were provided by Dr. Bruno Goud (Institut Curie, Paris, France). The polyclonal antibodies against mannosidase II and β-COP were obtained from Drs. Marilyn Farquhar and Kelley Moremen (University of California, San Diego, CA, and University of Georgia, Athens, GA) and Jennifer Lippincott-Schwartz (National Institutes of Health, Bethesda, MD), respectively. The monoclonal antibody to SFV E2 glycoprotein (E2–1) was provided by Dr. Margaret Kielian (Albert Einstein College of Medicine, Bronx, NY). The polyclonal and monoclonal (P5D4) antibodies against the VSV G glycoprotein were gifts from Dr. Kai Simons (EMBL, Heidelberg, Germany) and Dr. Thomas Kreis (University of Geneve, Geneve, Switzerland), respectively. The monoclonal antibody against human transferrin receptor, which does not cross-react with hamster receptors, was provided by Dr. Thomas Ebel (Karolinska Institute, Stockholm, Sweden). The mouse monoclonal antibody against dinitrophenol (MADNP-1) was purchased from the Experimental Immunology Unit (University of Louvain, Louvain, Belgium), and the secondary antibodies (FITC-, TRITC-, or peroxidase-conjugated goat anti-rabbit or anti-mouse $F_{(ab)2}$-fragments) were from Immunotech (Marseille, France).

Cell Culture and Virus Infection

All cell culture materials were purchased from Life Technologies (Inchinnan, Scotland). Hep-2 and NRK cells were cultured in DMEM supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% (vol/vol) fetal bovine serum (FBS). BHK-21 cells were cultured in Glasgow MEM containing 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10% (vol/vol) tryptose phosphate broth, and 5% FBS. Chinese hamster ovary cells were maintained in Eagle's α-MEM containing 2 mM L-glutamine, penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% FBS. Rat pheochromocytoma (PC12) cells were cultured in RPMI 1640 medium containing 10% horse serum and 5% FBS and supplemented with L-glutamine, penicillin, and streptomycin as above. Differentiation was induced by adding nerve growth factor (100 ng/ml) to the culture medium for 2 d. Low-temperature (15°C) incubations were performed on a water bath in Na_2CO_3-free medium buffered with 20 mM HEPES (pH 7.2). Baf A1 was a gift from Dr. David Keeling (Astra Hässle, Göteborg, Sweden). A stock (1 mM) was prepared in DMSO and stored at −80°C. Staining of NRK cells with acridine orange showed that 10^{-6}-10^{-7} M Baf A1 was sufficient to neutralize acidic compartments. Concanamycin A and BFA were kindly provided by Dr. Akira Takatsuki (Riken, Saitama, Japan), and nocodazole was purchased from Sigma (St. Louis, MO).

The construction of the Semliki Forest virus (SFV) replicon-based vectors (obtained from Drs. Peter Liljeström and Henrik Garoff, Karolinska Institute, Stockholm, Sweden), expressing either the human transferrin receptor or the complete set of virus structural proteins, with a mutation in the E2 glycoprotein (Y8K), has been described earlier (Liljeström and Garoff, 1991; Zhao *et al.* 1994). The packaging of the genomic RNA constructs into SFV particles was performed in BHK-21 cells as described by Liljeström and Garoff (1991). The procedures for infection of cells with the temperature-sensitive mutant ts045 of vesicular stomatitis virus (VSV) have been described by Bergman (1989). Generally, virus stocks were diluted in MEM supplemented with 1% (wt/vol) BSA and 2 mM L-glutamine, and the viruses were adsorbed to BHK-21 cells at 37°C (SFV) or at 31.5°C (VSV ts045) for 1 h, followed by growth of the cells for 3–4 h at 37°C or 39.5°C, respectively. To synchronize the exit of the VSV ts045 G protein from the ER, the cells were shifted to the permissive temperature (32°C) for the indicated times.

Metabolic Labeling and Endo H Digestion of the VSV G Protein

VSV ts045-infected Chinese hamster ovary cells were first preincubated for 15 min at 39.5°C in methionine and cysteine-free DMEM supplemented with 10% dialyzed FBS. The cells were then pulse-labeled for 15 min at the same temperature using 50 μCi/ml of a mixture of ^{35}S-methionine and ^{35}S-cysteine (Promix, Amersham, Buckinghamshire, UK) and then chased for 30–90 min in complete medium containing 10 times the normal concentration of cold methionine and cysteine. At harvest the cells were transferred to ice and solubilized in lysis buffer (50 mM Tris, pH 8.0, 0.4 M NaCl, 5 mM EDTA, 1% Triton X-100), containing 1 mM PMSF, 2 μg/ml aprotinin and a protease inhibitor mixture (CLAP: 10 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin).

VSV ts045 G protein was immunoprecipitated from the cell lysates using polyclonal antibodies. Aliquots of the lysates (containing ~1×10^6 cpm) were incubated with the antibodies overnight at 4°C. Thereafter 100 μl of a 10% (vol/vol) slurry of protein A-Sepharose (Pharmacia-LKB, Uppsala, Sweden) was added, and the samples were incubated with rotation for 1 h at RT. The samples were washed four times with lysis buffer and twice with 0.2 M Na-citrate buffer (pH 5.5), after which they were divided in two aliquots and the protein A beads were pelleted. An equal volume (50 μl) of 0.2 M Na-citrate buffer, pH 5.5, 0.1% SDS, with or without 0.5 mU endoglycosidase H (Boehringer Mannheim, Mannheim, Germany), was then added. Digestion was performed for 16 h at 37°C and terminated by addition an equal volume of 2× SDS-PAGE sample buffer. Finally, the samples were analyzed in 10% SDS-PAGE slab gels. The extent of processing of the VSV G protein to the endo H-resistant form was determined from autoradiographic images by densitometric scanning.

Cell Fractionation

PC12 and NRK cells were cultured in 175 cm^2 bottles until they reached 70–80% confluency. The cells were washed twice with ice-cold PBS, detached by mechanical agitation, and pelleted by centrifugation for 5 min at 1000 rpm at 4°C. The cell pellets were resuspended in 1 ml of ice-cold homogenization medium (50 mM sucrose, 1 mM EDTA, 10 mM HEPES, pH 7.2, supplemented with 1 mM PMSF, 2 μg/ml aprotinin, and 10 μg/ml CLAP) and homogenized by passing through a ball-bearing cell cracker. The sucrose concentration was adjusted to 0.25 M immediately after homogenization. The nuclei and cell debris were removed by centrifugation for 10 min at 600 × *g* at 4°C using the Sorvall HB-4 rotor. The postnuclear supernatants were then centrifuged for 90 min at 100,000 × *g* using the Beckman TL-100 rotor to obtain the cytosol and total membrane fractions. The pelleted membranes were resuspended in the homogenization medium containing 0.25 M sucrose, and the fractions were frozen in liquid nitrogen and stored in aliquots at −80°C. To analyze the distributions of p58, rab1p, and β-COP in the fractions, proteins were separated by SDS-PAGE (10% gels), transferred to nitrocellulose, and subjected to immunoblotting. The bound primary antibodies were detected using ^{125}I-protein A (Amersham, Buckinghamshire, UK).

Immunofluorescence Staining and Confocal Microscopy

For immunofluorescence, the cells cultured on glass cover slips were fixed for 30 min at room temperature with 3% (wt/vol) paraformaldehyde, prepared in 0.1 M phosphate buffer, pH 7.4. Cells were then rinsed with PBS, followed by PBS containing 0.2% (wt/vol) BSA, and then permeabilized by incubating for 5 min with 0.2% (wt/vol) saponin (Serva), or in some experiments with 0.1% (vol/vol) Triton X-100, in PBS–BSA. The cells were then incubated with PBS–BSA–saponin containing 10% FBS (blocking buffer) to block nonspecific binding of antibodies, after which primary antibodies, diluted in blocking buffer, were added to the cells. After extensive rinsing, the cells were incubated with fluorochrome-coupled (FITC or TRITC) goat anti-rabbit or mouse $F_{(ab)2}$-fragments (diluted in blocking buffer). In double immunofluorescence, the incubations with the primary and corresponding secondary antibodies were carried out successively. Finally, the coverslips were placed on objective glasses on a small drop of mounting medium (Slow-Fade, Molecular Probes, Eugene, Oregon). To localize acidic organelles, control cells and cells treated with 5 μM nocodazole were incubated for 30 min at 37°C in culture medium containing 50 μM DAMP (Molecular Probes) before fixation and double-staining with antibodies against p58 and DNP, as described above.

The samples were viewed with a Bio-Rad MRC-1000 confocal laser scanning microscope (Bio-Rad, Hercules, CA) equipped with a krypton-argon laser, an inverted Zeiss Axiovert microscope, and a 100× oil immersion objective. The images were recorded, merged and processed using the Bio-Rad software. The thickness of the optical sections was 0.54 μm. Photographs were taken on Fuji Sensia 200 ASA film. The images shown in Figure 1, and those used to obtain the data in Figure 3, were processed using identical software parameters and photographed using the same exposure times.

