A Rab1 Mutant Affecting Guanine Nucleotide Exchange Promotes Disassembly of the Golgi Apparatus

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Abstract. The Golgi apparatus is a dynamic organelle whose structure is sensitive to vesicular traffic and to cell cycle control. We have examined the potential role for rabla, a GTPase previously associated with ER to Golgi and intra-Golgi transport, in the formation and maintenance of Golgi structure. Bacterially expressed, recombinant rabla protein was microinjected into rat embryonic fibroblasts, followed by analysis of Golgi morphology by fluorescence and electron microscopy. Three recombinant proteins were tested: wild-type rab, mutant rabla(S25N), a constitutively GDP-bound form (Nuoffer, C., H. W. Davidson, J. Matteson, J. Meinkoth, and W. E. Balch, 1994. *J. Cell Biol.* 125: 225–237), and mutant rabla(N124I) defective in guanine nucleotide binding. Microinjection of wild-type rabla protein or a variety of negative controls (injection buffer alone or activated ras protein) did not affect the appearance of the Golgi, as visualized by immunofluorescence of α-mannosidase II (Man II), used as a Golgi marker. In contrast, microinjection of the mutant forms promoted the disassembly of the Golgi stacks into dispersed vesicular structures visualized by immunofluorescence. When S25N-injected cells were analyzed by EM after immunoperoxidase labeling, Man II was found in isolated ministacks and large vesicular elements that were often surrounded by numerous smaller unlabeled vesicles resembling carrier vesicles. Golgi disassembly caused by rabla mutants differs from BFA-induced disruption, since β-COP remains membrane associated, and Man II does not redistribute to the ER. BFA can still cause these residual Golgi elements to fuse and disperse, albeit at a slower rate. Moreover, BFA recovery is incomplete in the presence of rab1 mutants or GTPγS. We conclude that GTP exchange and hydrolysis by GTPases, specifically rabla, are required to form and maintain normal Golgi stacks. The similarity of Golgi disassembly seen with rabla mutants to that occurring during mitosis, may point to a molecular basis involving rabla for fragmentation of the Golgi apparatus during cell division.

THE Golgi apparatus is an exceptionally dynamic organelle which at any one time contains abundant newly synthesized membrane and secretory proteins en route to the cell surface and to other cellular organelles (23). In most cells, the Golgi apparatus resembles a stack of flattened discs that form an extensive intercalated network. This organization is partially dismantled in cells which are treated with microtubule-depolymerizing agents such as nocodozol, colchicine, or vinblastine (33) and is disassembled to vesicular elements when cells enter mitosis (60). Rapid disassembly of the Golgi apparatus is also seen after cells are exposed to the lipophilic fungal toxin, brefeldin A (BFA).[1] In many cell types, BFA causes the fusion of so-called "homotypic" membranes which is assumed to be due to a loss of vesicular coat proteins (32, 64). The latter include the coatomer protein β-COP implicated in ER to Golgi as well as intra-Golgi trafficking (21) and γ-adaptin and clathrin (47), involved in traffic from the *trans*-Golgi network to endosomes and/or lysosomes.

It is now recognized that the rab and ARF family of GTPases regulate intracellular trafficking through both the exocytic and endocytic pathways. In particular, the rab family of proteins has now grown to over 25 members (39). A combination of genetic, biochemical, and immunolocalization evidence suggests that these small GTPases mediate

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1. *Abbreviations used in this paper*: BFA, brefeldin A; GEP, guanine nucleotide exchange protein; Man II, α-mannosidase II; NRK, normal rat kidney; RT, room temperature; VSV-G, vesicular stomatitus virus glycoprotein.

different aspects of vesicle budding, targeting, and/or fusion at specific sites in the cell's complex "highway" system (4, 26, 46).

Analysis of the function of members of the rab family has benefited from the extensive mutational studies of cellular ras which identified point mutations that limit the ability of mutated *ras* protein to assume different conformations brought about by exchange of GTP for GDP, or those that lower the intrinsic rate of hydrolysis of GTP which returns a protein to a GDP-bound state (6). Rab proteins are proposed to confer directionality and/or specificity to transport vesicle function through their role as molecular switches to control the vectorial interaction of components involved in budding, targeting, and fusion. The necessity for cycling of rab and other GTPases involved in vesicular traffic is underscored by the sensitivity of many vesicular transport steps to the introduction of nonhydrolyzable analogues of GTP into cell-free or permeabilized cell transport assays (8, 41). In particular, mutational analysis of closely related rabla and rablb proteins (14, 45) and ARF1 (13) strongly implicate them in control of trafficking events from the ER to the Golgi apparatus and between early Golgi compartments (14, 42, 45).

To understand the role of the Golgi complex in the secretory pathway it is important to define the relationships between its structure and vesicular traffic. In this study we explore the possibility that rabla protein is not only actively involved in anterograde vesicular traffic, but also is critical for maintaining the structural integrity of the Golgi apparatus. This work follows the observations that BFA may inhibit the guanine nucleotide exchange protein that is critical for recruitment and/or maintenance of ARF on Golgi membranes (17, 28), and that GTPγS (which targets all guanine nucleotide binding proteins) and AlF_4^- (believed to target heterotrimeric G proteins [31]) interfere with Golgi apparatus disassembly after exposure to BFA (18, 34, 53).

To avoid the use of these general reagents, we take a more direct molecular approach using mutants of the rabl protein defective in guanine nucleotide exchange to explore the specific role of rabl in this process. We find that microinjection of selected mutant rabl proteins directly into living cells triggers the complete disassembly of the Golgi stack without a BFA-like collapse into the ER. Thus we observed that extensive vesicle budding occurs in the presence of rabl mutants with an apparent absence of downstream fusion. Nevertheless, distinctive Golgi elements persist, suggesting they represent a possible template for assembly of the Golgi stack. Our findings indicate that normal function of rabl in control of vesicular traffic is essential for the integrity of the Golgi stack. Furthermore, multiple interactive GTPases are critical for this process since rabl mutants markedly inhibit the disassembly of the Golgi apparatus by BFA. The relationship of these results to the molecular basis for fragmentation of the Golgi apparatus during mitosis is discussed.

Materials and Methods

Cell Culture

REF-52 cells were cultured in DME (University of California at San Diego [UCSD] core facility, San Diego, CA) supplemented with 10% fetal bovine serum (GIBCO BRL, Gaithersburg, MD), 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin sulfate (100 μg/ml). Cells were passaged twice per week, using brief trypsin digestion (ATV solution; UCSD core facility), and were maintained at 37°C in a 95% air, 5% CO_2 atmosphere. For microinjection experiments, cells were grown for 2–3 d on 12-mm circular glass coverslips (Fisher, Pittsburgh, PA) or in 35-mm plastic dishes (Costar Corp., Cambridge, MA). Both slips and dishes were scored with a diamond knife to mark a small circular or rectangular area for injection.

Immunoreagents

The specificity and characteristics of rabbit antisera to the catalytic domain of the Golgi-specific enzyme, α mannosidase II (Man II), were previously described (57). Mouse Man II antibodies were prepared from ascites induced with $5CF_3$ hybridoma cells as described by Burke et al. (12). Rabbit antisera to a peptide derived from β-COP was also prepared in this laboratory by Dr. Linda C. Hendricks using as immunogen the original EAGE sequence reported by Duden et al. (21), synthesized as a multiple antigenic peptide (52) (UCSD Center for Molecular Genetics Core Facility). Secondary antibodies were obtained from the following sources: FITC-conjugated goat anti-rabbit $F(ab')_2$ and anti-mouse IgG from Zymed Labs. Inc. (S. San Francisco, CA); Texas red–conjugated donkey anti-rabbit, rhodamine-conjugated goat anti-mouse, and AMCA-conjugated goat anti-guinea pig IgG as well as affinity-purified guinea pig IgG, used as an injection marker, were from Jackson ImmunoResearch Labs. Inc. (West Grove, PA). Fab fragments of goat anti-rabbit IgG conjugated to HRP were from Biosys (Compiégne, France).

Preparation of Recombinant rabl Protein

The purification of histidine-tagged canine rabla, rabla(N124I), and rabla(S25N) protein from *Escherichia coli* cell lysates is described in detail elsewhere (42, 45). For these experiments, rabla wild-type or mutant proteins were concentrated by ultrafiltration to a final concentration of ~0.3–0.5 mg/ml and stored in aliquots at −70°C.

Microinjection

In each experiment, 50–150 cells were injected with GTPγS (0.5–5 mM) or recombinant rabl protein in injection buffer (10 mM sodium phosphate, pH 7.4, 100 mM KCl or 25 mM Hepes-KOH, pH 7.2, 125 mM KOAc). Injection solutions also contained affinity-purified guinea pig IgG (2 mg/ml) or BSA-colloidal gold (1.5 mM) to serve as a marker for injected cells for fluorescence and electron microscopy experiments, respectively. These concentrations were diluted ~10–20-fold by injection into the cell cytoplasm. Colloidal gold concentrations were estimated by absorption at 520 nm (7). In some cases the cells were pretreated with 1.25 μg/ml BFA (Epicentre Technologies, Madison, WI) at 37°C for 30 min before injection. Alternatively, cells were injected and incubated at 37°C for specific intervals, followed by incubation with BFA for the times indicated. Incubations were terminated by immediate addition of fixative for either fluorescence or electron microscopy.

