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The small GTP-binding protein rab6p is redistributed in the cytosol by brefeldin A

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SUMMARY

Rab6 protein belongs to the Sec4/Ypt/rab subfamily of small GTP-binding proteins involved in intracellular membrane trafficking in yeast and mammalian cells. Its localization both in medial and *trans*-Golgi network prompted us to study the effects of brefeldin A (BFA) on rab6p redistribution. By two techniques, indirect immunofluorescence and cell fractionation, we investigated the fate of rab6p and compared it to other Golgi or *trans*-Golgi network markers in BHK-21 and NIH-3T3 cells. BFA, at 5 μg/ml, induced redistribution of rab6p according to a biphasic process: during the first 10-15 minutes, tubulo-vesicular structures - colabelled with a bona fide medial Golgi marker called CTR 433 - were observed; these structures were then replaced by punctate diffuse staining, which was stable for up to 3 hours. The 110 kDa peripheral membrane protein β-COP was released much more rapidly from the Golgi membranes, whereas the *trans*-Golgi network marker TGN 38 relocated to the microtubule organizing center. The kinetics of reversion of BFA action on these antigens was also followed by immunofluorescence. Consistent with these results, rab6 antigen, originally found as 40% in the cytosolic versus 60% in the particulate (P 150,000 *g*) fraction, became almost entirely cytosolic; moreover, it partitioned in the aqueous phase of Triton X-114 whereas the membrane fraction was detergent-soluble. Rab6p did not become part of the coatomers after its BFA-induced release from Golgi structures. Three requirements seemed to be necessary for such a release: integrity of the microtubules, presence of energy, and a hypothetical trimeric G protein, as revealed by the respective roles of nocodazole, ATP depletion, and sensitivity to aluminium fluoride. Finally, we have shown that BFA does not prevent attachment of newly synthesized rab6p to membranes.

Key words: brefeldin A, rab6 protein, small GTP-binding protein

INTRODUCTION

Since the pioneering work of G. Palade in the 1960's, who first established the relationship between ER, Golgi and secretory storage vesicles in regulated secretion, much progress has been made on the molecular dissection of the secretory pathway of eukaryotic cells (for a recent review, see Rothman and Orci, 1992; Mellman and Simons, 1992). One important discovery in the area of intracellular protein transport was the finding that many organelles involved in exocytic and endocytic membrane traffic have one or more ras-like small GTP-binding proteins on their cytoplasmic surface, which are probably specific for each membranous compartment (Bourne et al., 1991; Goud and McCaffrey, 1991; Pfeffer, 1992). This makes these proteins attractive candidates as regulators of vesicle transport: formation, accurate delivery to the correct target membrane (docking), and subsequent fusion with the acceptor membrane. More than twenty different genes coding for proteins related to yeast Sec4p and Ypt1p, involved in transport between the ER and the Golgi complex, and the Golgi complex and plasma membrane, respectively, have now been characterized. These mammalian homologues originally cloned in rat brain have been grouped under the term rab (Touchot et al., 1987), but human (Zahraoui et al., 1989) and canine (Chavrier et al., 1990b) equivalents have since been isolated. The rab proteins are about 21-25 kDa in mass, and 30% identical to ras protein (Touchot et al., 1987). They all possess a putative effector domain homologous to that of Ypt1p and Sec4p, and the highly conserved blocks of sequence that comprise a guanine nucleotide-binding motif. The rab proteins have distinct subcellular localizations, being associated with nearly every organelle (Chavrier et al., 1990a; recently reviewed by Takai et al., 1992; Pfeffer, 1992). Among these, rab6p has been localized on the cytoplasmic face of the medial and *trans* cisternae of the Golgi complex (distributed over their entire surface) in a variety of cell lines (Goud et al., 1990). By confocal and electron microscopy, rab6p was also found at the exit of the Golgi complex, in a tubulo-vesicular network referred to as *trans*-Golgi network (TGN) and also on some small vesicles (Antony et al., 1992). While rab6p function is still unknown, recent studies have unravelled the roles of several other rab proteins in specific steps of vesicular traffic,

in good agreement with their main cellular localization: rab1 and rab2 regulate vesicular transport between ER and successive Golgi stacks of cisternae (Plutner et al., 1991; Tisdale et al., 1992), rab3A controls fusion of synaptic vesicles during exocytosis (Fischer von Mollard et al., 1991; Oberhauser et al., 1992), rab4 an early sorting event in endocytosis (Van der Sluijs et al., 1992b), rab5 controls early endosomal fusion in vivo (Bucci et al., 1992) and in vitro (Gorvel et al., 1991), rab9 functions in transport between late endosomes and the *trans*-Golgi network (Lombardi et al., 1993), and rab17, specific for epithelial cells, might be involved in transcellular transport (Lütcke et al., 1993). From its intra- and post-Golgi localizations, we can put forward the working hypothesis that rab6p is involved in budding and/or targeting/fusion events at these levels of protein transport.

It then seemed interesting to study the effects of brefeldin A (BFA) on rab6p localization, since this macrocyclic fungal antibiotic is known to rapidly disorganize the Golgi complex (for recent review, see Klausner et al., 1992). The first reported effects of BFA on arrest of secretion of newly synthesized proteins in rat hepatocytes by Ikehara's group (Misumi et al., 1986; Fujiwara et al., 1988) were rapidly followed by a large number of studies mainly centered on the disruption of the Golgi complex, as such, and redistribution of *cis*-, medial, and *trans*-Golgi markers into the ER (Doms et al., 1989; Lippincott-Schwartz et al., 1990, 1991; Donaldson et al., 1990, 1991b). The action of BFA has been explained by an inhibition of the anterograde vesicular traffic from ER to Golgi and between Golgi stacks, without effect on retrograde transport (Lippincott-Schwartz et al., 1989). This leads to the microtubule-dependent formation of an extensive tubular network connecting previously separate cisternae of Golgi and pre-Golgi compartments (Lippincott-Schwartz et al., 1990). More recently, BFA has been shown to be able to perturb the TGN of certain cell lines. Reaves and Banting (1992), using antibodies to an integral membrane protein of the TGN, called TGN 38, have reported BFA-induced morphological changes of the TGN of normal rat kidney cells, in which the majority of the TGN collapses around the microtubule organizing center. They suggest a lack of tubular retrograde transport from the TGN to the *trans*-Golgi and put forward the hypothesis that BFA dissociates a kinesin-like motor from the TGN. Following the fate of the mannose 6-phosphate receptor, Wood et al. (1991) demonstrate that BFA specifically pertubs a recycling pathway between TGN and early endosomes. BFA also prevents post-Golgi movement in the direction of the cell surface both in constitutive and regulated pathways, but not return traffic (endocytosis) (Miller et al., 1992), as previously described (Misumi et al., 1986).