Immunoelectron Microscopy

Rat PC12 cells were grown to ~70% confluency on 35-mm-diameter culture dishes. A preembedding immunoperoxidase procedure was used for staining of control and Baf A1-treated cells with affinity-

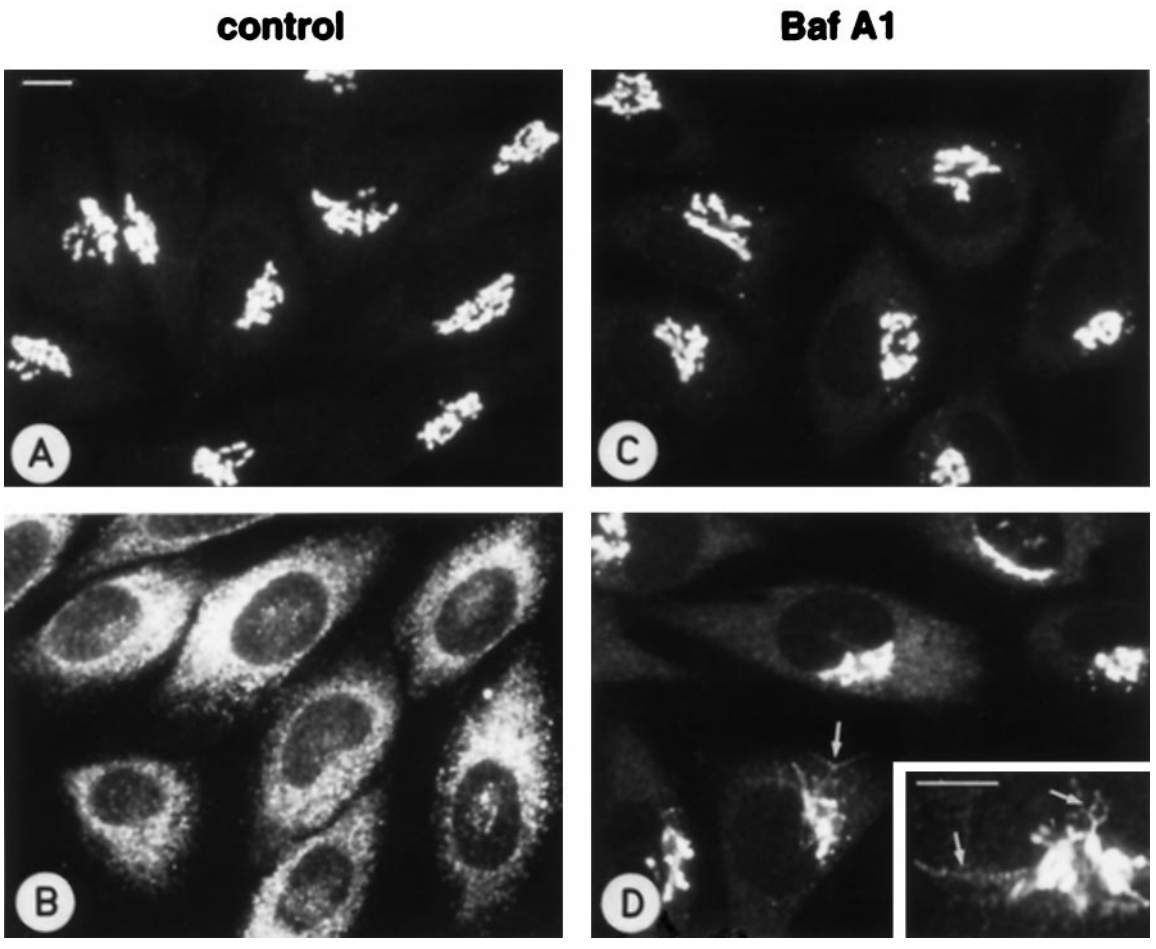

Figure 1. Baf A1 inhibits the BFA-induced redistribution of Golgi mannosidase II to the ER. Control NRK cells (A and B) and cells pretreated for 3 h in medium containing 10^{-6} M Baf A1 (C and D) were either fixed directly (A and C) or after a further 10 min incubation in the presence of BFA (1 μg/ml) (B and D), followed by staining with antibodies against mannosidase II. In control cells, BFA causes an efficient relocation of mannosidase II to the ER (B), whereas in Baf A1-treated cells the redistribution of the protein is considerably slowed down (D). Accordingly, tubular intermediates of the transfer process can be observed (D and inset; arrows). The cells in D also display weak ER-like staining, indicating that the retrograde transport of mannosidase II is not completely blocked by Baf A1. Bars, 10 μm.

purified antibodies against p58 and rab1p (Saraste *et al.*, 1987, 1995; Saraste and Svensson, 1991). After the cells were embedded in Agar 100 (Agar Scientific, Essex, England), thin (~50 nm) and thick (~200 nm) sections were cut horizontally. The thin sections were stained with lead citrate. The samples were examined in JEOL CX II electron microscope and photographed on Kodak EM film. Quantitation of the p58-containing ~80 nm vesicles was performed on 30–35 cells using thin sections cut across the central Golgi region.

RESULTS

Baf A1 Inhibits Retrograde Golgi to ER Transport

Previous studies measuring Golgi-specific processing of newly synthesized proteins have described variable effects of different H^+-ATPase inhibitors on forward transport between the ER and the Golgi complex (Klionsky and Emr, 1989; Muroi *et al.*, 1993; Yilla *et al.*, 1993; Palokangas *et al.*, 1994). In addition, our further biochemical and morphological experiments demonstrated that the transport of VSV G protein between the ER and *cis*/medial Golgi is only slightly affected by Baf A1 (our unpublished results) (see Figure 7, E and G). To determine whether organelle acidification is required for retrograde transport processes at the ER–Golgi boundary, we studied the effect of Baf A1 on the redistribution of Golgi proteins that takes place in BFA-treated cells (Klausner *et al.*, 1992). NRK cells were first treated for 3 h with Baf A1 (10^{-6} M), before addition of BFA (0.2–1 μg/ml) to the medium, followed by staining for confocal fluorescence microscopy using antibodies against the Golgi enzyme mannosidase II. As shown in Figure 1, A and C, Baf A1 alone did not affect the distribution of mannosidase II. In control cells exposed to BFA (1 μg/ml), the protein

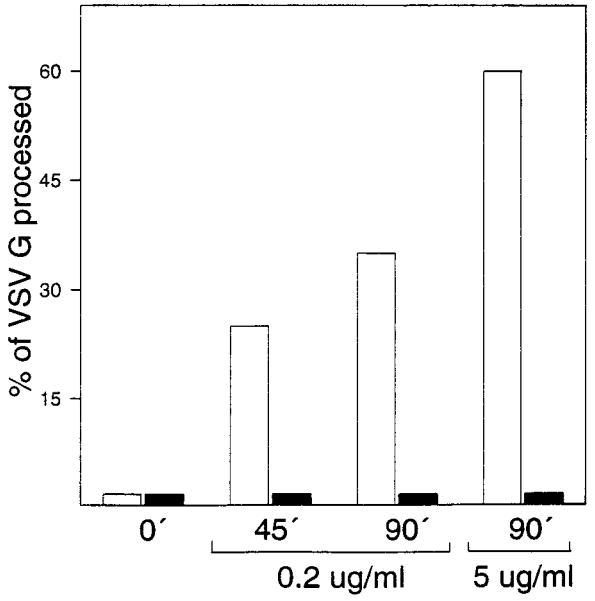

Figure 2. Baf A1 inhibits the BFA-induced redistribution of Golgi glycosyltransferases as measured by processing of the VSV tsO45 mutant G protein retained in the ER at the restrictive temperature (39.5°C). Chinese hamster ovary cells were infected with VSV tsO45 and, after virus adsorption, cultured for 3 h at 39.5°C in the presence or absence of Baf A1. The cells were pulse-labeled at 39.5°C with ^{35}S-methionine/cysteine and then either transferred directly on ice or chased for 45–90 min at 39.5°C in the presence of different concentrations of BFA (0.2 or 5 μg/ml). The pulse–chase of Baf A1-pretreated cells was performed in the continuous presence of the drug. The tsO45 G protein was immunoprecipitated from cell lysates and analyzed, either undigested or after digestion with endo H, by SDS-PAGE. The amount of conversion of the G protein to the endo H-resistant form was quantitated as described in MATERIALS AND METHODS. White columns, Control cells; black columns, Baf A1-treated cells.