Microscopy

For analysis by immunofluorescence microscopy, cells were fixed for 30 min at room temperature (RT) with 2% paraformaldehyde in 0.075 M phosphate buffer (pH. 7.4), permeabilized for 10 min with 0.01% Triton X-100 in PBS, processed with appropriate primary and fluorophore-tagged secondary antibodies, and mounted in Vectashield medium (Vector Labs, Burlington, CA). The cells were observed and photographed with a 100× (1.3 numerical aperture) oil-immersion lens on a Zeiss Axiophot microscope equipped for fluorescence microscopy (Carl Zeiss, Inc., Thornwood, NY).

For immunoperoxidase labeling, cells were fixed in 2% paraformaldehyde, 75 mM lysine 10 mM periodate fixative for 4 h at RT or 3% formaldehyde, 0.5% glutaraldehyde in 0.1 M phosphate buffer for 1 h at RT. Fixed cells were permeabilized with 0.1% saponin in PBS/1% BSA and sequentially incubated with primary antibodies to Man II; followed by HRP-conjugated secondary antibodies diluted in PBS/1% BSA containing 0.1% saponin. The cells were washed in 0.1 M cacodylate-HCl, pH 7.4, 7.5% sucrose, followed by postfixation with 4% glutaraldehyde in 0.1 M cacodylate-HCl buffer for 1 h, incubation in diaminobenzidine, and postfixation in 0.1 M cacodylate buffer containing 1% reduced OsO_4, 1% KFeCN as described (10). Cells were dehydrated and embedded in epon directly on the plates. The scored areas were then cut from the dish and sectioned on

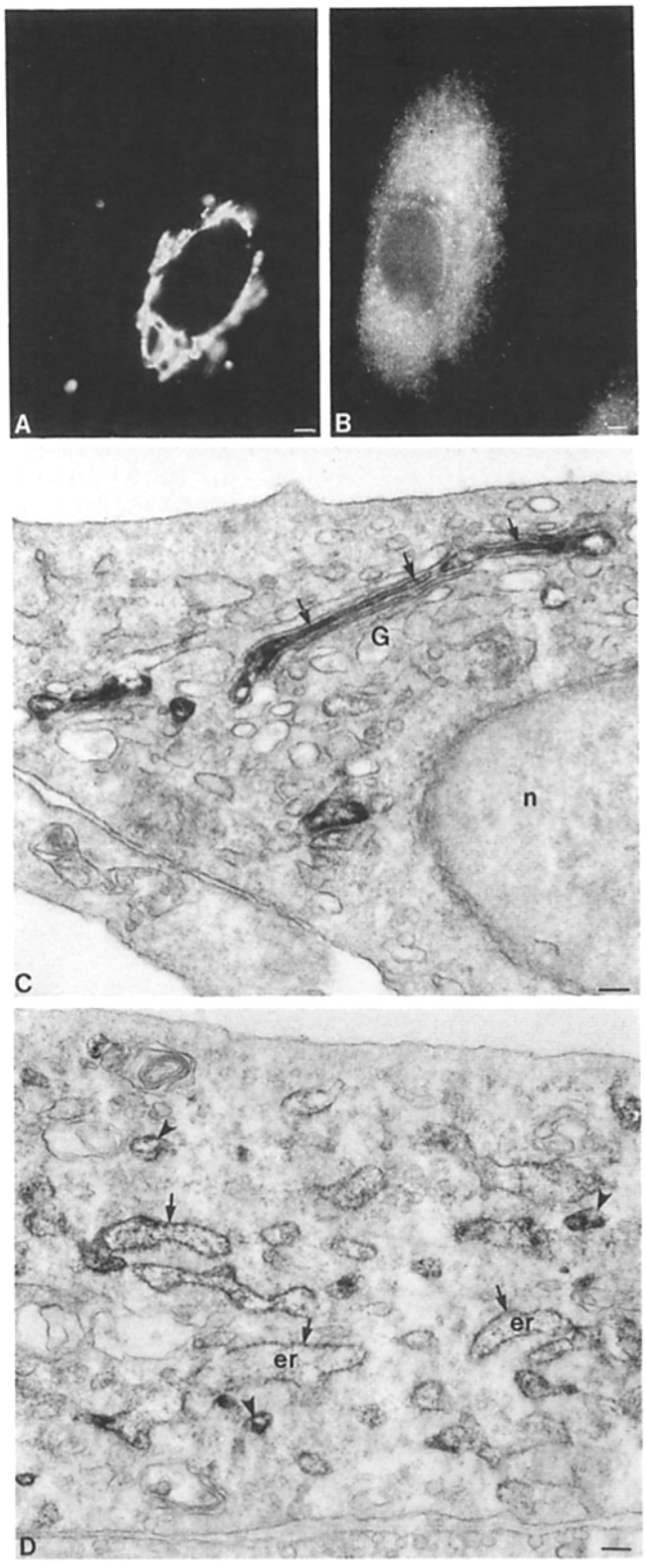


Figure 1. Man II localizes to middle Golgi cisternae in REF-52 cells and redistributes to the ER after BFA. Indirect immunofluorescence of Man II showing its Golgi localization in normal rat embryonic fibroblasts (*A*) and its dispersal following 30 min exposure to BFA (*B*). By immunoperoxidase, Man II is predominantly found in middle Golgi cisternae (*G*) in untreated cells (*C, arrows*). After 30 min in BFA, Man II is redistributed to the ER (*D, arrows*). Man II is also occasionally detected in a few scattered vesicular structures in BFA-treated cells (*D, arrowheads*). Bars: (*A* and *B*) 4 μm; (*C* and *D*) 0.05 μm.

a Reichert Ultracut E ultramicrotome. Sections were stained with lead nitrate. Micrographs were taken on either a Philips CM 10 (Philips Sci., Mahwah, NJ) or JEOL 1200EX-II electron microscope (JEOL USA, Peabody, MA).

Results

α-Mannosidase II as a Golgi Structural Marker

Previous studies have established that the Golgi enzyme Man II, varies in its distribution across Golgi cisternae and that this distribution is specific to the cell type examined (57). To establish its utility in the current studies as a marker for Golgi integrity, we first determined Man II distribution in REF-52 rat embryonic fibroblasts, the principal target cells for microinjection in this study. REF-52 cells have an elaborate Golgi apparatus, as shown by the fluorescence signal obtained using anti-Man II antibodies (Fig. 1 *A*). When examined by immunoelectron microscopy using HRP-conjugated secondary antibodies and standard staining procedures, we found Man II to be concentrated in the middle one or two cisternae of REF-52 cells (Fig. 1 *C*). It could occasionally be detected in adjacent *cis* and *trans* cisternae (not shown).

Rabla Mutants Alter Golgi Structure in the Absence of BFA

Given the critical role of rabla in ER to Golgi (55) and intra-Golgi (14) transport, we examined its possible role in the organization of the Golgi stack. For these experiments, recombinant wild-type and mutant rabla proteins were isolated from bacterial lysates after expression in *E. coli*. Nucleotide-binding properties of rabla proteins purified in this manner are described elsewhere (42, 45). Two mutants were tested in our microinjection assay system. The first, rabla(S25N), is analogous to H-ras(S17N) which has a preferential affinity for GDP (24, 42) and in the case of H-ras is growth inhibitory due to its sequestration of the ras-specific guanine nucleotide exchange protein (22, 24). The second mutant, rabla(N124I) fails to bind either GDP or GTP at detectable levels in vitro, similar to the analogous transforming H-ras(N116I) mutant (45, 58). These bacterially expressed rab proteins lack posttranslational modifications—such as prenylation, acylation or methylation—which are normally a prerequisite for the functional association of ras-related proteins with membranes (37). The microinjection protocol avoids these problems, since the recombinant proteins are expected to be appropriately modified by endogenous enzymes after microinjection. It has recently been shown that the rabla(S25N) mutant is inhibitory only after prenylation (42).

REF-52 cells were microinjected directly into the cytoplasm with wild-type rabla or one of two mutants, N124I or S25N, at a concentration of 0.3–0.5 mg/ml. Injection solutions were routinely supplemented with 2 mg/ml affinity-purified guinea pig IgG in order to positively identify the injected cells via immunofluorescence microscopy. The cells

were then incubated for up to 1.5 h to allow sufficient time for cellular enzymes to posttranslationally modify the recombinant proteins and to achieve a new steady state distribution, and then fixed and processed for immunofluorescence microscopy to assess effects on Golgi structure. Microinjection of wild-type rabla had no effect upon Golgi structure (not shown). Other controls which had no effect on Golgi structure included injection buffer alone and activated ras protein (data not shown).

In contrast to the lack of effects by microinjection of the wild-type rabla or ras proteins, microinjection of the S25N or N124I mutants caused a dramatic breakup of the Golgi apparatus within 1.5 h after injection. These results are documented in Fig. 2, *A–D*, where photographic pairs show the double staining technique used to unequivocally identify injected cells (Fig. 2, *A* and *C*) and Golgi structure (Fig. 2, *B* and *D*) in injected and noninjected cells. To explore the effect of the GDP-bound S25N mutant in more detail, we examined the Golgi organization in microinjected cells by electron microscopy. Cells to be injected were first targeted within a small circle or rectangle scored on the bottom of a 35-mm plastic dish using a diamond pen. The injection solution was supplemented with 1.5 mM BSA-colloidal gold (12 nm) to serve as a marker for injected cells in ultrathin sections (25). After fixation and processing for immunoperoxidase localization of Man II, the scored region was excised from the dish, embedded, and sectioned for transmission electron microscopy. In some cases, the colloidal gold was omitted, but all cells within the scored area of the dish were injected to ensure that sections through the region contained predominantly injected cells. At the electron microscope level, the dispersed Golgi resembled dilated ministacks, short, isolated cisternal elements, or large (200–300 nm) vesicles (Fig. 3, *A–D*). In REF-52 cells these altered Golgi structures were often clustered and stained heavily with peroxidase reaction product, indicating the presence of the Golgi enzyme Man II. Numerous smaller (40–80 nm) vesicular carriers (Fig. 3 *C*, *V*) which did not stain for the presence of the Golgi enzyme were often in the near vicinity.