One of the earliest events observed (within one minute after BFA addition) is the dissociation of a 110 kDa protein from Golgi membranes (Donaldson et al., 1990). This protein has been identified as β-COP, which localizes primarily to either transitional ER, Golgi stacks, or *trans*-Golgi network, but it is also part of the Golgi-derived (non-clathrin) coated vesicles (Duden et al., 1991; Serafini et al., 1991b). These vesicles are thought to play a role in the bulk flow of constitutive secretory and membrane proteins between the different stacks of Golgi cisternae (Orci et al., 1986). Golgi membranes indeed possess a transiently attached coat. This comprises α, β, γ and δ coat proteins, which also exist in the cytosol along with other low molecular mass proteins, and a small GTP-binding protein, the ADP-ribosylation factor ARF. These components form the coatomer, the protomeric unit of the non-clathrin coated vesicles (Waters et al., 1991; Serafini et al., 1991a). Interestingly, ARF and γ-adaptin, the adaptor protein of the clathrin-coated vesicles associated with the TGN, rapidly dissociate from Golgi and TGN membranes respectively, upon addition of BFA, in a manner indistinguishable from β-COP (Donaldson et al., 1991a; Robinson and Kreis, 1992). Another Golgi membrane-associated protein of 200 kDa with many properties in common with β-COP has been identified, which also rapidly leaves Golgi membranes upon BFA treatment (Narula et al., 1992). It then appears that the non-clathrin coat (and the clathrin coat associated with TGN) play a critical role in budding of Golgi-derived transport vesicles or in regulating the formation of tubules involved in Golgi transport. To our knowledge, with the noticeable exception of ARF, no effect of BFA on small GTP-binding proteins has been reported.

In this paper, we followed rab6p after BFA treatment of BHK-21 and NIH-3T3 cells both by the indirect immunofluorescence technique and by cell fractionation. As rab6p has been found in two distinct localizations (medial and *trans* cisternae of the Golgi complex, and TGN), relocation of rab6p was compared to that of a resident protein of the medial Golgi cisternae, called CTR 433 (Jasmin et al., 1989) and of TGN 38, a bona fide marker of the TGN in the same cells (Luzio et al., 1990). We also studied the fate of β-COP. We characterized the BFA-induced redistribution of rab6p in terms of energy requirement, microtubule dependence and activation of GTP-binding proteins.

MATERIALS AND METHODS

Reagents and antibodies

BFA was obtained from Epicentre Technologies (Madison, WI), and stored frozen as a 10 mg/ml stock solution in ethanol. It was used at 5 μg/ml final concentration.

Antibodies used for immunofluorescence included an affinity-purified rabbit antibody against human rab6p (Goud et al., 1990), M3A5, a mouse monoclonal antibody against β-COP kindly donated by T. Kreis (Allan and Kreis, 1986), CTR 433, a mouse monoclonal antibody against the medial compartment of the Golgi apparatus, kindly provided by M. Bornens (Jasmin et al., 1989), and a rabbit polyclonal antibody against TGN 38, an integral membrane protein of the TGN, a kind gift of P. Luzio (Luzio et al., 1990). Antisera directed against *E. coli*-produced human rab1Ap, rab2p, rab4p and rab6p, and used for immunoblot detection have been previously characterized (Goud et al., 1990; Maridonneau-Parini et al., 1991). Anti-ER antibodies used for immunodetection were provided by Dr D. Louvard (Louvard et al., 1982). For convenience, we shall refer to the antigen recognized by CTR 433 antibody as 'CTR 433'.

Cell culture and immunofluorescence

BHK (clone 21) cells were grown in Glasgow minimal essential medium (Gibco Life Technologies Ltd., Paisley, Scotland) containing 10% fetal calf serum and 10% tryptose phosphate broth

(Gibco). NIH-3T3 cells were grown in αMEM (Gibco) containing 10% fetal calf serum. For reasons of convenience, all the immunofluorescence microscopy studies presented here were realized on NIH-3T3 cells, but BHK-21 cells gave the same results. Note, however, that anti-TGN 38 antibodies did recognize the mouse antigen, but not the hamster one.

Cells were seeded on glass coverslips (12 mm diameter) in 24-well dishes 48 hours before use. They were fixed with 4% paraformaldehyde in PBS-Ca^{2+}-Mg^{2+} for 15 minutes at room temperature, then permeabilized by either a 15 minute incubation with 0. 05% saponin in PBS-Ca^{2+}-Mg^{2+} containing 0. 2% BSA (for CTR433, rab6 and TGN 38 antibodies) or by a 4 minute incubation with 0. 1% Triton X-100 and 0. 05% SDS in PBS-Ca^{2+}-Mg^{2+} (for M3A5). In the latter case, an extra 15 minute incubation in the presence of 0. 2% BSA was necessary. The cells were then incubated for 40 minutes in the presence of the various antibodies, washed three times and incubated for a further 40 minutes with the appropriate sheep or donkey anti-immunoglobulin antibodies labeled with either fluorescein or Texas Red (Amersham, UK). The coverslips were mounted on Mowiol (Hoescht) and examined with a Zeiss epifluorescence microscope equipped with the appropriate filters.

Subcellular fractionation

Subconfluent cells were washed once with PBS, trypsinized, pelleted and resuspended in 4 volumes of cold 10 mM Tris-HCl, pH 7.4, 5 mM sucrose containing 1 mM PMSF and a mixture of protease inhibitors (0. 1 μg/ml final concentration of leupeptin, chymostatin, pepstatin, antipain and aprotinin) (buffer A). All subsequent steps were done at 4°C. The cells were broken by repeated passages through a ball-bearing homogeneizer (Bernitech Engineering) (minimal volume 800 μl). Unbroken cells and nuclei were removed by a 10 minute spin at 600 ***g*** and the resulting post-nuclear supernatants (PNS) were ultracentrifuged at 150, 000 ***g*** in a Kontron TST 55. 5 rotor for 1 hour. Supernatants and membrane pellets (carefully resuspended in the cytosol volume of buffer A) were used as such or partitioned in Triton X-114 to determine by immunodetection the quantities of rab6p and other proteins. If not used immediately, samples were quickly frozen in liquid nitrogen and stored at −80°C.

Triton X-114 extraction

Extraction in 1% (final) Triton X-114 was done essentially as described by Bordier (1981): 5 minutes at 4°C, 2 minutes at 30°C, centrifugation 1 minute at 1, 200 ***g*** and the pellet containing the detergent phase carefully resuspended in initial volume of buffer A.

Sucrose gradient centrifugation

Pre-confluent BHK-21 cells (2 dishes of 100 mm diameter for each control and BFA-treated cells) were fractionated as described above. The cytosolic fractions (about 1 mg protein as determined by Bradford assay (Bradford, 1976)) were loaded on top of 5%-30% (wt/vol) continuous sucrose gradients (10 ml each) made in 15 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM DTT, 0. 5 mM PMSF and the mix of protease inhibitors as described above. A separate gradient was loaded with marker proteins (bovine serum albumin, human immunoglobulins G and thyroglobulin). The gradients were centrifuged in a SW41 rotor at 275,000 ***g*** for 16 hours at 4°C. One ml fractions were collected from the top, and proteins were precipitated with 10% trichloracetic acid. After washing of the pellets with acetone, the samples were carefully resuspended in 100 mM Tris base, 10 mM EDTA and 23% glycerol containing bromophenol blue, and processed for analysis by SDS-PAGE and immunoblot detection.