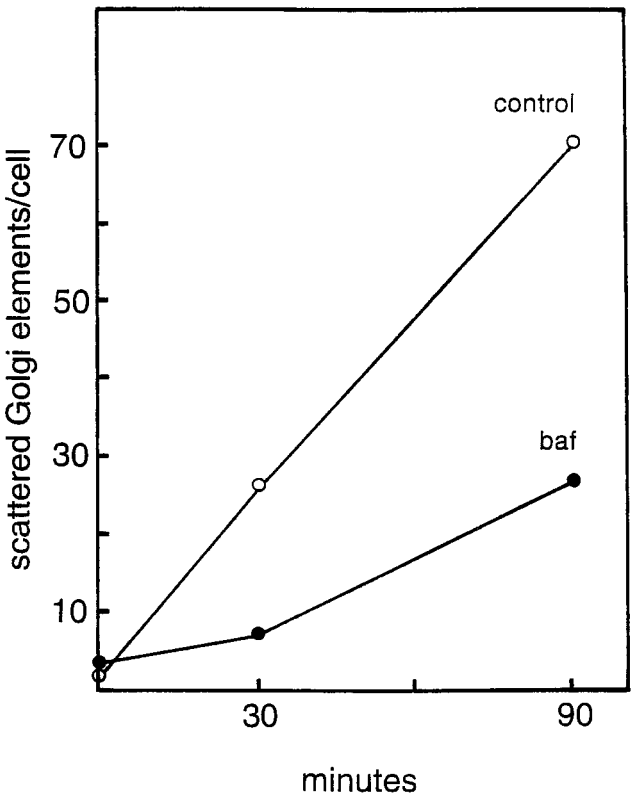

Figure 3. Baf A1 inhibits the nocodazole-induced redistribution of Golgi enzymes. Control and Baf A1-treated NRK cells were either fixed directly or after a further 30 or 90 min incubation in the presence of the microtubule-depolymerizing drug nocodazole (5 μM). The cells were stained for immunofluorescence microscopy using antibodies against Golgi mannosidase II, and the number of scattered, peripheral Golgi structures was determined as described in MATERIALS AND METHODS. Figure 1, A and C, also illustrates the typical distribution of mannosidase II in control and Baf A1-pretreated cells, respectively, before the addition of nocodazole (0 min time point).

rapidly assumed an ER-like distribution (Figure 1B), indicating extensive fusion of Golgi elements with the ER. In contrast, the Golgi structures in Baf A1-pretreated cells remained largely resistant to BFA (Figure 1D), and long, mannosidase II-positive tubules were seen to extend from the Golgi complex (Figure 1, D and inset). In addition, weak, ER-like staining was seen in the Baf A1-treated cells (Figure 1D). These results indicate that Baf A1 considerably slows down the BFA-induced retrograde transport of Golgi proteins to the ER.

Because the effect of BFA results from the dissociation COPI (coatomer) coats (Donaldson *et al.*, 1990) that predominantly associate with the cytoplasmic surface of IC and *cis*-Golgi membranes (Oprins at el., 1993; Griffiths *et al.*, 1995), we also examined whether Baf A1 affects the redistribution of coatomer using immunolocalization with antibodies directed against the β-COP subunit. These results indicated that the effect of Baf A1 on Golgi redistribution in BFA-treated cells correlates with an inhibition of COPI release from membranes (our unpublished results).

To quantitate the effect of Baf A1 on BFA-induced retrograde transport and fusion of Golgi elements with the ER, we used the temperature-sensitive mutant tsO45 of VSV. When the mutant-infected cells are grown at the restrictive temperature (39.5°C), the tsO45 G protein is produced normally but accumulates in the ER in a misfolded state (Doms *et al.*, 1987). Because the activity of *cis*/medial Golgi processing enzymes is not significantly affected by Baf A1 (our unpublished results), the conversion of the tsO45 G protein-bound glycans to the endoglycosidase H-resistant form at 39.5°C could be used to measure the effect of this drug on BFA-induced relocation of these proteins to the ER (Doms *et al.*, 1989). In control cells, the tsO45 G protein became partly endo H-resistant when 0.2 μg/ml BFA was used, whereas in Baf A1-

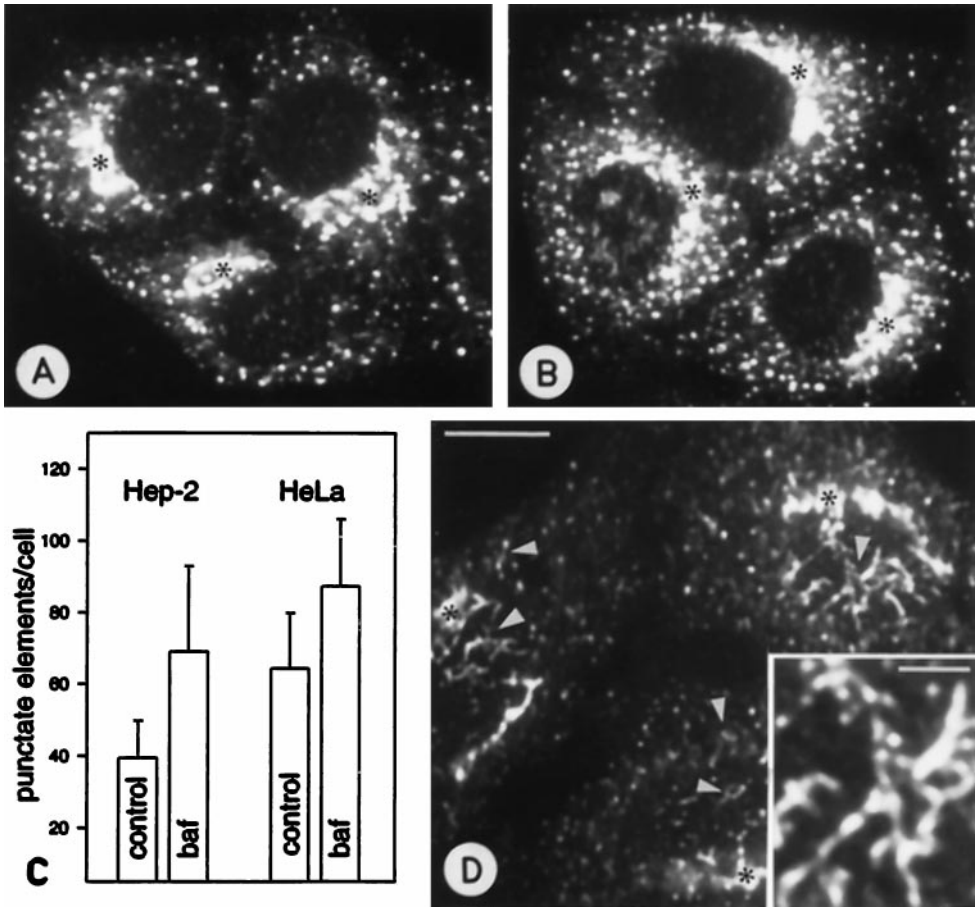

Figure 4. Accumulation of ERGIC-53 in globular and tubular pre-Golgi structures in Baf A1-treated cells. Human Hep-2 and HeLa cells were incubated for 3 h in the presence or absence of Baf A1 and immunostained for ERGIC-53 using monoclonal (G1/93) antibodies. (A and B) Confocal immunofluorescence images from the middle plane of control and Baf A1-treated Hep-2 cells, respectively. (C) Quantitation showing that Baf A1 significantly increases the number of detectable, ERGIC-53-positive pre-Golgi structures in both Hep-2 and HeLa cells. (D and inset) Optical section from the top of the nuclei, which best illustrates the accumulation of ERGIC-53 in both centrally and peripherally located pre-Golgi tubules (arrowheads) in Baf A1-treated Hep-2 cells. Bars, 10 μm (A, B, and D) and 2.5 μm (inset in D).

pretreated cells no conversion was detected (Figure 2). At higher concentrations of BFA (5 μg/ml), the maturation of the ER-retained G protein was enhanced, but only in control cells. The finding that with time mannosidase II obtained a more ER-like distribution also in Baf A1-pretreated cells exposed to BFA (our unpublished results) indicates that Baf A1 either inhibits the fusion of Golgi-derived tubules with the ER or results in the segregation of the G protein and Golgi enzymes within the ER system.

Because recent results of Cole and coworkers (1996) have supported the idea that the formation of peripheral Golgi structures in cells treated with microtubule inhibitors (e.g. nocodazole) involves retrograde transport of Golgi components to the ER, we also examined the effect of Baf A1 on nocodazole-induced redistribution of Golgi proteins. Control and Baf A1-pretreated NRK cells were incubated for 30–90 min in the presence of nocodazole (5 μM) and stained for confocal fluorescence microscopy using antibodies against mannosidase II. The quantitation of the data (Figure 3) shows that the redistribution of mannosidase II to peripheral Golgi structures was considerably (65–75%) inhibited by Baf A1 in these cells. Moreover, as compared with control cells, many of the peripheral Golgi structures observed in Baf A1-treated cells were relatively weakly positive for mannosidase II (our unpublished results).

Accumulation of Pre-Golgi Tubules in Baf A1-treated Cells

To determine further the effect of Baf A1 on the function of the early secretory compartments, we examined the distribution of p58/ERGIC-53 in the drug-treated human and rodent cells. This homo-oligomeric integral membrane protein that continuously cycles between the ER, IC, and *cis*-Golgi (Saraste *et al.*, 1987; Saraste and Svensson, 1991; Schweizer *et al.*, 1988; Lippincott-Schwartz *et al.*, 1990; Lahtinen *et al.*, 1992) binds both COPII coats and coatomer (Kappeler *et al.*, 1997; Tisdale *et al.*, 1997) and appears to act as a receptor for mannose-containing glycoproteins (Fiedler and Simons, 1994; Arar *et al.*, 1995; Itin *et al.*, 1996, Nichols *et al.*, 1998). Because of these properties, it was expected to provide a sensitive marker for testing the luminal conditions of the compartments it traverses.