The role of these two rabl mutants has been explored extensively in normal rat kidney (NRK) cells where, in the case of the rabla(S25N) mutant, there was a striking correlation

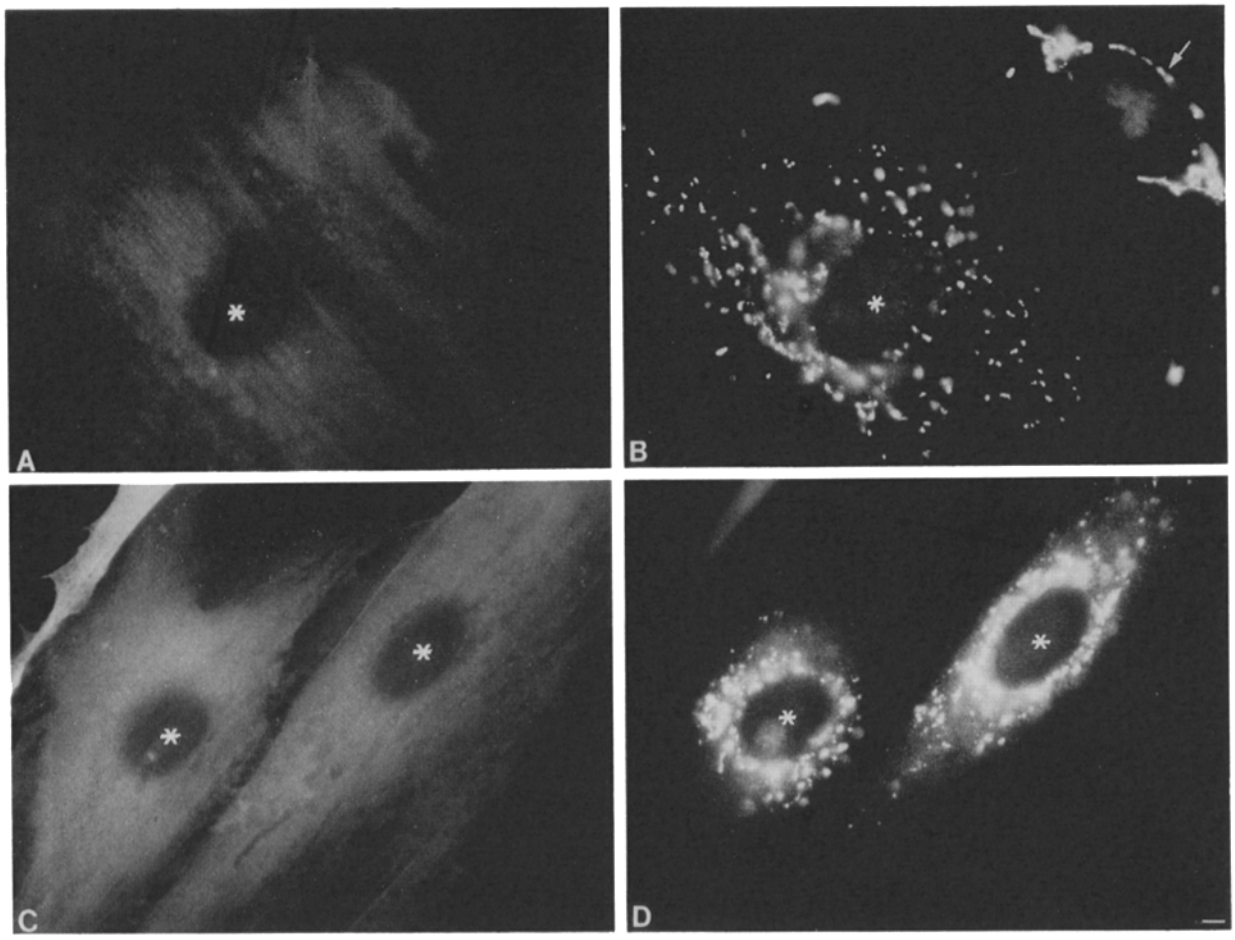


Figure 2. Microinjection of rabla mutants causes vesiculation of the Golgi apparatus. REF-52 cells were injected with recombinant rabla mutants, N124I (*A* and *B*) or S25N (*C* and *D*) and guinea pig IgG, incubated at 37°C for 90 min, and subsequently fixed for double immunofluorescence staining. Coverslips were processed with polyclonal anti-Man II antiserum (1:800), followed by FITC-conjugated goat anti-rabbit secondary antibody and AMCA-conjugated goat anti-guinea pig IgG. Microinjection of both mutants (*asterisks*) resulted in a redistribution of Man II (*B* and *D*) into many vesicular structures scattered away from the perinuclear region of the cell. A noninjected cell (*B*, *arrow*) shows the normal Golgi appearance. Guinea pig IgG (2 mg/ml) diffuses in the cytosol and serves as a marker for microinjected cells (*A* and *C*). Bars, 4 μm.

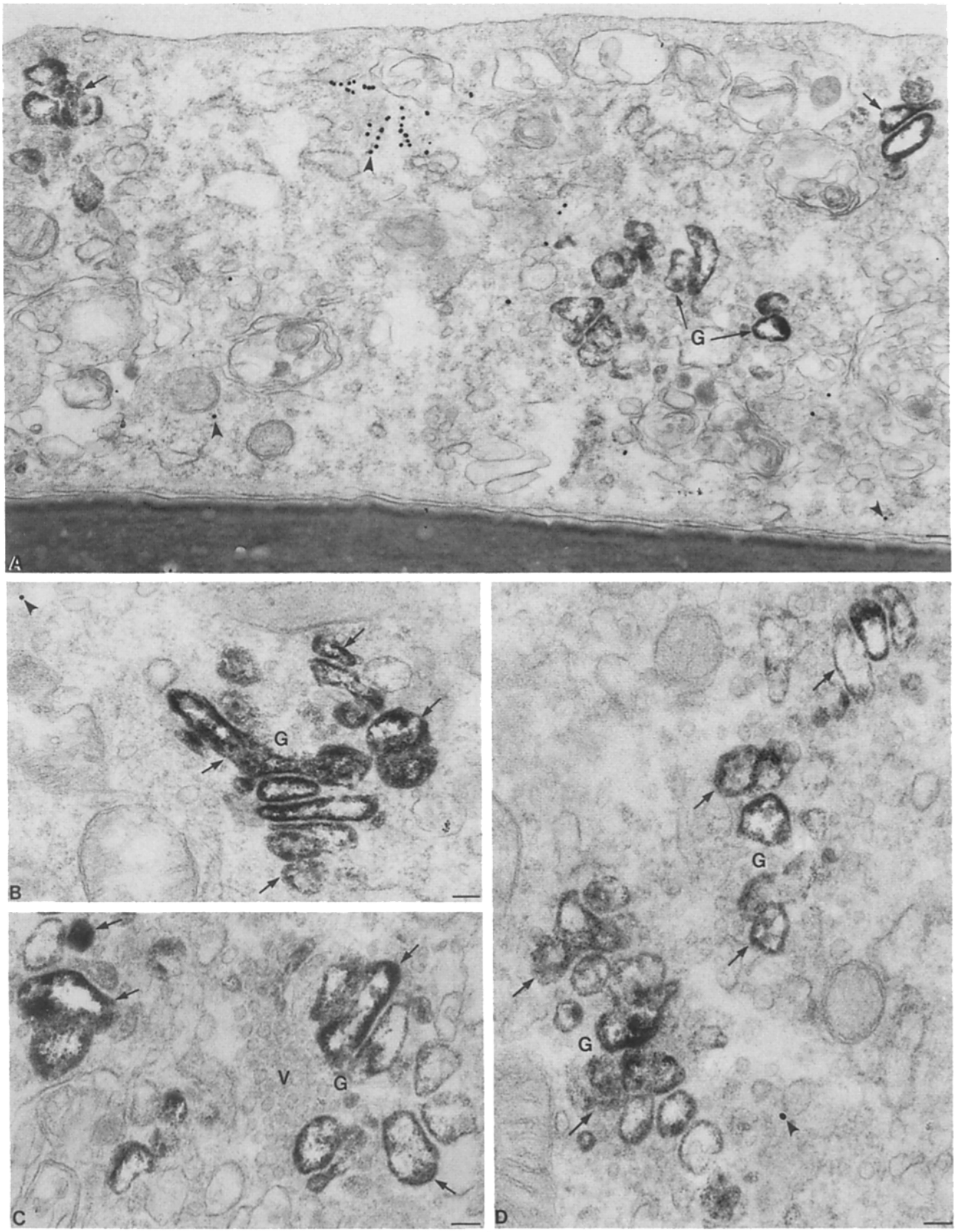


Figure 3. Man II is found in dispersed Golgi elements in rabla(S25N)-injected REF-52 fibroblasts. Injection solutions containing rabla(S25N) were supplemented with 12 nm colloidal gold coupled to BSA (1.5 μM), and injected cells were incubated for 90 min at 37°C before fixation for immunoperoxidase localization of Man II. Man II staining is found in clusters of "Golgi elements" (*G, arrows*) scattered throughout the cytoplasm. Clusters of smaller (40–80 nm), unstained vesicles can also be seen in close proximity to some of the Golgi elements (*V* in *C*). Refer to Fig. 1 *C* for the normal Golgi appearance and Man II distribution in REF-52 cells. Arrowheads point to colloidal gold used as a marker for injected cells. Bar, 0.1 μm.