In vivo labelling

Subconfluent NIH-3T3 cells (2 dishes of 100 mm diameter for each control and BFA-treated cells) were washed twice with PBS and incubated for 1 hour in RPMI 1640 without cysteine or methionine (ICN-Flow). BFA (5 μg/ml), was added for the last 30 minutes. Then 400 μCi of a [^{35}S]methionine and [^{35}S]cysteine mixture (New England Nuclear) were added per dish for exactly 5 minutes at 37°C. Cells were rinsed twice at 4°C with complete medium containing an excess of cysteine and methionine (10 mM each). Half of the cells were processed directly for cytosol/membrane fractionation (see below), while the other two plates were incubated an extra hour at 37°C in the chase medium. BFA (5 μg/ml) was added again during all the chase period in one of the dishes. Cells were detached in the absence of liquid with a rubber policeman, and the plates were rinsed with 500 μl of 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 1 mM PMSF, and a mixture of protease inhibitors as detailed above (buffer B). Cells were broken in a Dounce homogenizer, and post-nuclear supernatants were used to fractionate cytosol and membrane as described above.

Immunoprecipitation, electrophoresis and immunoblot detection

Polypeptides of the fractions issued of the pulse/chase experiment were denatured in SDS and immunoprecipitated as described (Roa and Boquet, 1985). In order to distinguish between unmodified and prenylated forms of rab6p, gels (15% acrylamide) were prepared from stock solutions of 30% acrylamide/0. 016% bisacrylamide. In most cases, when unmodified versus modified forms of rab6p need not be separated, regular stock solutions (30% acrylamide/0. 8% bisacrylamide) were used. Reduced proteins were separated by SDS-PAGE (12% polyacrylamide), and transferred onto nitrocellulose filters for immunoblotting. The filters were incubated with antibodies and labeled bands were visualized by the alkaline phosphatase method (alkaline phosphatase-conjugated second antibody from Promega, 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium from Sigma Chemical Co., St. Louis, Mo) or by iodinated Protein A-Sepharose if quantification was necessary. Care was taken to load on gels equivalent amounts of total protein from the PNS fractions when comparing control to BFA-treated cells, as estimated by the Bradford technique. As membrane pellets were routinely redissolved in the original volume of cytosol, and as the volumes of cytosol and membrane loaded on gels were equal to PNS volume, it follows that the signal immunodetected in PNS should represent the sum of the signals detected in cytosol and membrane fractions.

RESULTS

Kinetics of redistribution of rab6p in cells treated with brefeldin A

Previous immunocytochemical studies both at light and electron microscope levels in various cell types have shown that BFA treatment for an appropriate time induces the resorption of Golgi cisternae and concomitant redistribution of Golgi content and membrane into the ER (reviewed in Klausner et al., 1992). TGN morphological alterations have also been attributed to BFA effect, at least in some cell lines (Reaves and Banting, 1992). As rab6p has been shown to be concentrated predominantly on the medial and *trans*-Golgi cisternae (Goud et al., 1990) and also on the TGN (Antony et al., 1992), we treated subconfluent NIH-3T3 cells with 5 μg/ml BFA for various periods of time to deter-

mine the fate of rab6p. In all the immunofluorescence experiments presented in this manuscript, a marker for a resident protein of the medial Golgi, called CTR 433 (Jasmin et al., 1989), has been examined by double labelling with rab6p. The fates of TGN 38, a specific marker of the TGN (Luzio et al., 1990), and of β-COP, the 110 kDa protein of Golgi membrane, which contributes to the coat of non-clathrin coated vesicles (Duden et al., 1991) were also followed by immunofluorescence microscopy on permeabilized cells, as described in Materials and Methods.

As shown in Fig. 1, the pattern of rab6 staining in control cells revealed a typical Golgi, with extensively interconnected stacks of cisternae surrounding the nucleus. The pattern of CTR 433 staining was superimposable to that of rab6p as seen in double-label immunofluorescence, except for small dots stained with anti-rab6p antibodies and spread all over the cytoplasm, and absent with CTR 433 antibodies (see Fig. 1, control lane). TGN 38 was in typical perinuclear caps, as reported by Luzio et al. (1990). β-COP was predominantly associated with the Golgi region, although less tightly packed to it than rab6p and CTR 433, as already observed by Donaldson et al. (1990). It was, in addition, localized to peripheral grains, most probably transitional ER or cytosolic coatomers (Duden et al., 1991).

Addition of BFA to NIH-3T3 cells had a dramatic effect on all these markers. As already described (Donaldson et al., 1990), β-COP was widely dispersed throughout the cytoplasm as early as 2 minutes after the drug was added (Fig. 1, lower panel), and it remained as such throughout BFA treatment (data not shown). At that time (2 minutes), rab6p, CTR 433 and TGN 38 began to move from a juxtanuclear localization into continuous tubular processes, in most of which rab6p and CTR 433 were found to be common. The tubules through which TGN 38 passes were reported to be distinct from the ones emanating from *cis* and medial cisternae (Reaves and Banting, 1992). This structure, already described in particular for mannosidase II, a *cis*-medial cisternae marker, as beaded necklace-like tubular processes extending from the Golgi apparatus (Lippincott-Schwartz et al., 1990) persisted for 10 minutes, although an isolated cell occasionally still contained them, as exemplified here at time 60 minutes for rab6p/CTR 433 (Fig. 1). At 15 minutes of BFA addition though, the fates of rab6p and CTR 433 interestingly diverged: while CTR 433 redistributed to a fine punctate/reticular pattern typical of the ER and was stably located there for 3 hours (data not shown), rab6p acquired a discrete punctate and diffuse staining, scattered all over the cytoplasm. Between 30 and 60 minutes, rab6p staining seemed to fade as compared to CTR 433 for example. TGN 38, by 5 minutes of BFA addition, acquired a punctate pattern of dispersion, while by 10 minutes, and still more clearly by 15 minutes, a compact center of TGN 38 staining appeared at the nuclear membrane. This stucture is probably the microtubule organizing center (MTOC), as previously identified in NRK cells (Reaves and Banting, 1992). These MTOC structures were stabilized for at least 3 hours of BFA treatment (data not shown). Considering again the rab6p pattern of staining, it should be noted that all cells at times 15 and 30 minutes, but not at 60 minutes, also exhibited a compact center reminiscent of the MTOC. As anti-sera against rab6p and TGN 38 were both raised in rabbit species, we were unable to confirm by double-label immunofluorescence the colocalization of the transient compact structures visualized at times 15 and 30 minutes with anti-rab6 antibodies, with the MTOC revealed by anti-TGN antibodies. Similar results have been obtained using BKH-21 cells at the same dose of BFA (5 μg/ml). Thus, rab6p, to the extent so far examined, is uniquely affected by BFA treatment.