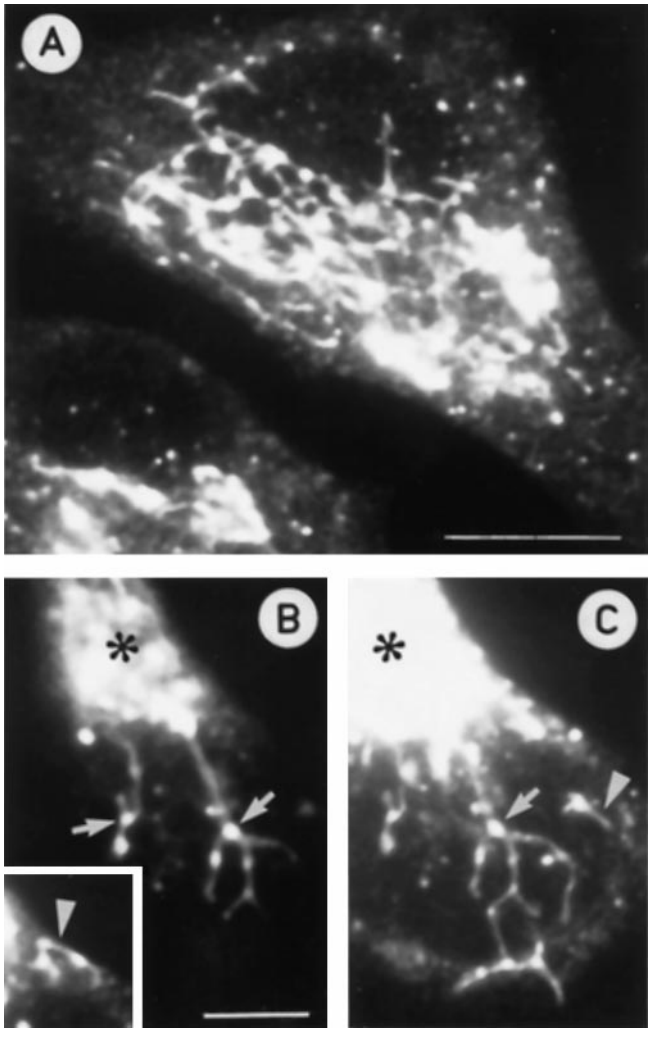

Figure 5. Visualization of the pre-Golgi tubules in Baf A1-treated PC12 cells using antibodies against rab1p. (A) Confocal immunofluorescence image demonstrating tubulation of the pre-Golgi structures in the drug-treated cells and the formation of an extensive, partly continuous reticulum. (B and C) Two partly overlapping confocal sections from the same cell showing details of a rab1p-positive reticulum that extends from the Golgi region (asterisks) towards the cell periphery. Note the localization of the globular IC domains to the branchpoints of the reticulum (arrows). The arrowheads indicate rab1p-positive tubules extending from and connecting the peripheral, globular pre-Golgi structures. Bars, 10 μm (A) and 5 μm (B).

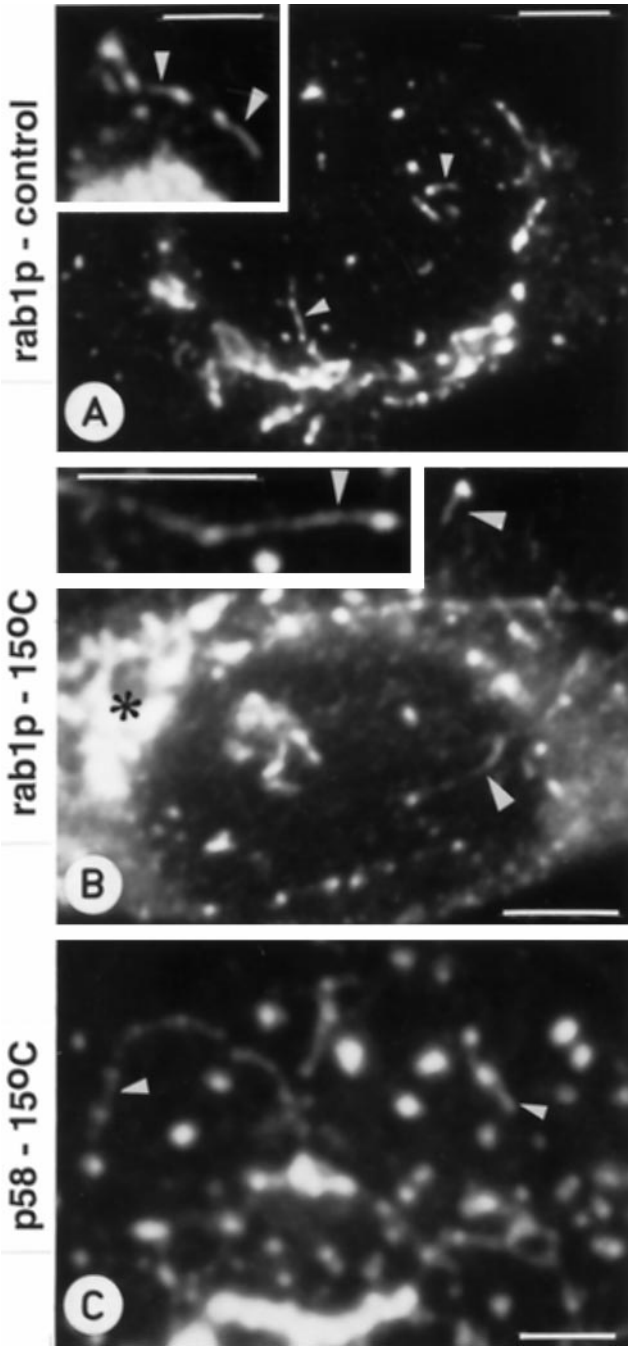

Figure 6. Pre-Golgi tubules can also be detected in control and 15°C-treated cells. Control NRK (A) and PC12 cells (A, inset) and NRK cells incubated for 2 h at 15°C (B and inset) were stained with antibodies against rab1p. Note the tubular extensions and connections of the pre-Golgi structures (arrowheads) that become more pronounced in response to the low-temperature treatment. The asterisk in B denotes the Golgi region. (C) The pre-Golgi tubules accumulating in NRK cells at 15°C also contain p58. Bars, 5 μm (A, B, and insets) and 2.5 μm (C).

The localization of the human ERGIC- 53 in control and Baf A1-treated (10^{-6} M) Hep-2 cells is shown in Figure 4, A and B, respectively. Although the overall distribution of the protein, i.e. its presence in peripheral pre-Golgi and central Golgi structures, did not dramatically change in response to Baf A1, the detection of ERGIC-53 in scattered pre-Golgi structures significantly increased as compared with control cells (Figure 1C). Interestingly, confocal fluorescence mi-