between loss of transport function and apparent disappearance of the Golgi stack by immunofluorescence (42). When NRK cells were examined at the EM level after microinjection of the S25N mutant, the findings were similar to those in REF-52 cells except that we found a more extensive disassembly of the Golgi. Widely dispersed Golgi remnants composed of vesicular structures 50–300 nm in diameter containing Man II peroxidase reaction product (Fig. 4, *A* and *B*, *arrows*) were accompanied by an extensive profusion of small 40–80-nm vesicles, many of which lacked reaction product. The normal distribution of Man II in NRK Golgi is shown for comparison (Fig. 4 *C*). The presence of gold particles confirmed that cells were injected with the mutant rabla(S25N). Disruption of the Golgi stack was detectable within 30 min after injection of mutant rabla, and it persisted up to 6 h. This is in contrast to REF-52 cells in which 50%

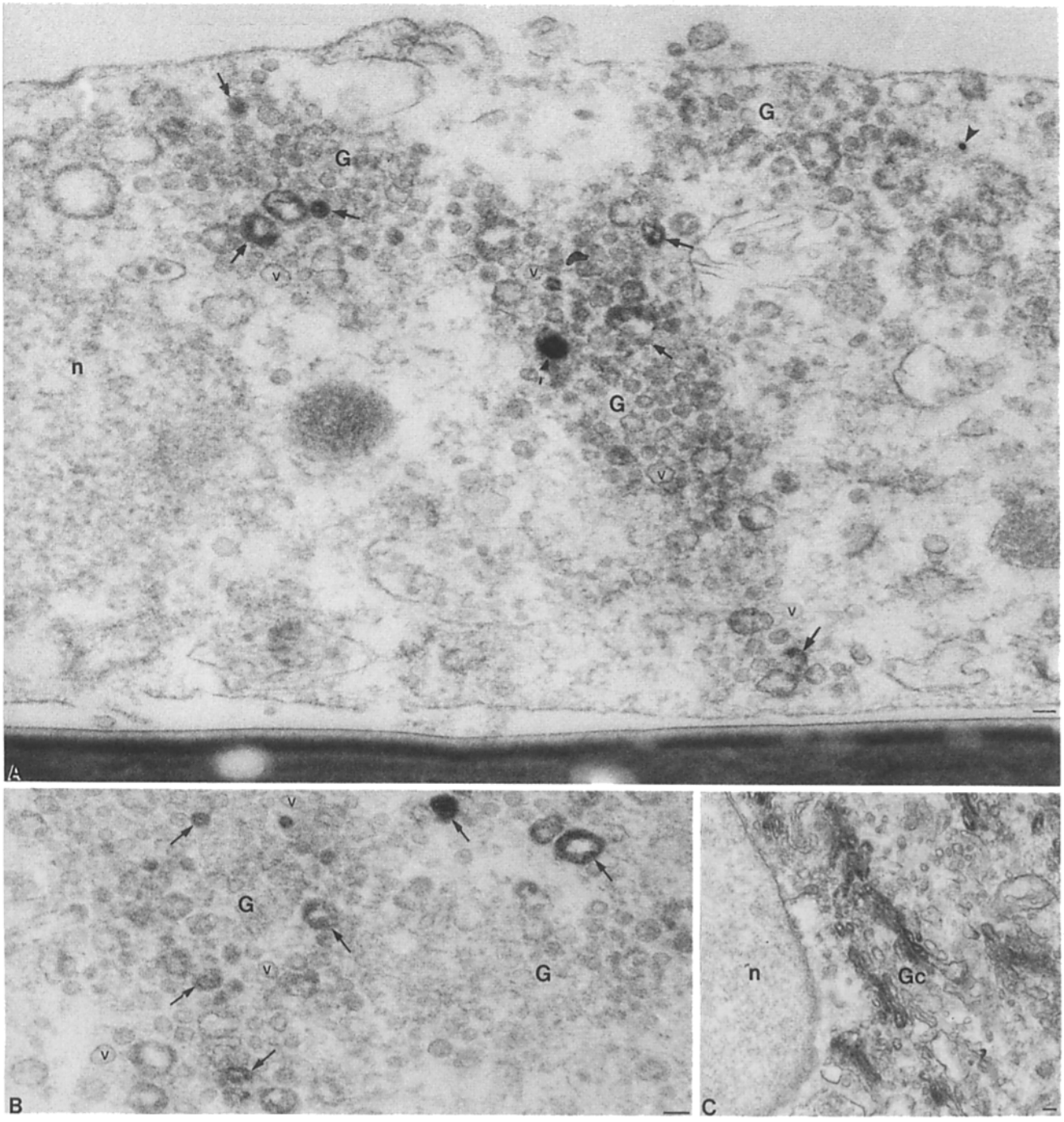


Figure 4. Dispersal of Golgi apparatus in rabla(S25N)-injected NRK cells (prepared as in Fig. 3). (*A* and *B*) At 1.5 h after S25N injection into NRK cells Man II-containing structures (*arrows*) were typically smaller (50–100 nm in diameter) than in REF-52 cells and were not recognizably stacked (*G*, *arrows*). A large number of vesicles resembling carrier vesicles (*V*) with little or no peroxidase reaction product were consistently seen in the vicinity. (*C*) Normal Golgi distribution of Man II in noninjected NRK cells is shown for comparison. *Gc*, Golgi complex; *n*, nucleus. Bars, 0.1 μm.

of the microinjected cells began to recover normal Golgi structure by 3 h after injection. The difference in sensitivity of the two cell types is probably due to the fact that NRK cells have ~1/10 the volume of REF-52 cells. Thus, the rate and extent of disassembly appears to be proportional to the fractional ratio of the injected rabla(S25N) mutant relative to the endogenous rabl pool. This is consistent with the competitive nature of its inhibition as demonstrated in experiments where the Golgi was protected when equal amounts of wild-type and mutant rab were injected (not shown). Alternatively, it could be explained by differences in the degradation rates of mutant protein to endogenous rab. In no case could the Man II peroxidase reaction product be detected in the ER of either cell line, indicating that the mutant protein was not inducing a BFA-like phenotype. This is consistent with the finding that newly synthesized VSV-G protein fails to be processed to Golgi forms in the presence of the S25N mutant (42).

Both the Dispersal of the Golgi with BFA and Its Reassembly after BFA Washout Are GTP-dependent Processes

To compare the disassembly of the Golgi induced by rabl mutants to that caused by BFA, a putative inhibitor of an ARF-specific GDP exchange protein (GEP) that triggers collapse of Golgi elements to the ER (17, 28), we examined the effects of BFA on Golgi integrity in REF-52 cells. As described for many (but not all) cell types (30, 32, 56), the application of BFA (1.25 μg/ml) to REF-52 cells results in rapid dispersal of Golgi membranes and redistribution of Golgi resident enzymes to isolated vesicles and to the ER. This process is complete within 20–30 min after the addition of BFA, as visualized by a nuclear envelope and diffuse cytoplasmic immunofluorescence signal for Man II (Fig. 1 *B*). This was also documented by the detection of Man II–immunoperoxidase reaction product in the rough ER and vesicular structures by immunoelectron microscopy (Fig. 1 *D*). The effects of BFA are rapidly reversible, and the Golgi completely reassembles within 1 h after addition of BFA-free media (see below).

While the disassembly of BFA-treated Golgi is inhibited by the nonhydrolyzable guanine nucleotide analog GTPγS in permeabilized cells (20, 53), this has not been demonstrated in vivo. To determine the extent to which GTPases control BFA-induced Golgi disassembly and reassembly, we introduced GTPγS into the cytoplasm of REF-52 cells via microinjection. Injection of GTPγS in the absence of BFA failed to dramatically alter the Golgi apparatus, as visualized 30 min after injection by immunofluorescence staining for Man II (not shown). However, microinjection of GTPγS 10–15 min before the addition of BFA to the media inhibited the BFA-induced dispersal of the Golgi (data not shown). Effective concentrations of GTPγS in the injection pipette were 0.5–5 mM. Assuming that injection solutions are diluted 10–20-fold in the cell cytoplasm (43) this indicates that GTPγS need not be in large excess over endogenous guanine nucleotide (estimated at 0.1–0.5 mM) to be an effective reagent in the intact cell.