Reassociation of rab6p with Golgi apparatus after BFA wash out

All previously reported effects of BFA on the redistribution of markers have been shown to be fully reversible: for example, mannosidase II (Lippincott-Schwartz et al., 1989), β-COP (Donaldson et al., 1990), γ-adaptin (Robinson and Kreis, 1992) and TGN 38 (Reaves and Banting, 1992) begin to return to their original location(s) within minutes after drug removal, although with noticeable differences in intermediate patterns of staining when compared in double-label experiments. We therefore treated NIH-3T3 cells for 30 minutes with 5 μg/ml BFA in order to get a complete effect on redistribution of our selected markers. We removed the drug by three washes and fixed, permeabilized, and prepared the cells for indirect immunofluorescence. As seen in Fig. 2, as soon as 2 minutes after drug removal from the incubation medium, rab6p, TGN 38 and β-COP (Fig. 2c, lower panel) began to move to their original location, while for CTR 433, it took longer to be mobilized. By 7 minutes, β-COP was localized in discrete vesicles, which had enlarged (Fig. 2d, lower panel), and by 30 minutes it became more compact, resembling the pattern of β-COP staining of control cells (data not shown). TGN 38, from an original MTOC location after 30 minutes of BFA treatment, acquired a more elongated pattern of staining, less compact and finely tubulated (at times 2 and 5 minutes of drug removal). At times 10 and 15 minutes, it began to recondense again close to the nucleus, and only after 1 hour of BFA wash out was the recovery close to complete.

Concerning rab6p and CTR 433, as expected from the kinetics of BFA action shown in Fig. 1, the examination of the pattern of recovery from a 30 minute BFA treatment displayed two phases: an initial phase (2-15 minutes) where both antigens had an apparently distinct localization, and a second phase (30-60 minutes) where they colocalized, although not still completely, in the Golgi apparatus. Moreover, it seemed that rab6p was mobilized faster than CTR 433: only between 15 and 30 minutes of drug removal did this last marker leave its ER location. Rab6p acquired a patchy staining with sometimes interconnected tubules as soon as 5 minutes after drug removal. These patchy structures seemed to concentrate at one end of the nucleus at 15 minutes. At time 30 minutes, they had condensed to form recognizable Golgi elements, now colocalized with CTR 433 staining. At 60 minutes, these structures got still more compact, resembling closely the Golgi of control cells. From these data, we conclude that the effect of BFA upon the localisation of rab6p, in common with other markers already described as being affected by BFA treatment, is fully reversible. Moreover, the pattern of staining and the kinetics of relocation to the Golgi apparatus were distinct

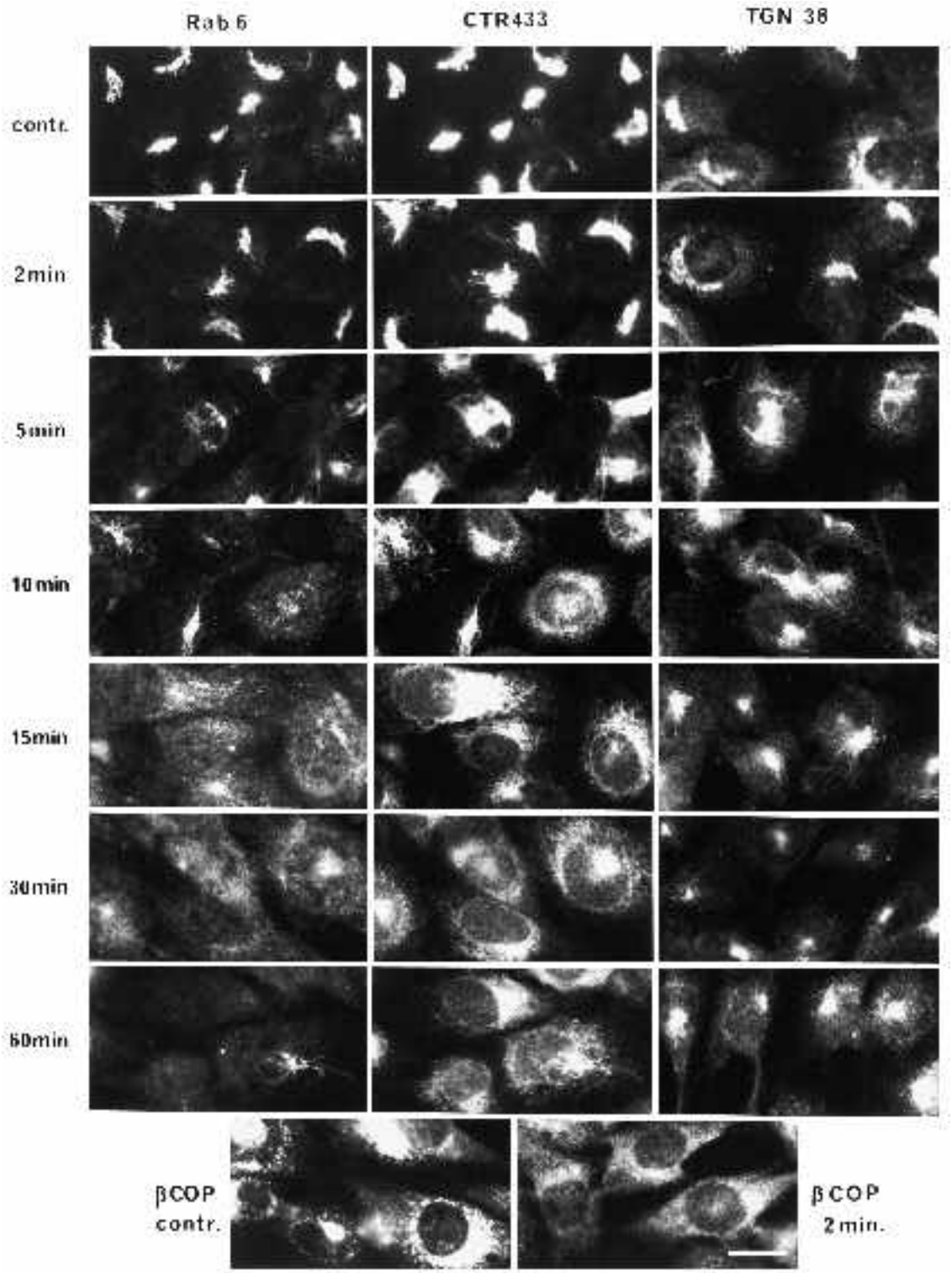

Fig. 1. Time course of redistribution of rab6p, CTR 433, TGN 38 and β-COP in BFA-treated cells. The distributions of a Golgi/TGN marker, rab6p, a medial Golgi marker, CTR 433, and a TGN marker, TGN 38, were examined by indirect immunofluorescence in NIH-3T3 cells after 0, 2, 5, 10, 15, 30, and 60 minutes of BFA treatment (5 μg/ml) at 37°C. In the case of β-COP, only one time of BFA action (2 minutes), is shown. The cells were fixed immediately after BFA treatment, permeabilized, and prepared for indirect immunofluorescence microscopy as described in Materials and Methods. Note that in the case of rab6p and CTR 433, double labeling has been performed. Bar, 10 μm.