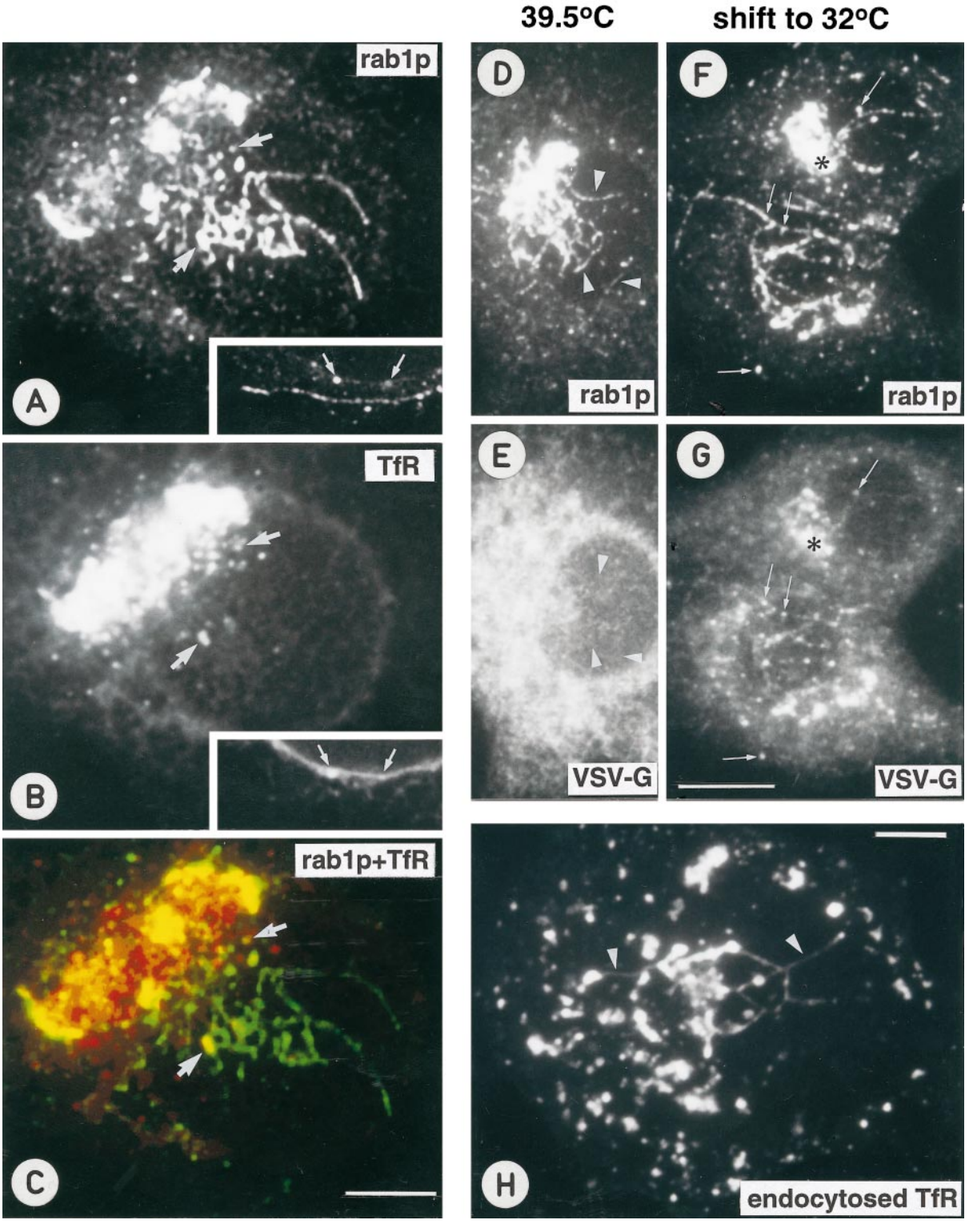

Figure 7. Newly synthesized, anterogradely transported proteins are not concentrated in the pre-Golgi tubules accumulating in Baf A1-treated cells. (A–C) BHK-21 cells were infected with an SFV vector encoding the human TfR, treated with Baf-A1, and double-stained for rab1p (A) and TfR (B). C shows the merged confocal image. Partial colocalization of rab1p and TfR is observed in the punctate pre-Golgi structures (arrows), whereas both central and peripheral (see insets in A and B) rab1p-positive tubules do not contain detectable amounts of TfR. (D–G) Double-localization of rab1p (D and F) and the VSV-G protein (E and G) in tsO45 mutant-infected, Baf A1-treated cells incubated

croscopy also revealed that extensive tubulation of the ERGIC-53–positive IC membranes takes place in the presence of Baf A1. Long, anastomosing tubules, which frequently connected the punctate pre-Golgi structures, were easily detected on top of the nuclei in the drug-treated Hep-2 cells, but tubule formation was also observed in more peripheral locations (Figure 1D). Baf A1 had a similar effect on the distribution of ERGIC-53 in HeLa cells (Figure 1C), as well as the localization of rat p58 in BHK-21, NRK, and PC12 cells. Moreover, induction of tubule formation was observed also at tenfold lower concentrations (10^{-7} M) of Baf A1 or when concanamycin A, another proton ATPase inhibitor (Muroi *et al.*, 1993), was used (our unpublished results).

The Pre-Golgi Tubules in Control and Baf A1-treated Cells Contain rab1p

The small GTP-binding protein rab1p is a regulatory component of the transport machinery that operates between the ER and the Golgi complex (Plutner *et al.*, 1991) and specifically associates with the cytoplasmic surface of IC and *cis*-Golgi membranes (Griffiths *et al.*, 1994; Saraste *et al.*, 1995). To further determine how inhibition of acidification affects the functional organization of the IC, immunolocalization of rab1p in control and Baf A1-treated cells was performed. As shown for differentiating PC12 cells (Figure 5), the drug induced the formation of long, rab1p-positive, tubular structures (see also Figure 7A, D, and F) that typically accumulated in central locations but were also found in the cell periphery. In part of the cells, the tubules formed an extensive reticulum that extended from the Golgi region over almost the entire cell body (Figure 5A). As described above for p58/ERGIC-53 (Figure 4D), the anastomosing, rab1p-positive tubules in these reticular structures connected the globular IC domains, which were also found at the branchpoints of the reticulum (Figure 5, B and C).

Importantly, using confocal fluorescence microscopy we could also detect rab1p-containing pre-Golgi tubules in untreated cells (Figure 6A). These, however, were less frequent and typically shorter than the tubules seen in the drug-treated cells. When cells were incubated at 15°C, which inhibits both forward transport and recycling through the IC (Saraste and Kuismanen, 1984; Saraste and Svensson, 1991), both rab1p and p58 (Figure 6, B and C, respectively) were found associated with long tubular and reticular structures, similar to those seen in Baf A1-treated cells. Furthermore, double-staining of 15°C-treated HeLa cells showed the presence of the two proteins in the same pre-Golgi tubules (our unpublished results). Interestingly, in contrast to rab1p, we failed to detect p58 in tubular structures in control cells. These results indicate that the extensive tubulation of the IC observed in Baf A1-treated cells represents an amplification of a normally occurring process and correlates with inhibition of membrane trafficking through the IC.

Anterograde Markers Are Not Enriched in the Pre-Golgi Tubules

To determine whether the pre-Golgi tubules accumulating in Baf A1-treated cells are part of the forward (anterograde) or retrograde transport pathway, we used SFV replicon-based vectors (Liljeström and Garoff, 1991) to obtain high-level expression of anterogradely transported marker proteins. After infection with an SFV vector encoding the human transferrin receptor (TfR), BHK-21 cells were incubated in the presence of Baf A1 and then double-stained with antibodies against rab1p and human TfR. In both uninfected (our unpublished results) and SFV-infected Baf A1-treated cells, rab1p was localized to punctate pre-Golgi structures and associated, long tubules (Figure 7A). Although partial colocalization of newly synthesized TfRs with rab1p was observed in the Golgi region and punctate pre-Golgi structures, the receptors could not be detected in the tubular, rab1p-positive IC domain (Figure 7, B and C). It is unlikely that this was due to a conformational specificity of the monoclonal antibodies used, because in addition to the punctate pre-Golgi elements, human TfRs could also be detected in the ER, including the nuclear envelope (Figure 7, B and inset). Moreover, immunolocalization of SFV E2 glycoproteins in cells infected with a mutant SFV construct that fails to form virus particles (Zhao *et al.*, 1994) gave similar results (our unpublished results).

To obtain more efficient loading of the early secretory compartments with anterogradely transported proteins, we used the ts045 mutant of VSV, which allows the synchronization of the G protein export from the ER (Bergman, 1989). After shift of cells from

Figure 7 (cont). for 3 h at 39.5°C (D and E) or shifted for an additional 5 min to 32°C in the continuous presence of the drug (F and G), to synchronize the export of the G protein from the ER. At 39.5°C, the bulk of the G protein remains arrested in the ER and is not detected in the rab1p-positive tubules (arrowheads in D and E). In cells shifted to 32°C, the G protein and rab1p partially colocalize in the Golgi region (asterisk) and many of the punctate pre-Golgi structures (arrows), whereas the rab1p-positive tubules remain mostly devoid of the G protein. (H) To study the effect of Baf A1 on endocytic compartments, BHK-21 cells expressing human TfR were incubated in medium containing mouse anti-TfR antibodies. After Baf A1-treatment the cells were fixed, and the internalized TfR-antibody complexes were visualized using fluorochrome-coupled secondary antibodies. Note the tubular connections between peripheral and central endocytic structures (arrowheads). Bars, 5 μm (A–C, H) and 10 μm (D–G).

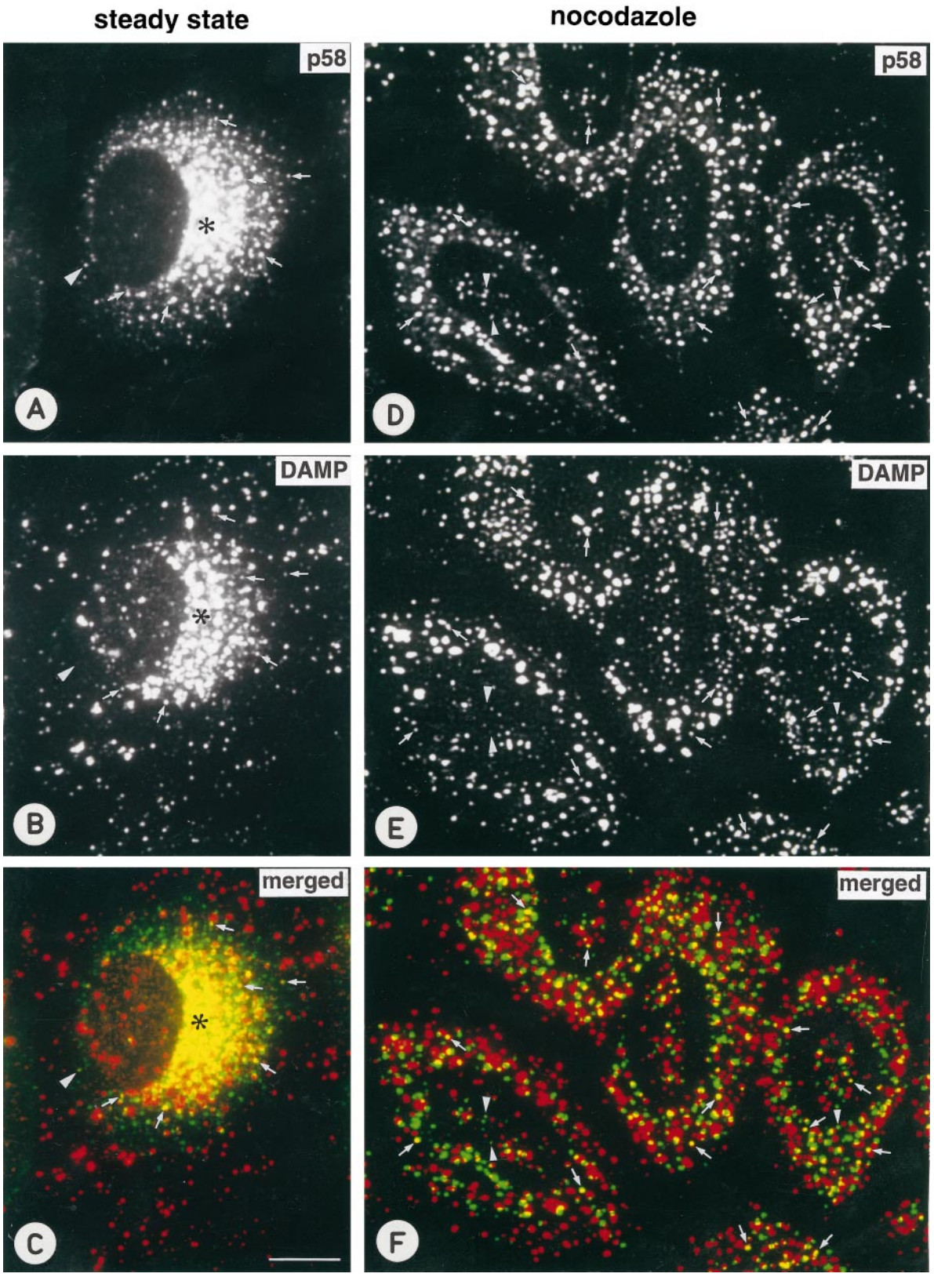
steady state
nocodazole
p58
p58
A
D
DAMP
DAMP
B
E
merged
merged
C
F