We also found that the recovery of the Golgi apparatus after removal of BFA is profoundly affected by the presence of GTPγS. For these experiments, REF-52 cells were pretreated at 37°C with BFA for 30 min and subsequently injected with 0.5 mM GTPγS. After a short (10–15 min) incubation at 37°C, the cells were washed free of BFA and incubated for 1 h to allow Golgi apparatus reassembly. In control cells (outside the injection circle) the Golgi apparatus reformed to its normal appearance, as seen by the fluorescence signal using anti-Man II antibodies. However, in GTPγS-injected cells Man II was dispersed in a dotlike pattern (not shown) suggesting that this Golgi enzyme is arrested in vesicular intermediates that exit the ER, but fail to organize into typical Golgi cisternae. From these results we conclude that both disassembly and reassembly of the Golgi complex following BFA treatment are dependent upon GTPases.

Golgi Elements Dispersed by the rabla(S25N) Have a Delayed Response to BFA

Since both rabla mutants and BFA caused disorganization of the Golgi apparatus, we wondered if the two mechanisms share a common or interactive pathway. To answer this question, REF-52 cells were injected with the S25N mutant and incubated for 1.5 h at 37°C to allow for the previously observed dispersal into isolated Golgi elements. We then supplemented the media with BFA and fixed the cells at intervals of 10, 20, and 30 min following exposure to the fungal metabolite. Noninjected cells on the same coverslips served as controls for the BFA effect (shown in Fig. 1, *C* and *D*). At 10 min after BFA addition Golgi dispersal was well advanced in uninjected cells, and thin, tubular Golgi structures could be observed in only a few scattered cells (not shown). By contrast, 10 min after BFA was added to S25N-injected cells, the isolated Golgi elements seemed to coalesce in the perinuclear region with a few fine tubular extensions (Fig. 5 *B*). At 20 and 30 min after BFA, when Golgi apparatus dispersal is complete in noninjected cells, in S25N-injected cells the Golgi marker was found in elaborate tubules or "beads on a string" (Fig. 5 *D*) similar to those previously described by Lippincott-Schwartz et al. (36). In noninjected REF-52 cells, they were seen transiently at much earlier times (5–10 min) after BFA addition. In electron micrographs (Fig. 6, *A–C*) where immunoperoxidase reaction product identifies the presence of the Golgi marker, Man II (57), these tubules appeared to form by fusion of isolated Golgi elements. After BFA addition, they expanded dramatically over 20 min (Fig. 6, *D* and *F*). Man II staining was also detected in large vesicular structures that were often far removed from the perinuclear area and likely to correspond to "beaded" vesicular carriers or tubules which cross through the plane of section (Fig. 6 *E*). These results show that Golgi elements, previously dispersed by injection of mutant rabla(S25N), can still fuse and begin redistribution to the ER in response to BFA. However, this process is greatly delayed since Man II reaction product could not be detected in the ER as late as 30 min after the addition of BFA.

Microinjection of S25N Mutant Alters Reorganization of the Golgi Apparatus Following Recovery from BFA

It was recently shown that the exit of newly synthesized viral protein out of the ER is blocked in the presence of the GDP-bound form of rabla, allowing only partial maturation of VSV-G from the ER form to pre-Golgi intermediates (42). We wondered if S25N would similarly block the movement of Golgi Man II from the ER (induced by exposure to BFA)

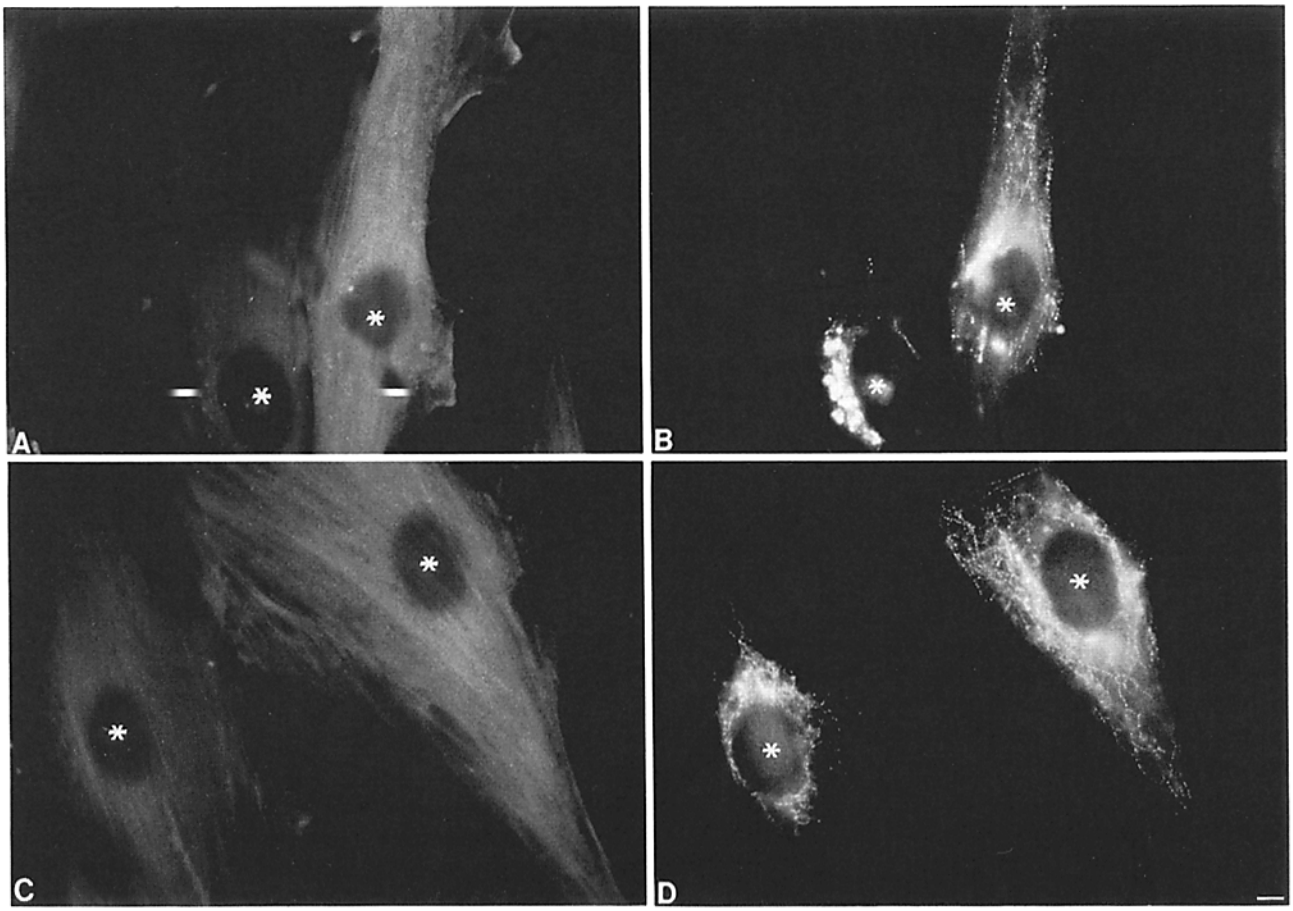


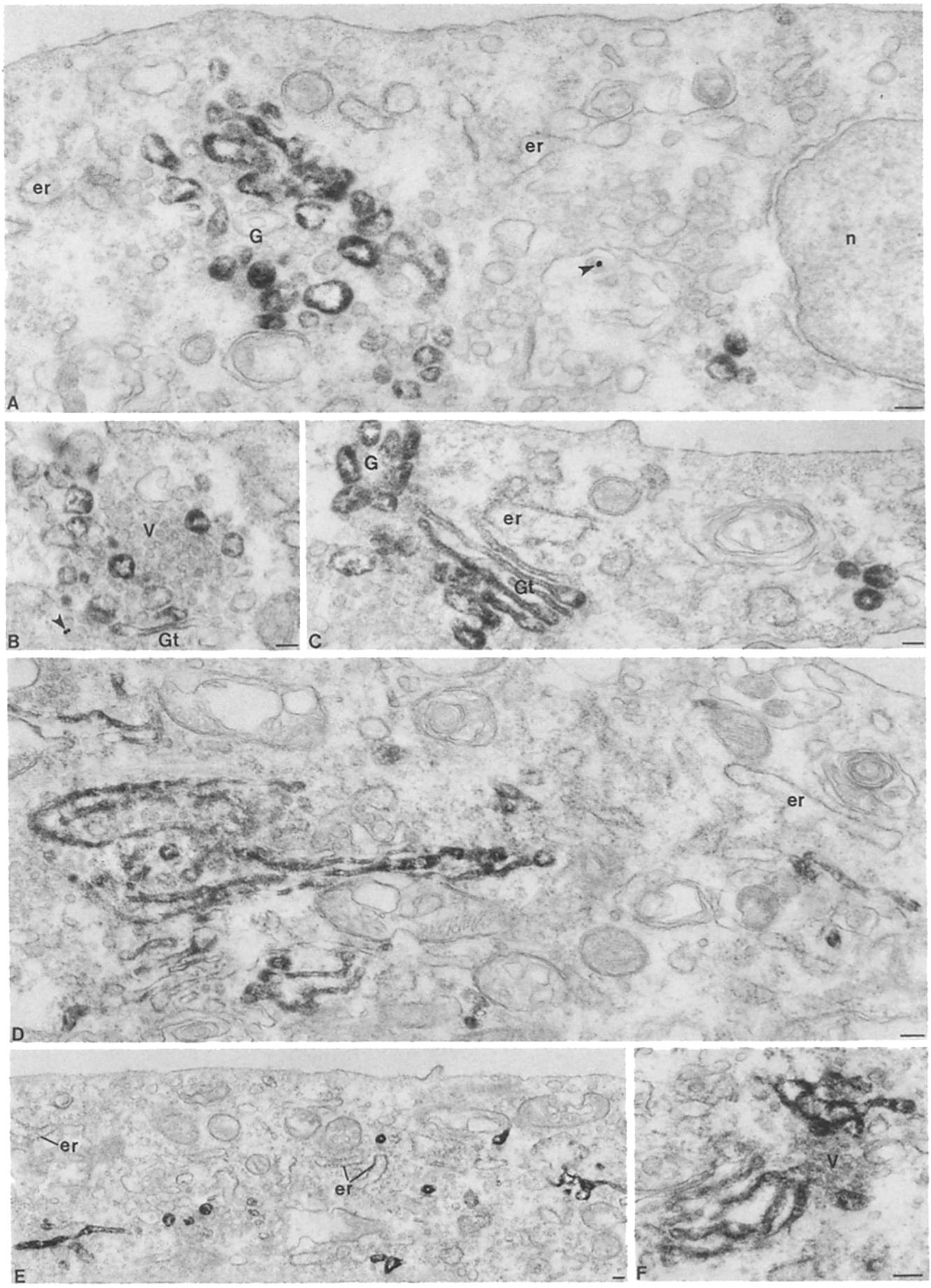
Figure 5. Golgi elements in S25N-injected fibroblasts remain responsive to BFA but tubulation and redistribution is slowed. REF-52 cells were injected with S25N rab mutant and incubated for 1.5 h at 37°C. BFA was then added and the cells fixed 10 or 20 min later and processed for immunofluorescence detection of guinea pig IgG (*A* and *C*) or Man II (*B* and *D*). All four cells in the figure (*A* and *C*, *asterisks*) stain positive for guinea pig IgG, used as marker for injected cells. (*A* and *B*) 10 min after BFA, Golgi elements coalesce and begin to extend fine tubules in S25N-injected cells. (*C* and *D*) By 20 min after BFA, Man II staining persists in long, elaborate tubules in the S25N-injected cells (*asterisks*). By contrast, in noninjected cells on the same coverslips Man II had almost completely dispersed after 10 min in BFA, and the process was complete at 20 min (not shown). Bars, 4 μm.