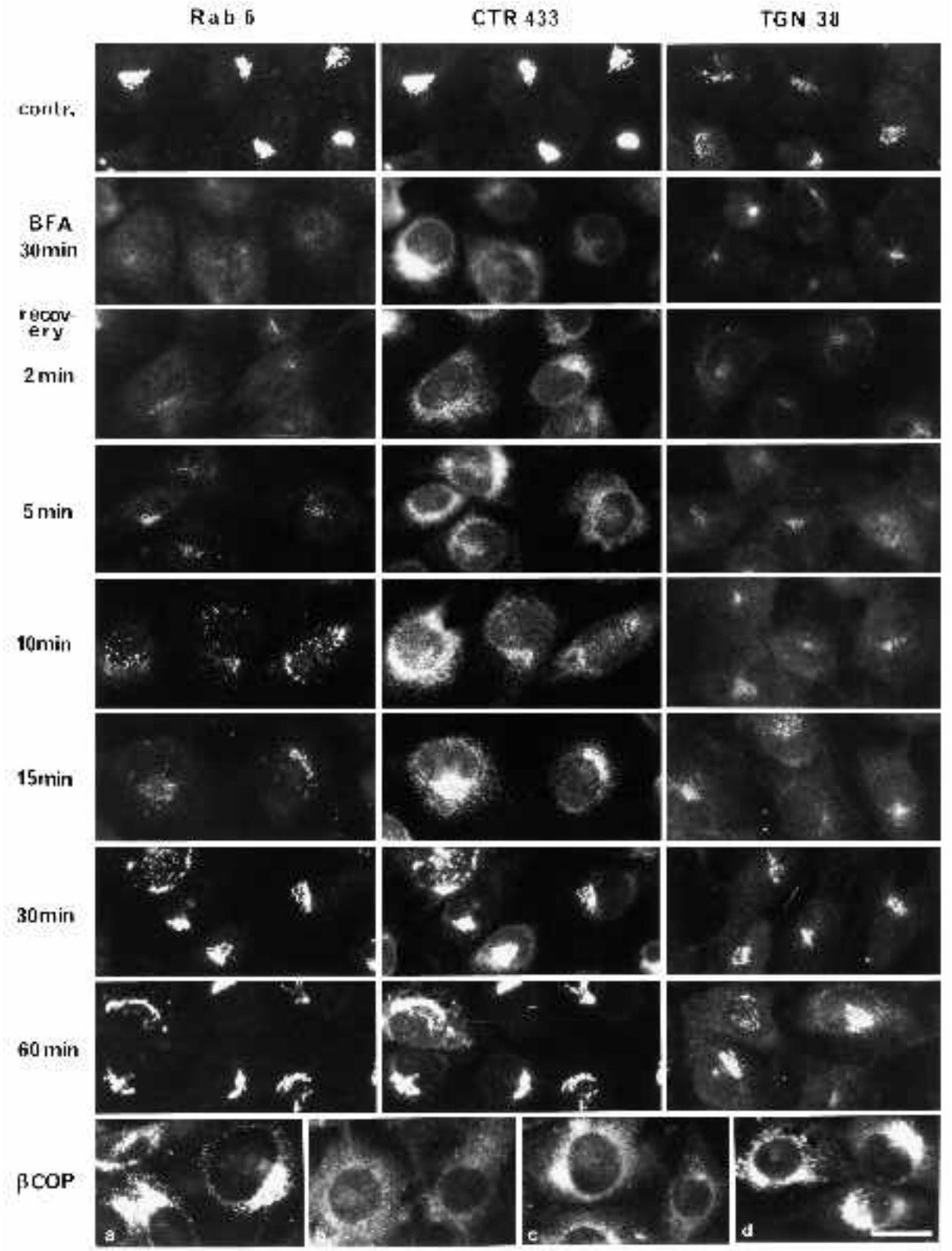

Fig. 2. Time course of recovery from BFA treatment. NIH-3T3 cells were incubated for 30 minutes with 5 μg/ml BFA and then chased in fresh medium for 0, 2, 5, 10, 15, 30 or 60 minutes. The cells were then fixed, permeabilized, and labeled with anti-rab6 or CTR 433 (double-label) or TGN 38 as described in Fig. 1. In the case of β-COP, only times 0, 2, and 7 minutes of recovery from BFA are shown (bottom line, b-d). A represents control cells labeled with anti-β-COP antibody. Bar, 10 μm.

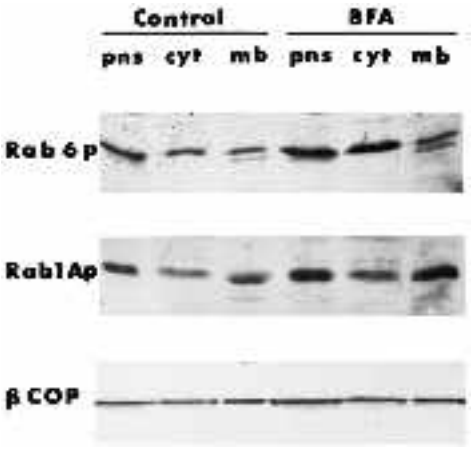

Fig. 3. Redistribution of rab6p in the cytosolic fraction after BFA treatment BHK-21 cells were exposed to BFA (5 μg/ml) for 30 minutes before being trypsinized and homogenized as described in Materials and Methods. The post-nuclear supernatant (pns), cytosol (cyt), and crude membrane (mb) fractions were processed for immunoblotting using anti-rab6, anti-rab1A, and anti-β-COP antibodies by the alkaline phosphatase method. There is a massive redistribution of rab6p in the cytosol of BFA-treated cells, although rab1Ap and β-COP appear essentially unaffected.

from those of a Golgi resident protein, CTR 433, of a TGN marker, TGN 38, and of β-COP.

Rab6p is redistributed in a cytosolic fraction after treatment of cells with BFA

In order to specify the new localization of rab6p as seen by indirect immunofluorescence, we decided to fractionate post-nuclear supernatants of BHK-21 cells treated for 30 minutes with BFA, and to compare the distribution of rab6p to that of untreated control cells. Post-nuclear supernatants were subjected to 150,000 ***g*** centrifugation, and both pellet and cytosolic fractions were electrophoresed in SDS-polyacrylamide gels, and subsequently immunoblotted as detailed in Materials and Methods. As shown in Fig. 3, rab6p in control cells was localized in both cytosolic and membrane fractions. After BFA treatment, rab6p was found mainly in the cytosolic pool. Quantification, by computer-assisted image analysis, of the material present in the bands confirmed this result: 38% of total rab6p in the cytosol of control cells, and 78% in the same fraction of BFA-treated cells. In a second quantified experiment, 54% of rab6p was detected in the cytosol of control cells, and 95% after BFA treatment. All the experiments performed have pointed to a massive redistribution of membrane-bound rab6p to the cytosolic pool upon treatment of cells with BFA. Similar results were obtained with both NIH-3T3 and BHK-21 cells (data not shown).

We checked that after removal of BFA and a further 30 minute incubation in the absence of the drug, rab6p goes back to its initial location, in the membrane fraction (data not shown). When probing the same fractions with the anti-β-COP antibody, no redistribution of the antigen was observed after BFA treatment (Fig. 3), although the immunofluorescence study indicated a scattering throughout the cytoplasm. Robinson and Kreis (1992) have reported similar findings in Vero cells for β-COP and γ-adaptin, suggesting that cytoplasmic coat proteins aggregate after BFA treatment. Quantification of the respective amounts of β-COP present in cytosolic and membrane pools from immunoblots revealed by the iodinated Protein A-Sepharose method were approximately 40% and 60%. Also, the rab1A protein, which has been localized in both ER and Golgi compartments (Goud and Saraste, unpublished), has been found to show the same distribution before and after a 30 minute treatment of cells with BFA: about 30% in the cytosol and 70% in the membranes. Two other small GTP-binding proteins of the rab family, rab2p, located in the intermediate or 'salvage' compartment (Chavrier et al., 1990a) and rab4p in early endosomes (Van der Sluijs et al., 1991) did not present any noticeable change in the same fractionation experiments after BFA treatment (data not shown). For technical reasons, it proved impossible to use CTR 433 antibody in immunoblotting experiments. We, however, could check that our method of cell breaking and of post-nuclear supernatant fractionation does not change the distribution of the ER compartment: after BFA treatment, anti-ER polyclonal antibodies labeled exclusively antigens present in the pellet (membrane) fractions, as expected (data not shown).