Figure 8.

39.5 to 32°C, normal folding of the G protein is resumed, and it is rapidly transported via the IC and the Golgi complex to the plasma membrane (Lotti *et al.*, 1992; Lippincott-Schwartz *et al.*, 1995). BHK-21 cells were infected with ts045 and incubated for 3 h at 39.5°C in Baf A1-containing or drug-free medium. Part of the cultures were then transferred to 32°C for an additional 5 min in the presence or absence of the drug, before double-staining with antibodies against the G protein and rab1p. In cells maintained at 39.5°C in the presence of Baf A1, the mutant G protein was predominantly localized to the ER and not detected in the rab1p-positive pre-Golgi and Golgi elements (Figure 7, D and E). After the brief shift of these cells to 32°C, efficient transport of the G protein to punctate IC structures and the Golgi complex took place, similar to that seen in untreated cells (our unpublished results). In contrast, even under these synchronized conditions, the mutant G protein was largely lacking from the tubular IC domains containing rab1p (Figure 7, compare F and G). Taken together, our results on the localization of a set of anterograde markers support the notion that the accumulation of pre-Golgi tubules in Baf A1-treated cells is due to an effect of the drug on retrograde transport.

When BHK-21 cells are infected with an SFV vector encoding the human TfR, the receptors become fully functional, and after reaching the plasma membrane they are internalized and delivered to the endosomal recycling pathway (Liljeström and Garoff, 1991). By adding the human TfR-specific antibodies to the culture medium and detecting the internalized, TfR-bound antibodies in fixed cells using fluorescent secondary antibodies, we could localize the receptors in endocytic compartments. In response to Baf A1 treatment, enhanced formation of TfR-containing tubules connecting central and peripheral endocytic structures (Figure 7H), and similar in morphology to the pre-Golgi tubules described above, was observed.

A Subpopulation of the Pre-Golgi Structures Are Acidic

To examine directly the luminal pH conditions of the pre-Golgi structures, we used DAMP, a weak base that accumulates in acidic organelles and has been previously localized to endocytic structures and *trans*-Golgi elements (Anderson *et al.*, 1984; Anderson and Pathak, 1985); however, the sites of DAMP accumulation have not been compared with the localization of pre-Golgi markers. NRK cells were incubated with 50 μM DAMP and double-stained with monoclonal anti-dinitrophenol and polyclonal anti-p58 antibodies. In cells fixed at steady state, DAMP could be detected in some of the more centrally located pre-Golgi structures, whereas the peripheral, p58-positive elements were mostly negative (Figure 8, A–C). To increase resolution, cells were treated with nocodazole before DAMP incubation to disperse the pre-Golgi elements to the cell periphery (Saraste and Svensson, 1991; Cole *et al.*, 1996). Double-staining of the nocodazole-treated cells showed that ~17% of the p58-positive elements also contained DAMP (Figure 8, D–F; the partly overlapping structures giving rise to a three-color image in Figure 8F were not included in these calculations). These pre-Golgi structures were typically small in size and only weakly positive for DAMP. These results exclude the possibility that the observed colocalization of the two markers in the Golgi region of steady-state cells (Figure 8C) is based solely on high organelle density.

Baf A1-induced Redistribution of β-COP to Pre-Golgi Structures

Because Baf A1 appeared to inhibit retrograde transport processes at the ER–Golgi boundary, it was of interest to examine its possible effect on the subcellular distribution of coatomer. Localization of β-COP in control and Baf A1-treated NRK cells indicated that the binding of coatomer to membranes as such is not affected by the drug (Figure 9, A and B); however, there was an apparent reduction in the staining in the Golgi region, and in parallel, enhanced association of β-COP with peripheral structures was observed (Figure 9C). Double-localization with newly synthesized proteins and p58/ERGIC-53 has shown that the β-COP–positive peripheral elements, detected by immunofluorescence microscopy, represent pre-Golgi structures, and increased association of β-COP with these elements also takes place at 15°C (our unpublished results) (Duden *et al.*, 1991; Saraste and Kuismanen, 1992; Griffiths *et al.*, 1995; Lippincott-Schwartz *et al.* 1995; Scales *et al.*, 1997); however, tubular elements, similar to those containing p58/ERGIC-53 and rab1p, could not be detected in control or transport-

Figure 8 (facing page). The lumenal pH of centrally located pre-Golgi structures is acidic. (A–C) NRK cells were incubated for 30 min at 37°C in medium containing the weak base DAMP (50 μM), followed by fixation and double-staining for p58 (A) and DAMP (B). C is the merged image, showing apparent colocalization of the two markers in the perinuclear Golgi region (asterisks). Some colocalization is also seen in a few more peripherally located pre-Golgi structures that can be distinguished as individual elements (arrows). (D–F) To disperse both endocytic and pre-Golgi structures, cells were treated for 2 h with 5 μM nocodazole, incubated in the presence of DAMP, and processed for confocal immunofluorescence microscopy as above. A number of the p58-positive structures colocalize with DAMP in these cells (arrows). The arrowheads indicate pre-Golgi structures that contain very weak DAMP labeling, giving rise to a light green color (instead of yellow) in the merged image. Bar, 10 μm.