into a recognizable Golgi stack after removal of BFA. To investigate this possibility, cells were first pretreated with BFA for 30 min at 37°C, microinjected with the rabla(S25N) mutant, and then incubated in the continued presence of BFA for 1.5 h. BFA was then washed out, the media replaced and incubation continued for 1 h to allow for Golgi apparatus reassembly. As shown by immunofluorescence (Fig. 7 *B*, *asterisks*), in S25N-injected cells the reorganization of the Golgi apparatus after washout of BFA appeared to be arrested in vesicular structures that were similar to those seen in cells injected with S25N alone (compare to Fig. 2 *D*). This was confirmed by electron microscopy of injected cells (Fig. 7 *C*), where Man II was again found in vesicles and ministacks that lack the typical flattened, stacked appearance of normal Golgi apparatus. Man II was not detected in the ER under these conditions. Similar results (not shown) were seen when N124I-injected cells were examined by immunofluorescence for Man II after BFA washout.

β-COP Codistributes with Man II in S25N-injected Cells

Loss of membrane association of the vesicular coat protein,

Figure 6. Electron micrographs of S25N-injected fibroblasts show a progression of Golgi element fusion and tubulation in response to BFA. (*A–C*) S25N-injected REF-52 cells were processed for immunoperoxidase localization of Man II, 10 min after addition of BFA. Isolated Golgi elements (*G*) are still present, but tubulation and fusion of these elements has begun (*Gt*). Small, unstained vesicles persist near some of the Golgi elements (*V*). Arrowheads in some of the photographs point to colloidal gold particles used to identify injected cells. In some cases, colloidal gold was found elsewhere in the same cell but was not present in the field shown. (*D*) Elaborate tubules are seen in S25N-injected cells processed for immunoperoxidase localization of Man II, 20 min after BFA. (*E*) The injected cell shows Man II localized in isolated tubules and vesicular structures. The latter may correspond to tubules in cross section or to the beaded structures seen by immunofluorescence (refer to 5 *D*). Bars, 0.1 μm.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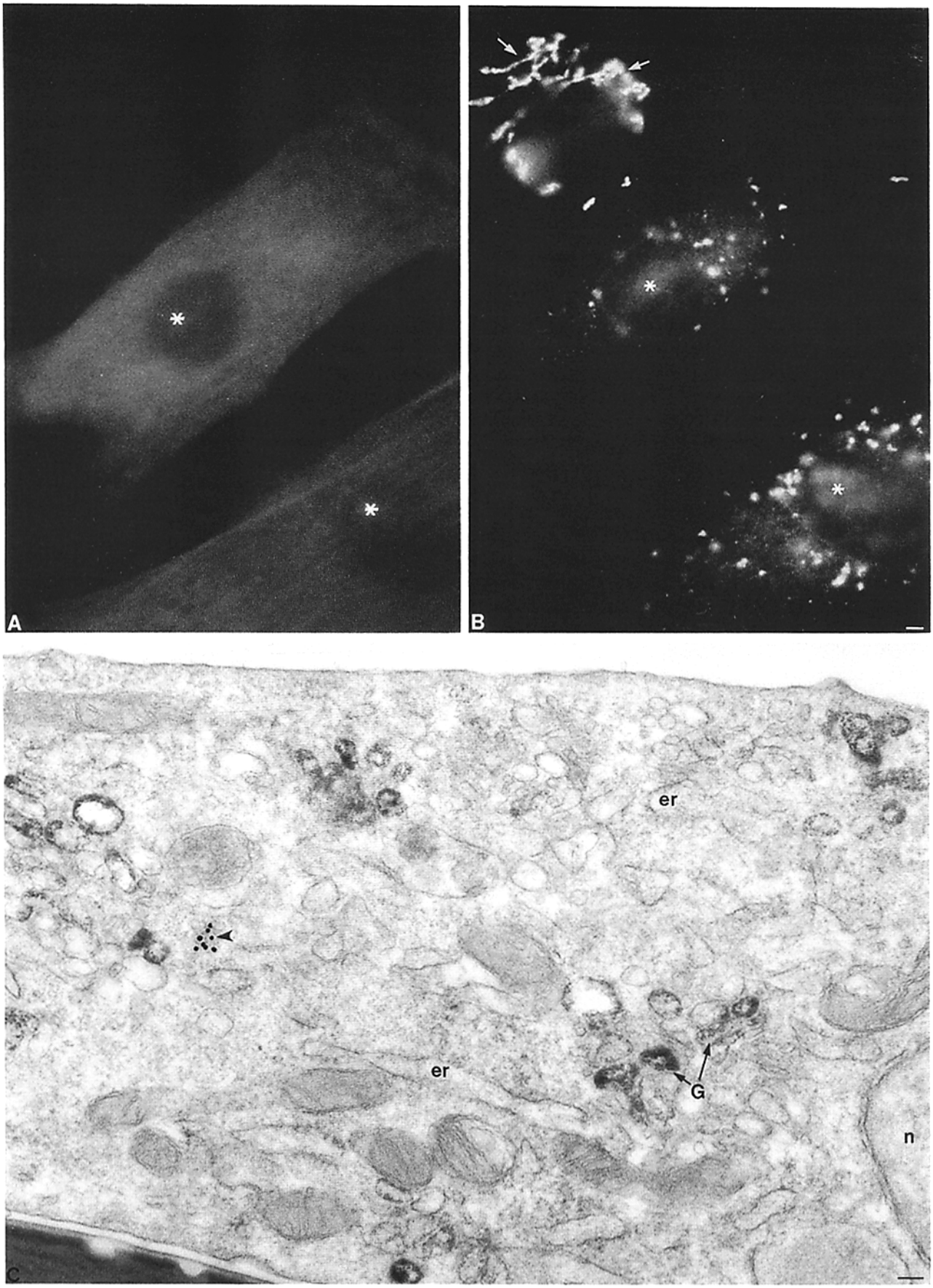
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β-COP, is an exceptionally rapid event preceding the dissolution of the Golgi apparatus with BFA (18, 19, 29). In contrast, we found that the breakup of the Golgi apparatus which occurs after S25N microinjection is not accompanied by loss of β-COP from the dispersed Golgi elements. This finding is illustrated in Fig. 8, *A–C*, where microinjected cells were triple labeled for guinea pig IgG to mark injected cells, for β-COP (using rabbit IgG 2881), and for Man II (using a mouse monoclonal antibody, 5CFC3 [12]). In these micrographs, the fluorescence patterns for β-COP and Man II are nearly identical. Interestingly, we also found that the time course of β-COP loss from Golgi membranes in response to BFA was significantly retarded in S25N-injected cells. At 10 min after BFA, β-COP was completely dispersed in the cytosol of control cells (not shown). In contrast, in the presence of S25N, some of the Man II-containing structures still contained β-COP (Fig. 7, *E* and *F*) at the same time point. At 20 min after BFA, when the extensive tubular structures are seen in S25N-injected cells, β-COP was not detectable on membranes (not shown). Thus, the vesicular disassembly of the Golgi stack induced by the rabla(S25N) mutant immobilizes a significant fraction of β-COP to a vesicular pool which has a markedly reduced sensitivity to BFA.