Microtubule integrity and energy are required for BFA-induced redistribution of rab6p

Redistribution of BFA-induced Golgi resident proteins has been shown to be inhibited by nocodazole, a microtubule-depolarizing agent (Lippincott-Schwartz et al., 1990; Donaldson et al., 1990). This suggests that microtubule integrity is required for retrograde transport. As rab6p is redistributed after BFA treatment, we studied the effect of an incubation of cells for 1 hour with 20 μM nocodazole before adding to the same medium 5 μg/ml BFA for an extra hour. As seen in Fig. 4, rab6 antigen remained in fragmented Golgi structures, ressembling the ones obtained by nocodazole treatment alone. However, these structures looked somewhat more patchy. Double-immunofluorescence staining revealed that CTR 433 colocalized in the very same structures. In the case of TGN 38, nocodazole alone gave rise to small fragmented structures dispersed all over the cytoplasm, while additional BFA treatment induced the appearance of vesicles concentrated at one end of the nucleus and dispersed staining, as described by Reaves and Banting (1992). Finally, β-COP was used as a control marker. We confirm in NIH-3T3 cells previous results obtained on NRK cells (Donaldson et al., 1990) that microtubule disruption did not inhibit the release of β-COP induced by BFA, while it remained in fragmented Golgi structures after nocodazole treatment alone. These results are in favor of the involvement of the microtubule network in the redistribution of rab6p after BFA treatment.

Other agents known to inhibit BFA-induced redistribution of Golgi proteins in the ER are metabolic poisons, such as the combination of 50 mM 2-deoxyglucose with 0. 05% sodium azide, which results in ATP depletion. It has been reported that ATP depletion alone disrupts the association of β-COP with the Golgi apparatus but not that of mannosidase II, the medial Golgi resident enzyme (Donaldson et al., 1990), nor TGN 38 (Reaves and Banting, 1992).

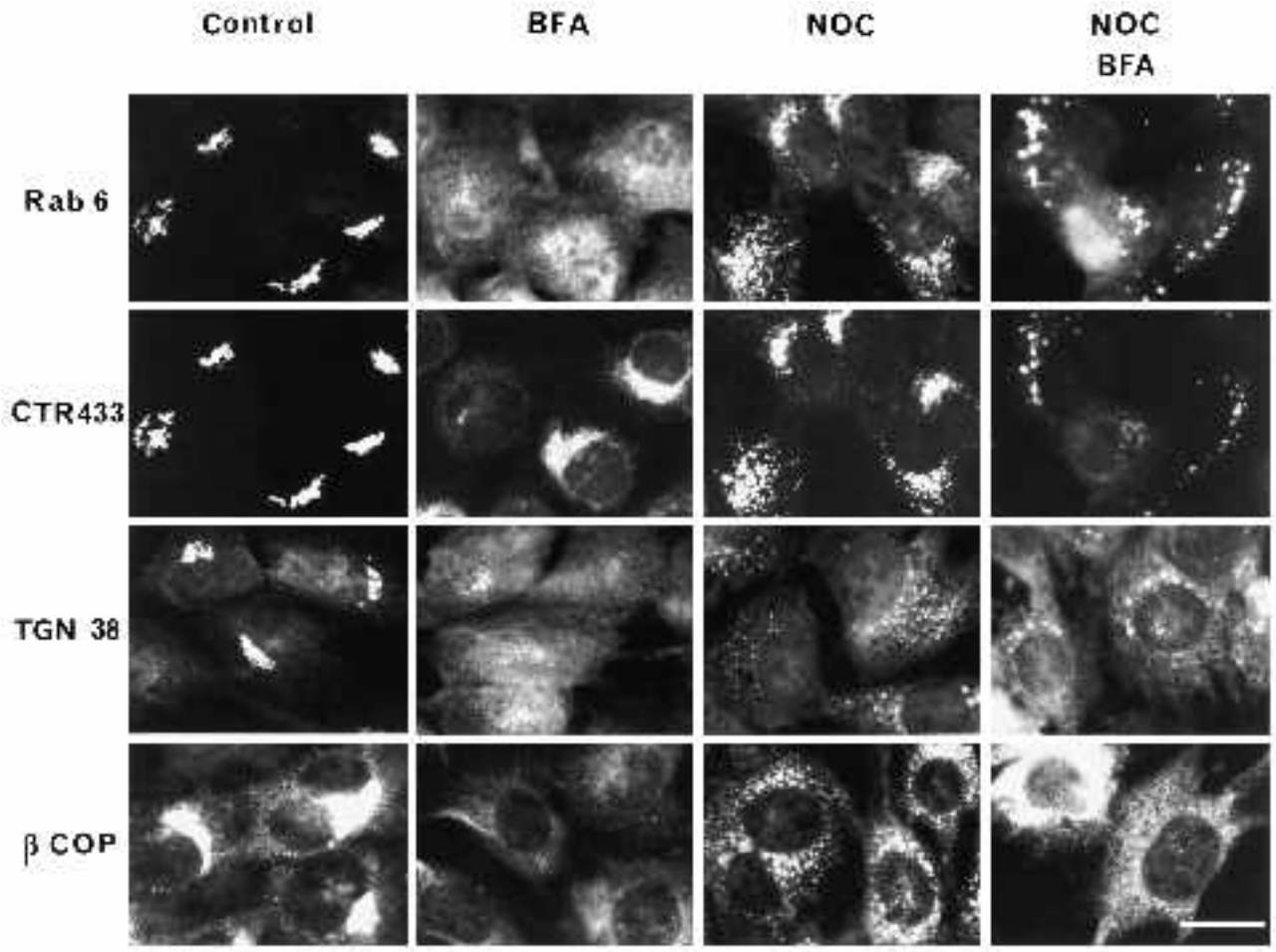

Fig. 4. Effect of nocodazole on BFA-induced redistribution of rab6p and other Golgi and TGN markers. NIH-3T3 cells were left untreated, or incubated with BFA (5 μg/ml) for 1 hour, or incubated with 20 μM nocoadazole (NOC) for 1 hour, or incubated with nocodazole for 1 hour and then nocodazole plus BFA for an additional hour. Cells were then fixed, permeabilized and stained for indirect immunofluorescence using antibodies directed against rab6p and CTR 433 (double labelling), TGN 38 or β-COP. Microtubule integrity is required for BFA-induced redistribution of rab6p, since this antigen remains in fragmented Golgi structures when cells are pretreated with nocodazole. Bar, 10 μm.