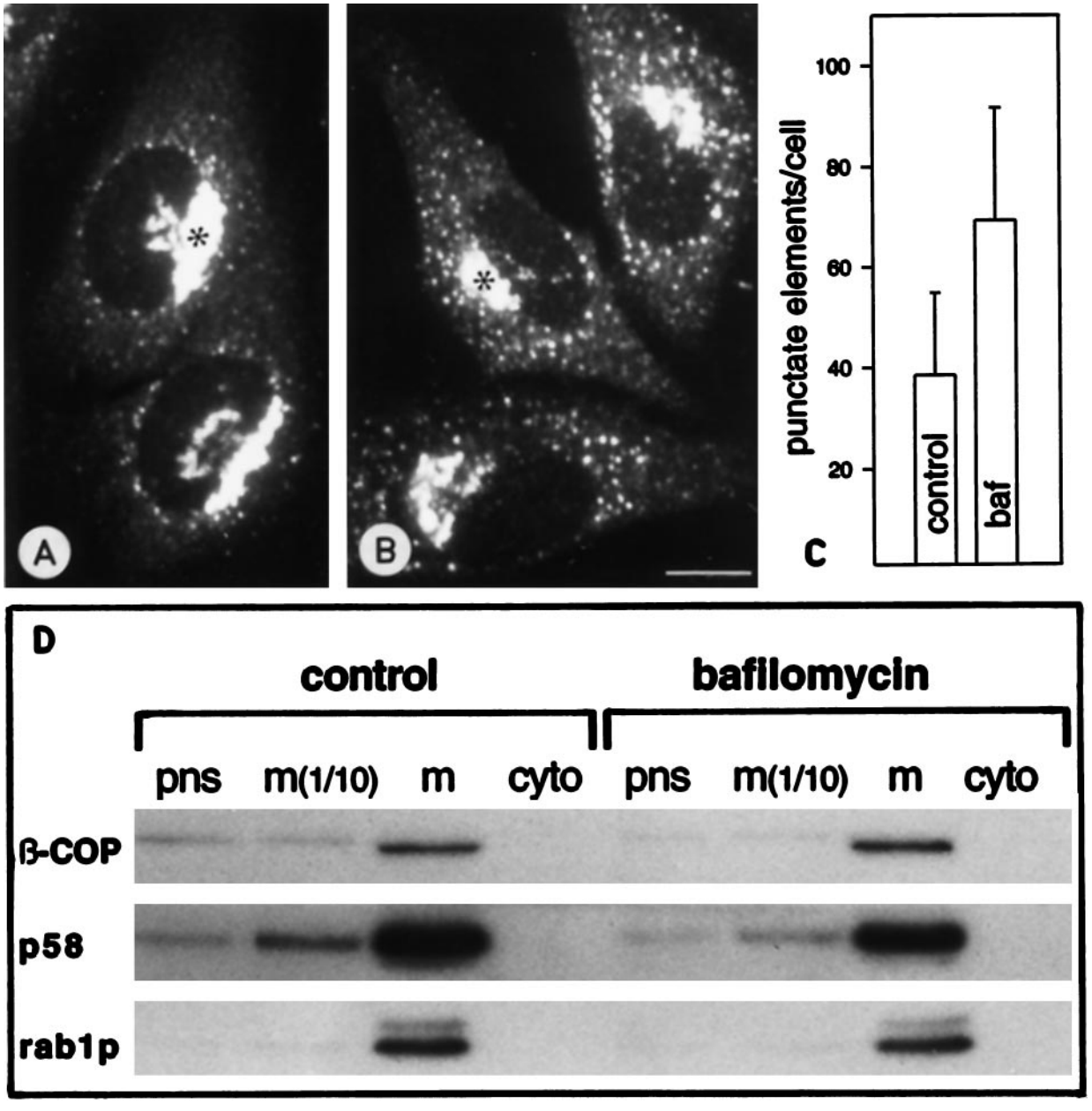

Figure 9. β-COP is redistributed in response to Baf A1 treatment, but its membrane-bound pool is not significantly affected. (A and B) Confocal immunofluorescence images from control (A) and Baf A1-treated NRK cells (B), stained with antibodies against β-COP, demonstrating an increased association of β-COP with peripheral pre-Golgi structures in response to Baf A1. Bar, 10 μm. The quantitation in C shows that Baf A1 causes an approximately twofold increase in the number of detectable pre-Golgi structures. (D) Association of β-COP with membranes. Postnuclear supernatant (pns), total membrane (m), and cytosol (cyto) fractions were prepared from control and Baf A1-treated PC12 cells. Equal amounts of protein from each fraction were run in SDS-PAGE, transferred to nitrocellulose, and analyzed for their content of β-COP, p58, and rab1p by quantitative immunoblotting.

arrested cells stained with antibodies against β-COP, suggesting that coatomer does not bind along the the length of such tubules.

To verify and quantitate the effect of Baf A1 on membrane association of β-COP, total membrane and cytosol fractions were prepared from control and drug-treated cells and subjected to immunoblotting using antibodies against β-COP, p58, and rab1p. Consistently, the drug treatment did not result in any significant change in the amount of membrane-bound β-COP in either PC12 (Figure 9D) or NRK cells (our unpublished results). In conclusion, our results indi-

cate that Baf A1 alters the subcellular distribution of coatomer but does not appear to affect its binding to membranes.

Baf A1 Affects the Formation of p58-containing (COPI) Vesicles

Previous immunoelectron microscopic studies have demonstrated the presence of p58 in pleiomorphic pre-Golgi structures, cisternal elements, and small vesicles in the *cis*-Golgi region (Saraste *et al.*, 1987; Saraste and Svensson, 1991; Lahtinen *et al.*, 1992). The p58-containing vesicles, which are expected to correspond to either coated or uncoated COPI vesicles (Tisdale at al., 1997), could be easily distinguished in rat PC12 cells because of their homogenous size (diameter ~80 nm) and intensive staining, and they were frequently seen to bud from weakly labeled *cis*-Golgi cisternae (Figure 10, A and inset). Treatment of the cells with Baf A1 resulted in dilatation of both *trans*-Golgi and p58-positive *cis*-Golgi cisternae, but the polarized organization of the cisternal stacks was still preserved (Figure 10, B and inset). Interestingly, although there was an apparent accumulation of p58 in the Golgi area, the number of p58-positive ~80 nm vesicles was significantly reduced (Figure 10E). In addition to *cis*-Golgi elements, p58 was localized to large vacuoles and tubular structures in the perinuclear region of the Baf A1-treated cells (Figure 10, C and D).

DISCUSSION

We have used well established marker proteins to examine the effect of Baf A1, a specific inhibitor of V-type H^+-ATPase (Bowman *et al.*, 1988), on bidirectional protein transport and the functional organization membranes at the ER–Golgi interface. We show here that low concentrations of Baf A1 exert distinct effects on the pre-Golgi IC without affecting the polarized organization of the Golgi complex and the distribution of mannosidase II. Moreover, many of the effects of this drug on the IC are similar to those that take place at 15°C, when protein transport and recycling through this compartment are inhibited. Both treatments enhance tubule formation and result in the accumulation of p58/ERGIC-53 as well as increased binding of coatomer to the IC membranes (Saraste and Kuismanen, 1984; Saraste and Svensson, 1991; Schweizer *et al.*, 1990; Duden *et al.*, 1991; Griffiths *et al.*, 1995; Lippincott-Schwartz *et al.*, 1995; Stinchcombe *et al.*, 1995; Tang *et al.*, 1995; present results); however, in addition to its membrane effects, temperature reduction also partially depolymerizes microtubules, which are required for the movement of the pre-Golgi structures (Saraste and Svensson, 1991; Presley *et al.*, 1997; Scales *et al.*, 1997), explaining why forward transport between the ER and Golgi is efficiently inhibited at 15°C (Saraste and Kuismanen, 1984), in contrast to the Baf A1-treated cells (Klionsky and Emr, 1989; Palokangas *et al.*, 1994; our unpublished results).

A Functional H^+-ATPase Is Required for Retrograde Transport

The present results indicate that Baf A1 primarily affects retrograde transport processes at the ER–Golgi boundary. First, the drug inhibited the transfer of Golgi components to the ER, occurring in cells treated with either BFA (Klausner *et al.*, 1992) or nocodazole (Cole *et al.*, 1996). In both cases the block was incomplete and Baf A1 only affected the kinetics of transport. Accordingly, mannosidase II was detected in long, Golgi-derived tubules in BFA-treated cells preexposed to Baf A1, similarly as when its redistribution in these cells is slowed down by incubation at 20°C (Lippincott-Schwartz *et al.*, 1990), and with time the protein assumed an ER-like distribution. Because the anterograde and retrograde pathways between the ER and Golgi are functionally connected (Aridor and Balch, 1996; Lewis and Pelham, 1996), the observed, partial inhibition of the latter also can explain the ongoing forward transport that takes place in the presence of Baf A1.

Second, Baf A1 treatment resulted in the accumulation of p58/ERGIC-53, which normally cycles between the ER, IC, and *cis*-Golgi, in peripheral pre-Golgi structures and the Golgi region. Because forward transport, i.e. the centralization of the pre-Golgi structures themselves, is largely unaffected by Baf A1, these results suggest that it inhibits the retrograde transport of the protein to the ER. This conclusion is also supported by our results showing that the number of p58-containing ~80 nm Golgi vesicles was considerably reduced in the drug-treated cells. On the basis of recent data (for review, see Aridor and Balch, 1996), these represent COPI vesicles that operate in the retrieval of the protein from post-ER compartments. Namely, the cytoplasmic tail of p58/ERGIC-53 contains a double-lysine motif (Schindler *et al.*, 1993; Lahtinen *et al.*, 1996) that binds coatomer (Kappeler *et al.*, 1997; Tisdale *et al.*, 1997), and antibodies against the tail have been reported to inhibit both coatomer binding and the recycling of p58 from post-ER compartments (Tisdale *et al.*, 1997). Because p58/ERGIC-53 is an abundant membrane protein (Lahtinen *et al.*, 1992), its accumulation in the peripheral pre-Golgi structures also provides one possible explanation for the observed redistribution of coatomer in Baf A1-treated cells.

Our results suggest that Baf A1 inhibits retrograde transport at the ER–Golgi boundary by affecting the function of the COPI coats. The drug did not inhibit the association of β-COP (coatomer) with membranes as such but resulted in its redistribution because of