Discussion

In the present study we have taken a molecular approach to identify, for the first time, a specific role for rabla protein in controlling the structural integrity of the Golgi stack. We observed that either the S25N or N124I mutant forms trigger Golgi disassembly. These two mutants differentially restricted the rabla protein into either the GDP-bound form (S25N) or a conformation that has a high rate of guanine nucleotide exchange (N124I), thus disrupting the normal GDP/GTP cycle involved in the function of these proteins as molecular switches.

Previously, both mutants were found to be potent inhibitors of ER to Golgi and intra-Golgi traffic (14, 42, 45). By analogy to the equivalent mutation in H-ras (S17N) (24), the rabla(S25N) mutant is likely to bind in a competitive fashion to a rab-specific GEP and prevent the normal function of this protein in promoting GDP/GTP exchange and recruitment of wild-type rabl (42, 44). In this case, the phenotype we observed is that the S25N mutant markedly reduced the extent of vesicle budding in vivo and in vitro (42). The H-ras(S17N) mutant remains unable to stimulate a downstream effector protein in vitro even upon binding of GTPγS (22), implying that the rabl mutant may also be restricted to an inactive conformation. Since vesicles still accumulate which are deficient in targeting/fusion, it is therefore likely that they contain the mutant rabla(S25N) which is incapable of undergoing a key conformation change(s) associated with GTP-binding required to initiate these events (42). On the other hand, the N124I mutant is equivalent in principle to the oncogenic (activated) H-ras (N116I) mutant (58). While it has no measurable effect on vesicle formation, it quantitatively inhibits vesicle targeting/fusion (45). The rabla(N124I) mutant is likely to noncompetitively bind to an effector critical for fusion competence (42, 45). Thus, the defects in the two mutant proteins reinforce the importance of conformational changes associated with either GTP binding or hydrolysis as being essential to promote the fusion of carrier vesicles to downstream acceptor compartments.

Dismantling of the Golgi by Rabla Mutants Does Not Trigger a BFA-like Phenotype

In this report we provide evidence that both the BFA-induced collapse of the Golgi apparatus and the reverse process, Golgi reassembly, are GTP-dependent in vivo. A surprisingly large number of GTPases localize to the Golgi region, including both small molecular weight G proteins (rabl, rab2, rab6, ARF, and rho) (27, 40, 48) and at least one member of the heterotrimeric G protein family ($G_{\alpha i3}$) (50, 62, 63). Many of these GTPases remain potential candidates for GTPγS-sensitive retrograde movement of membranes to the ER as demonstrated in permeabilized cells (20, 55) and here in microinjected cells. That GTPγS partially blocks reassembly of the Golgi apparatus, seen by the presence of Man II in scattered vesicular structures, is consistent with the proposal by Alcalde et al. (1) that reconstruction of the Golgi apparatus from BFA-treated cells is a two step process involving reexport of Golgi-specific proteins from the ER followed by cisternal assembly. Our results imply that GTP hydrolysis is specifically needed for cisternal assembly and Golgi stack organization. Since the rabla mutants also blocked Golgi assembly after BFA washout, GTP hydrolysis by rabl is at least one of the components involved in this process.

It is has been proposed that BFA mediates Golgi disassembly through the inhibition of a specific GEP required for the recruitment of ARF (17, 28, 35). ARF is critical for the binding of the β-COP–containing coatomer complex (16, 28) as well as a number of other peripherally associated Golgi proteins (35). In their absence, receptors and fusion-related factors—normally only operational in the context of a coated vesicle cycle—are exposed, triggering an extensive fusion of the ER and early Golgi compartments. In the presence of the rabla(S25N) or the rabla(N124I) mutants, we do not detect the redistribution of Man II to the ER either morphologically (shown here) or biochemically (42, 45). In contrast, recent evidence suggests that an ARF mutant (ARF1[T31N]) which is restricted to a GDP-bound conformation (like the rabla-[S25N] mutant), triggers the collapse of the Golgi apparatus to the ER in a fashion which is virtually indistinguishable from that of BFA (13). Thus, by analogy with ras mutants the functional and morphological consequences of inhibiting

Figure 7. Injection of mutant rabla(S25N) arrests Golgi recovery from BFA. REF-52 cells were treated for 30 min with BFA, which redistributes the Golgi enzyme Man II to the ER, and then injected with S25N mutant rab protein. After 90-min incubation, BFA was washed out, incubation continued for 1 h, and cells fixed for double-labeling immunofluorescence (*A* and *B*) or for immunoperoxidase (*C*) using Man II-specific antibodies. Noninjected cells (*B, arrows*) display a normal Golgi appearance, indicating full recovery from BFA. In S25N-injected cells, Man II was found in isolated Golgi elements (*G*) that were seen either by immunofluorescence (*B, asterisks*) or immunoelectron microscopy (*C*) to be similar to those in cells injected with S25N alone (refer to Figs. 3 and 4). Bars: (*A* and *B*) 2 μm; (*C*) 0.1 μm.

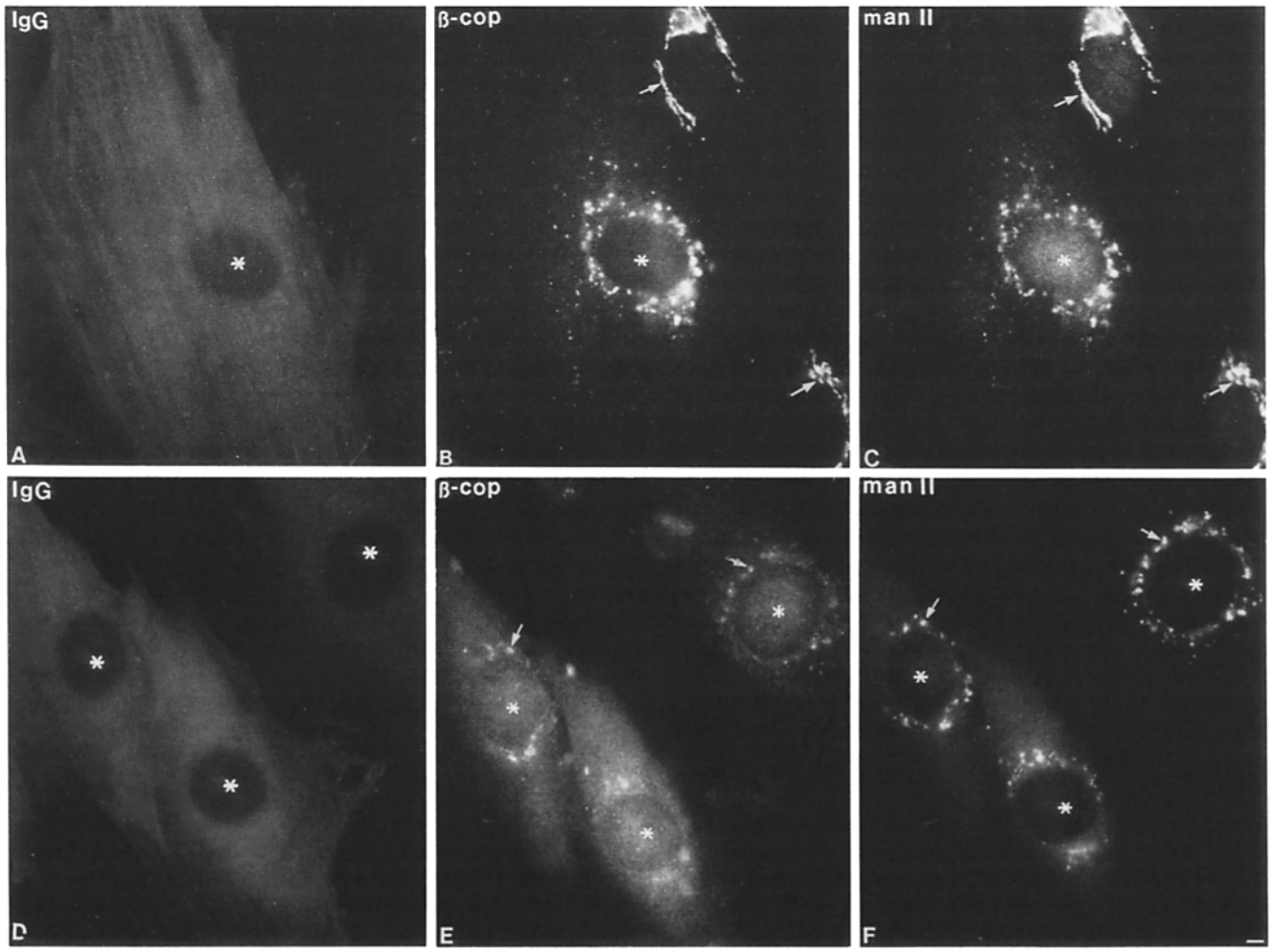

Figure 8. The ER-Golgi coatamer protein, β-COP , localizes to isolated Golgi elements in S25N-injected cells and slowly dissociates when BFA is added. Coverslips containing S25N-injected REF-52 cells were processed for triple-labeling immunofluorescence microscopy to identify (*a*) injected cells (using AMCA-conjugated antibodies to anti-guinea pig IgG); (*b*) distribution of β-COP (using rabbit anti-β-COP IgG, followed by FITC-conjugated antibodies to rabbit IgG); and (*c*) Man II distribution (using monoclonal antibodies to Man II, followed by Texas Red-conjugated antibodies to mouse IgG). In *A–C*, cells were fixed 90 min after injection of mutant rab protein. A single cell (*A*, see IgG marker, *asterisk*) in this field is injected; β-COP (*B*) and Man II (*C*) show almost identical imunofluorescence patterns corresponding to dispersed Golgi elements (see Fig. 2). Arrows in *B* and *C* point to intact Golgi apparatus in noninjected cells. In *D–F*, cells were injected with S25N, incubated for 90 min and then exposed to BFA for 10 min. All three cells in the field were injected (*D*, see IgG marker), and show persistent Golgi elements by anti-Man II immunofluorescence (*F*). β-COP can still be seen to colocalize with a few of these structures (*E*, *arrows*). In noninjected cells in adjacent fields β-COP IgG did not stain discrete structures after 10-min exposure to BFA (not shown). Bars, 2.5 μm.