However, ATP depletion blocks the ability of BFA to induce return of mannosidase II to the ER (Donaldson et al., 1991b) but has a much less sensitive effect on the concentration of TGN 38 on MTOC in NRK cells (Reaves and Banting, 1992). β-COP is still found dispersed after sequential action of metabolic poisons and BFA (Donaldson et al., 1991b). These observations were confirmed in NIH-3T3 cells as shown in Fig. 5, with a slightly more sensitive effect of ATP depletion on TGN 38: alone, this treatment for 10 minutes induced the beginning of the redistribution of the antigen, which sometimes could be seen in tubulo-vesicular structures, and if BFA was added for an extra 30 minutes, TGN 38 was found dispersed in the whole cytoplasm and not so concentrated at one end of the nucleus as expected for an MTOC localization. We have studied the effect of 2-deoxyglucose and sodium azide on rab6p and CTR 433. If added to cells for 10 minutes, these compounds had no clear effect as compared to control cells. On subsequent addition of BFA, retrograde movement of CTR 433 was partially impaired, the antigen being found in long tubulo-vesicular structures, like the ones present at early stages of BFA action (compare with Fig. 1). Rab6p was equally affected by such a treatment and also found in tubulo-vesicular structures, most of them being labelled with CTR 433 in double immunofluorescence. The same results were obtained when BFA was added for 10 minutes instead of 30 minutes (data not shown). These results support the hypothesis that ATP is required not only for BFA-induced return of resident Golgi proteins (mannosidase II, CTR 433) to the ER, but also for the redistribution of rab6p.

Aluminium fluoride inhibits BFA-induced redistribution of rab6p

Donaldson et al. (1991a,b) have reported a protective effect of aluminium fluoride ($AlFn^-$) on dissociation of β-COP induced by BFA (or by ATP depletion) and of mannosidase II (Donaldson et al., 1991b), but not of ARF (Donaldson et al., 1991a). $AlFn^-$ (n=3-5), the active species of NaF and $AlCl_3$, potently and reversibly activates trimeric G proteins, but not small GTP-binding proteins (Sternweis and Gilman, 1982; Kahn, 1991). $AlFn^-$ mimics the γ phosphate of GTP when bound to the GDP-bound form of the

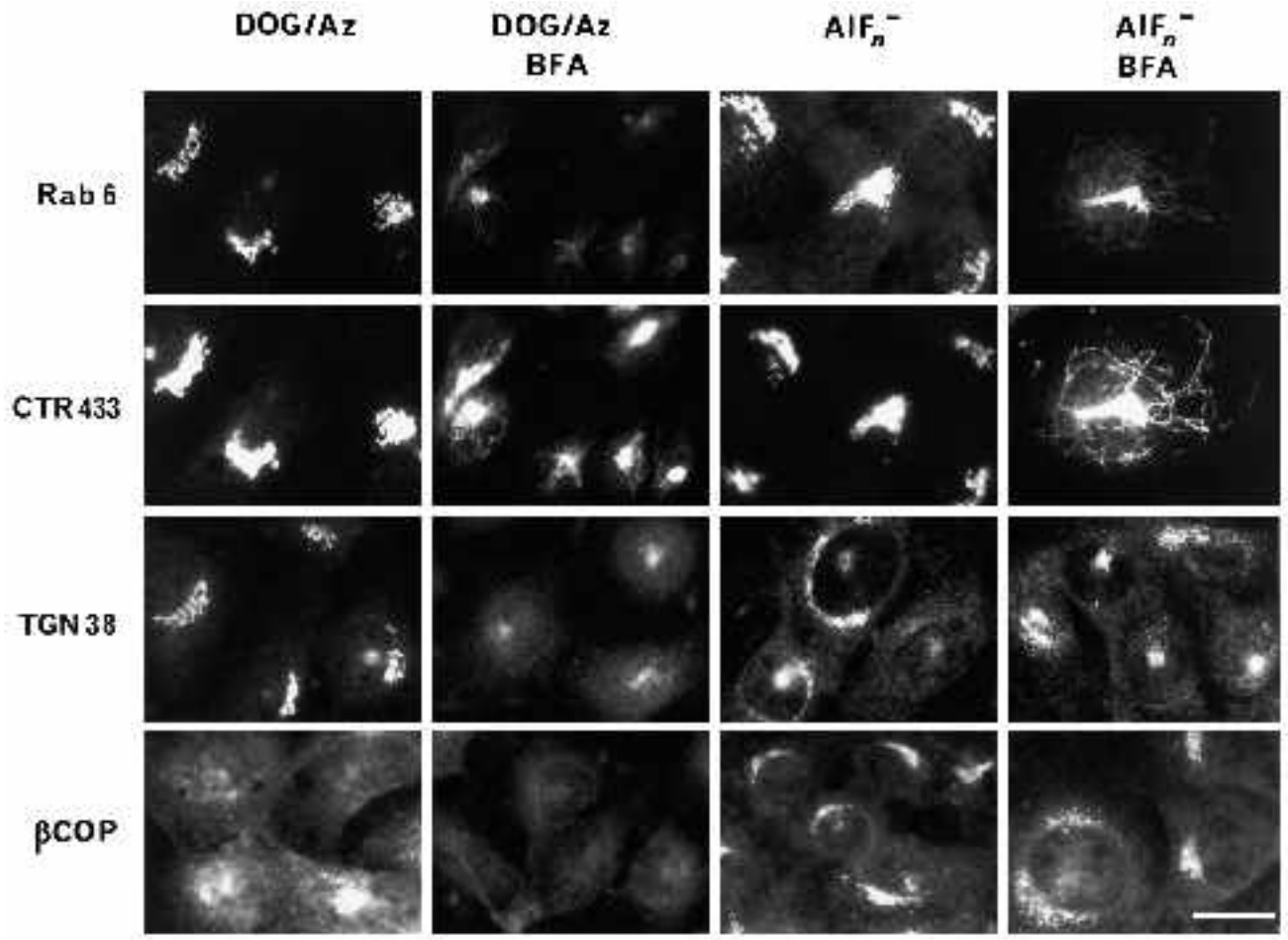

Fig. 5. Energy poisons and aluminium fluoride inhibit BFA-induced redistribution of rab6p. NIH-3T3 cells were depleted in ATP with 50 mM 2-deoxyglucose plus 0. 05% sodium azide for 10 minutes (DOG/Az), or were depleted in ATP for 10 minutes followed by BFA-treatment in the presence of DOG/Az for 30 minutes. ATP depletion partially protects rab6p from being redistributed in the cytosol after BFA. CTR 433 is also found in tubulovesicular structures. The same treatment has no effect on TGN 38, found in the microtubule-organizing center as after BFA-treatment alone. β-COP is already dispersed in the cytosol by ATP depletion alone. $AlFn^-$, when added alone in the form of 30 mM NaF and 50 μM $AlCl_3$ for 10 minutes has no effect on localization of rab6p, CTR 433 nor β-COP, and a slight dispersing effect on TGN 38. If this treatment is followed by addition of BFA for 30 minutes (or 10 minutes, data not shown), redistribution of rab6p and CTR 433 in the cytosol and the ER, respectively, is largely impaired, β-COP is totally protected, while TGN 38 is unprotected and found in the microtubule-organizing center. Bar, 10 μm.