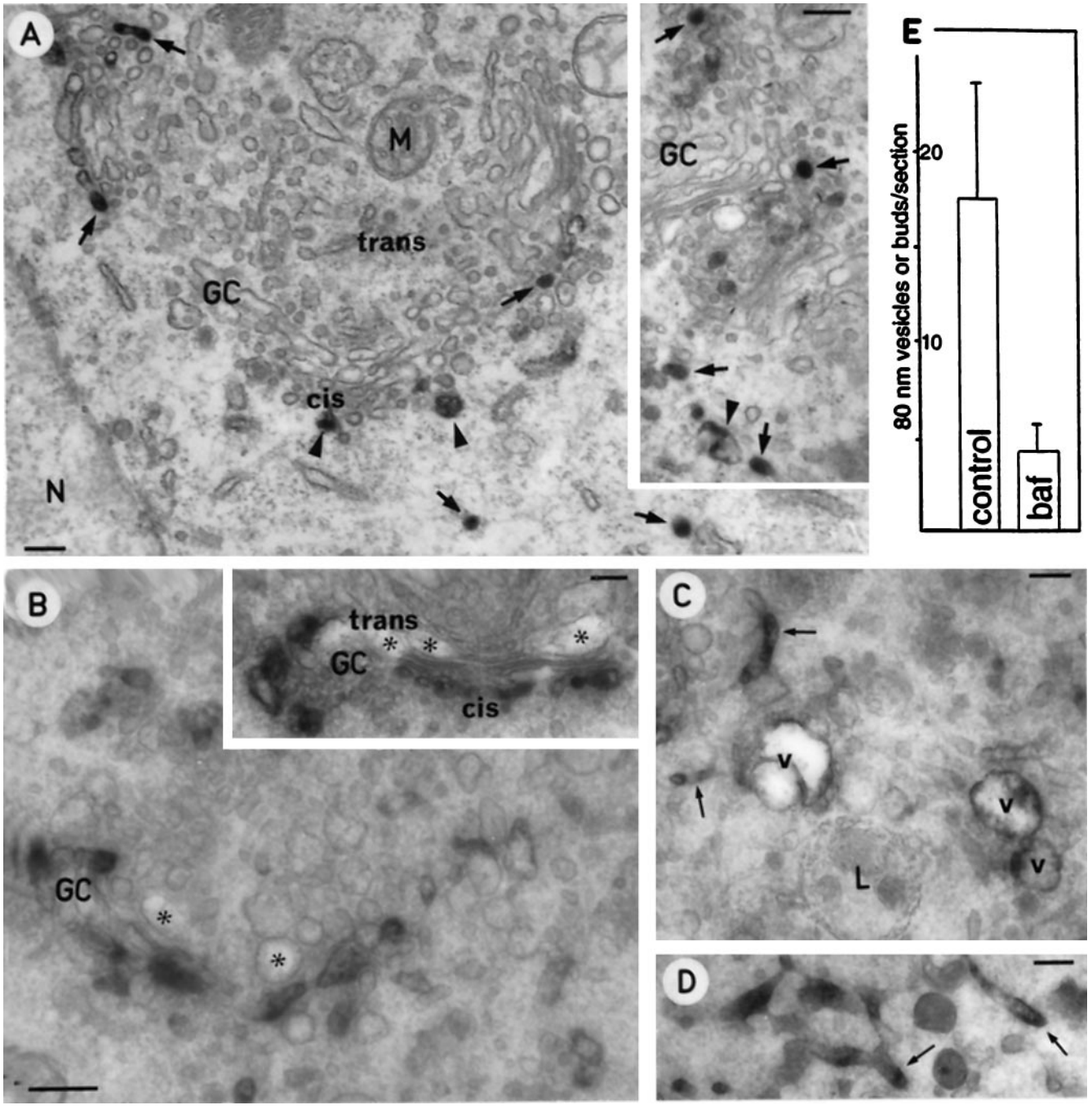

Figure 10. Baf A1 inhibits the formation of p58-containing 80 nm (COPI) vesicles. Control and Baf A1-treated PC12 cells were stained with antibodies against p58 and processed for immunoperoxidase electron microscopy. (A and inset) Control cells showing the presence of p58 in both cisternal and pleiomorphic elements (arrowheads) and a number of ~80 nm vesicles or buds (arrows) at the *cis* face of the Golgi complex (GC). (B–D) In Baf A1-treated cells the cisternal organization and polarity of the Golgi are maintained, but both *trans*-elements (asterisks) and p58-positive *cis*-elements appear dilated. In spite of the more intensive staining of the *cis*-Golgi cisternae (B and inset), the p58-positive 80 nm vesicles are largely absent. C and D show p58-positive vacuoles (v) and tubules (arrows) observed in the drug-treated cells. (E) Quantitation showing that Baf A1 considerably reduces the number of p58-positive 80 nm vesicles and buds. A (inset) and B (inset)–D correspond to ~50 nm and ~200 nm sections, respectively. N, Nucleus; M, mitochondria. Bars, 0.2 μm.

apparent, premature binding to peripheral pre-Golgi structures. In addition, it interfered with the formation of p58-containing Golgi (COPI) vesicles. Regarding the latter effect, the proton pump could be involved in

creating the lumenal conditions required for the detachment of vesicles (or short tubules) (see also Sciaky *et al.*, 1997), explaining the observed accumulation of the long pre-Golgi tubules in the drug-treated cells. Furthermore, the coats could remain bound to the tips of the tubules and inhibit their fusion with the ER membrane.

The Role of Pre-Golgi Tubules

Previous studies of BFA-treated cells have demonstrated the function of tubular intermediates in retrograde transport and fusion of Golgi membranes with the ER (Lippincott-Schwartz *et al.*, 1990). BFA induces the dissociation of COPI coats (Donaldson *et al.*, 1990), which is a prerequisite for the observed organelle fusion (Scheel *et al.*, 1997), whereas the release of these coats, and loss of Golgi identity, does not take place in Baf A1-treated cells, and accumulation of pre-Golgi tubules is observed under conditions in which retrograde transport, rather than being induced, is partly inhibited. Thus, together with recent studies on living cells (Presley *et al.*, 1997; Sciaky *et al.*, 1997), the present results on the localization of endogenous marker proteins support the idea that the pre-Golgi tubules are authentic transport intermediates (Klausner *et al.*, 1992).

The pre-Golgi tubules in the drug-treated cells contained the recycling protein p58/ERGIC-53 but were mostly devoid of anterogradely transported proteins, indicating that they participate in retrograde transport. The weak labeling of some of the tubules in tsO45-infected, Baf A1-treated cells shifted to 32°C, where anterograde transport of the G protein to the Golgi took place efficiently (Figure 7G), and could be due to those mutant G proteins that remain incompletely folded and are therefore recycled to the ER (Hammond and Helenius, 1994). Similar considerations could explain the localization of tsO45 G protein–green fluorescent protein chimeras in tubular domains of the pre-Golgi structures in cells expressing high amounts of the fusion protein (Presley *et al.*, 1997). In addition, the appearance of the mutant G protein in pre-Golgi tubules has been observed in cells microinjected with antibodies against β-COP (Pepperkok *et al.*, 1993), which block both anterograde and retrograde transport at the ER–Golgi boundary (Scheel *et al.*, 1997).

Interestingly, the pre-Golgi tubules could be visualized best in both control and transport-arrested cells using antibodies detecting the A and B isoforms of the small GTP-binding protein rab1p. Previous studies, e.g. using mutant proteins, have demonstrated that these rab1 isoforms are part of the transport machinery operating between the ER and the Golgi complex (for review, see Nuoffer and Balch, 1994), but their precise functions remain unknown. The present results, which demonstrate the association of rab1p with the tubular domains of the pre-Golgi structures, raise the possibility that this protein regulates the movement of these elements along microtubules and thus could functionally resemble rab6p, another small GTPase, which has recently been shown to interact with a kinesin-like protein within the Golgi complex (Echard *et al.*, 1998).

Confocal fluorescence microscopy revealed the that the IC assumed a reticular shape in Baf A1- and 15°C-treated cells, indicating that the pre-Golgi structures are capable of homotypic fusion. Moreover, in Baf A1-treated cells, the tubules extending from the Golgi region apparently fused with the globular domains of the peripheral pre-Golgi structures, giving rise to the observed organization with branchpoints (Figure 5). In conclusion, the present results further emphasize the structural similarity between the pre-Golgi IC and early endosomes (Hopkins *et al.*, 1990; Tooze and Hollinshead, 1991) and suggest that the transit of proteins through the pre-Golgi structures, as in endosomes (for review, see Mukherjee *et al.*, 1997), involves their segregation between different membrane domains.

Pre-Golgi Acidification

Baf A1 binds to the the membrane-spanning sector of the vacuolar H^+-ATPase that harbors the proton-conducting activity (for review, see Stevens and Forgac, 1997). On the basis of recent studies of both mammalian and yeast cells, the assembly of this V_0 domain, as well as the peripheral V_1 domain, takes place in the early secretory pathway, most likely in the ER (Myers and Forgac, 1993; Graham *et al.*, 1998), and the present results further support the idea that the pre-Golgi IC already contains a fully assembled, functional H^+-ATPase. At steady state, DAMP was detected in predominantly centrally located p58-positive elements, indicating that their lumen is sufficiently acidic to result in the accumulation of the weak base but most likely does not have a pH below 6.0 (Boulay *et al.*, 1987); however, Baf A1 also affected the peripheral pre-Golgi structures, as judged by their increased tubulation and the accumulation of p58/ERGIC-53 and β-COP. It is possible that an active H^+-ATPase in these peripheral elements, instead of resulting in detectable acidification generates a membrane potential (for reviews, see Mellman *et al.*, 1986; Stevens and Forgac, 1997) that regulates, e.g. the oligomerization of proteins such as p58/ERGIC-53 and the association of cytoplasmic coats. As the pre-Golgi structures translocate toward the central Golgi region and change membrane properties, their internal pH becomes more acidic, resulting in efficient binding of the COPI coats. Accordingly, the pre-Golgi structures in this respect could also resemble endosomes, whose lumenal pH gradually decreases.

Recently, Aniento and coworkers (1996) showed that Baf A1 inhibits the association of certain COPI isoforms to the cytoplasmic surface of early endosomes. In contrast, and in agreement with the present results, these authors reported that in the same in vitro conditions the drug did not affect the binding of coatomer as such to biosynthetic membranes (Aniento *et al.*, 1996); however, although the detailed mechanisms (involving e.g. kinetics of acidification and pump density as well as other membrane properties) that regulate the binding of the different types of coat complexes to endosomal and pre-Golgi membranes appear to differ (and remain the subject of further studies), the present results suggest a common principle: the requirement for a functional H^+-ATPase.

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