ARF-GEP as opposed to rab-GEP are distinct. However, the fact that rabla(S25N) was found to dramatically reduce the tubulation and retrograde movement of Golgi membranes in response to BFA, is consistent with the notion that the rabla mutants may also mediate—directly or indirectly—assembly/disassembly of β-COP–containing complexes from the Golgi complex (44).

Mechanism of Rabla Mutant Disassembly of the Golgi Stack

How does the Golgi stack disassemble in the presence of excess mutant rabla? One possibility is that Golgi stack disorganization may be a consequence of the progressive loss of membrane (from the combined effects of membrane exiting the Golgi apparatus in anterograde direction as well as membrane returning to the ER via the retrograde pathway) that fails to be replaced because the arrival of new membrane from the ER is blocked. If this were the case, one would expect a net loss of Golgi membranes. Although it is not technically practical to perform morphometric measurements on microinjected cells due to sampling limitations, the extensive population of Golgi-derived remnant mini-cisternae and vesicles that accumulate in the cell intuitively argue against this point.

A second and more likely possibility, given the importance of rabl in intra-Golgi transport (14, 42), is that dismantling occurs as a direct consequence of ongoing vesicle budding which is subsequently deficient in fusion (42, 45). This interpretation is attractive because it would explain the following observations: (*a*) The coat protein β-COP remains

associated with the residual Golgi elements and vesicles which form in the presence of rabla(S25N), consistent with previous evidence that rabl and β-COP may function coordinately during vesicle assembly (44); (*b*) the dissociation of β-COP by BFA is delayed, and BFA-induced tubulation of the Golgi apparatus and the pursuant retrieval to the ER is greatly slowed. This would be predicted by the observations of Klausner et al. (32) that tubulation is constrained by the presence of coat proteins involved in vesicle budding; and (*c*) there is often an extensive accumulation of 40–60-nm carrier vesicles near the Man II-containing Golgi remnants in S25N-injected cells. These vesicles are reminiscent of the carrier vesicles seen at the periphery of the Golgi stack during normal transport. They are particularly prominent in NRK cells microinjected with S25N which are expected to have a higher ratio of mutant to endogenous rabl. Furthermore, we found that Man II (previously redistributed to the ER by BFA) could apparently exit the ER in the presence of S25N mutant and accumulate in dispersed Golgi elements that appear indistinguishable from those in cells injected with S25N alone. It is conceivable that Golgi resident proteins (including Man II) might be targeted to subdomains of the ER (transitional elements) in the course of BFA treatment and begin to bud and assemble as soon as Golgi-specific peripheral proteins reassociate after BFA is washed out.

It is intriguing that injection of either S25N (GDP-bound mutant) or N124I (guanine nucleotide-minus mutant) caused fragmentation of the Golgi stack, as assessed at the light microscopic level. Studies are currently in progress to compare the effects of the N124I mutant with those of the S25N mutant in more detail at the EM level. However, in preliminary experiments analyzed by fluorescence microscopy, we found at least one distinct difference between the two mutants: as described in Fig. 5, *A–D*, injection of S25N prior to the addition of BFA resulted in arrest of Golgi enzyme in extensive beads on a string. This distinctive morphology was not seen in N124I-injected mutants, although the Golgi apparatus often failed to completely redistribute to the ER after BFA was added as seen by a more compact juxtanuclear stain for Man II (data not shown), suggesting a critical role for a rabl effector protein in this process.

The Structural Organization of the Golgi Stack

One of the most intriguing possibilities suggested in the present study comes from the observation that the stacked Golgi cisterna can be drastically reduced to vesicles and remnant structures. Remnant structures contain for the most part, the Man II marker protein. These Man II-containing Golgi elements may represent the fundamental structural units that provide the template for reconstruction of individual Golgi compartments (30). The vesicular elements formed in the presence of the rabla(S25N) were apparently transport vesicles devoid of Man II. This interpretation, in turn, implies that a large fraction of the membrane associated with the Golgi stacks of mammalian cells is composed of lipid and protein in transit. This would also account for the extensive coat of transport-related components (β-COP, ARF, rabl, etc.) on the cisternae of the Golgi stack and would also account, in part, for the effects of BFA upon removal of these coats. Thus, the extensive and variable architecture of the Golgi stack found in higher and lower eukaryotes reflects a dynamic equilibrium between the anterograde and retrograde transport to and from individual compartments. These results may also provide an explanation for our recent observations that export from the ER involves sorting and concentration, while transport through the Golgi compartments is nonselective (5).

Our studies suggest that rabl plays a key role in modulating traffic through the stack by controlling the rate of vesicle targeting and fusion between compartments. These results are strikingly similar to the effect of rab5 in controlling the structure of the early endosome (11). Overexpression of a rab5 mutant (5Ile133) caused an accumulation of small tubules and vesicles at the periphery of the cells, apparently derived from early endosomes whose lateral fusion was impaired. In contrast, overexpression of the wild-type protein led to a marked elaboration of the endosomal compartment. These results imply, that like traffic control by rabl, the steady state morphology of early endosomes is also maintained by a balance of vesicles entering and exiting this organelle whose kinetics are controlled by the activity of a rab GTPase(s).

What are the implications from the present studies on the inhibition of transport and fragmentation of the Golgi stack during mitosis? During mitosis, intracellular transport is blocked (61), and the Golgi stack is fragmented (60). These events are controlled by the phosphorylation cascade triggered by activation of $p34^{cdc2}$ kinase upon entry into mitosis. Indeed, intra-Golgi transport in vitro can be directly inhibited with $p34^{cdc2}$ kinase (51). In addition, it has been demonstrated that the phosphatase inhibitor okadaic acid induces Golgi apparatus fragmentation (2, 38, 54) and arrests ER to Golgi transport (15) in a manner similar to that observed during mitosis. The inactivation of phosphatases 1 and 2A by okadaic acid has been shown to activate $p34^{cdc2}$ kinase and trigger other events associated with mitosis (65). Alternatively, okadaic acid may indirectly activate other kinases whose activity is kept in check during interphase by phosphatases.

The strong biochemical and morphological similarities between the inhibition by mutant rabl and onset of mitosis identifies at least one potential step which may be controlled by mitotic kinases. Interestingly, Warren (59) has previously proposed that the disassembly of the Golgi apparatus during mitosis may be due to an incomplete cycle in which vesicles can bud from the Golgi stack, but are incompetent in fusion. Our results provide support for, but do not prove, that this may be the case. Intriguingly, rabla was previously shown to be phosphorylated by $p34^{cdc2}$ kinase, and its membrane association slightly enhanced in mitotic HeLa cells (3). However, we have been unable to obtain any further evidence relevant to this point from analysis of rabla mutants defective in phosphorylation (Davidson, H. W., and W. E. Balch, unpublished observations). Any of the other transport components involved in vesicle targeting or fusion (9, 49) would also be expected to be excellent targets for phosphorylation control.

Taken together, our observations support the concept that the overall organization of the Golgi apparatus at all stages of the cell cycle is largely maintained by components controlling vesicular traffic. Rabla plays a critical role in this process, most likely via the regulation of coat components and/or vesicle fusion in both ER to Golgi and intra-Golgi

transport. It may also be either directly or indirectly critical for partitioning of the Golgi apparatus during cell division. If so, the process of preparation of the Golgi apparatus for cell division is, in principle, more a matter of reducing it to its basic structural elements, than physical fragmentation.

The authors thank Benjamin Jewell and Pamela Lewis for technical assistance and Dr. George Palade (UCSD) for helpful discussions during the preparation of this work. We thank Dr. Linda Hendricks (UCSD; now at Merck, Sharp & Dohme) for contributions to the preparation of Man II ascites and anti-β-COP antiserum, Dr. Dan Predescu (UCSD) for BSA-colloidal gold, and Dr. Brian Burke (Harvard University, Cambridge, MA) for 5CFC3 (anti-ManII) hybridoma cells.

Grant support for this project was provided by the National Institutes of Health to M. G. Farquhar (CA46128, CA58689, and DK17780), to W. E. Balch (GM33301, GM42336, and CA58689), and to J. R. Feramisco (CA39811 and CA58689), and by the California Tobacco Related Disease Research Program (2KT0030) to J. Meinkoth. B. S. Wilson was a postdoctoral fellow of the American Cancer Society (California Division) 1991–93. Claude Nuoffer is the recipient of a European Molecular Biology Organization Fellowship and a fellowship from the Swiss National Science Foundation.

Received for publication 3 August 1993 and in revised form 20 January 1994.

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