α subunits of large G proteins (Bigay et al., 1987). This suggests that a large G protein is involved in coat protein assembly and thus interfers with BFA action. We wondered if $AlFn^-$ would have such a protective effect on the dissociation of rab6p caused by BFA. Pretreatment of NIH-3T3 cells with 30 mM NaF and 50 μM $AlCl_3$ for 10 minutes before addition for 30 minutes of 5 μg/ml BFA prevented both rab6p and CTR 433 from complete dissociation from Golgi membranes. Instead, they were found colocalized in tubulo-vesicular structures extending away from a perinuclear focus and reminiscent of those observed at early times of BFA treatment (Fig. 5). We tried to reduce the time of action of BFA to 10 minutes in order to get a more complete protective effect of $AlFn^-$ on rab6p and CTR 433, but it resulted in the same pattern of staining as longer times of BFA treatment (data not shown). Alone, treatment of cells with $AlFn^-$ had no effect on rab6p nor CTR 433, which were located on the Golgi apparatus as in normal cells. We could confirm the protective effect of $AlFn^-$ on BFA-induced dispersion of β-COP as already found in NRK cells (Donaldson et al., 1991a), whereas in the same conditions, TGN 38 was unprotected and moved to what appeared as a microtubule organizing center at one end of the nucleus. Similar results have been obtained with NRK cells (B. Reaves and G. Banting, unpublished data). On the other hand, adding BFA before $AlFn^-$ could not inhibit BFA's effects (data not shown). Adding 30 mM NaF alone gave the same results (data not shown).

Characterization of cytosolic rab6p after BFA treatment.

Although β-COP was found to be dissociated from Golgi membranes more rapidly than rab6p after BFA treatment, we asked if rab6p could associate with β-COP-containing coatomers. Coatomers migrate as complexes of 11 S in bovine brain cytosol (Waters et al., 1991) or 13-14 S in rat

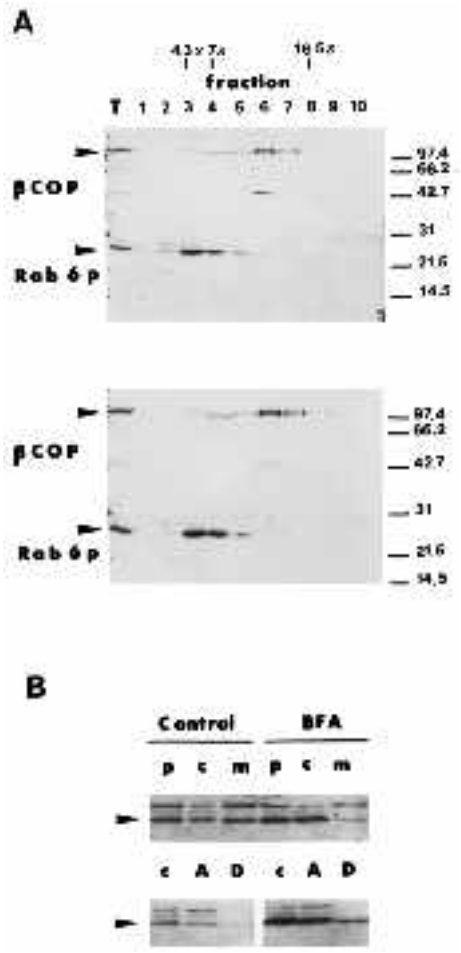

Fig. 6. The fraction of rab6p redistributed in cytosol after BFA-treatment is not associated with β-COP-containing coatomers and is mostly water soluble. Immunoblots of fractions of 5-30% sucrose gradients from control (Fig. 6A, top), and BFA-treated (Fig. 6A, bottom) BHK-21 cells show no co-sedimentation of coatomers (11 S, blotted with anti-β-COP antibodies), and rab6p (4.3 S, possibly complexed to some kind of GDI protein). T, total cytosol before centrifugation; material migrates from left to right. Markers: 4.3 S, bovine serum albumin; 7 S, human IgG; 16.5 S, thyroglobulin. Triton X-114 partitioning of cytosol (c) from control and BFA-treated NIH-3T3 cells gave rise to aqueous (A) and detergent (D) fractions, which were analyzed by SDS-PAGE and immunoblotted with anti-rab6 antibodies as explained in Materials and Methods (Fig. 6B). Almost all the cytosolic pool of BFA-treated cells partitions in the aqueous phase, as in the case of control cells. The upper band represents a contaminant protein also recognized by the pre-immune serum (Goud et al. 1990).

liver or Vero cells (Duden et al., 1991). Immunoblots of 5-30% sucrose gradient fractions from the cytosol of control and BFA-treated cells clearly showed no co-sedimentation of coatomers (as blotted with anti-β-COP antibodies) and rab6p (Fig. 6A). It should be noted at that point that rab6p, which migrates as a complex of 4.3 S, did not behave as a mere globular protein of 26 kDa, in which case it would be lighter. We do not know at present whether cytosolic rab6p forms multimers or is complexed to another protein. A good candidate for such a protein is the GDP-dissociation inhibitor protein (GDI), already characterized for three small GTP-binding proteins, including two rab proteins (rab3 Ap, Matsui et al., 1990; Sasaki et al., 1990; and rab11p, Ueda et al., 1991). Interestingly, in both control and BFA-treated cells, cytosolic rab6p seemed to migrate with the same S value.

We also performed Triton X-114 partitioning on both cytosolic and membrane fractions obtained from control cells, and compared them to similar fractions derived from BFA-treated cells. The various fractions were then run on SDS gels, and immunoblotted with anti-rab6p antibodies. As shown in Fig. 6B, rab6p from the cytosol of control NIH-3T3 cells was mostly water soluble. A small amount was repeatedly found to partition in the detergent phase. Conversely, rab6p from membranes of control cells was almost exclusively in the Triton X-114 phase (data not shown). It should be pointed out that cytosolic rab6p is probably isoprenylated, like the membrane-bound form (see below). We do not know at present why this pool then partitions in the aqueous phase of Triton X-114. One possibility could be that it is complexed to GDI or a GDI-like protein, which masks the hydrophobic moiety present on the carboxy-terminal end of rab6p. In BFA-treated cells, almost all the cytosolic pool partitioned in the aqueous phase, as did the control cytosol (Fig. 6B).

Taken together, the data on sucrose gradients and Triton X-114 partitioning suggest that cytosolic rab6p is in the same state in both control and BFA-treated cells.

Neosynthesized rab6p still attaches to membranes in the presence of BFA.

All ras-related small GTP-binding proteins so far examined are synthesized as cytosolic polypeptides on free ribosomes, and post-translationally modified at their carboxy terminal end, a condition that seems necessary for proper membrane attachment (see for example Hancock et al., 1991). Rab proteins are prenylated in the cytosol by a carbon 20 geranyl-geranyl group requiring a CC or a CXC (X = any amino acid) motif at the carboxy terminus of the molecule (Khosravi-Far et al., 1991; Kinsella and Maltese, 1992). The geranyl-geranyl transferase specific for rab proteins has recently been characterized and purified (Seabra et al., 1992a, 1992b). Interestingly, the A component of this enzyme shows striking similarity to the choroideremia protein, which itself resembles rab3 A GDI protein (Seabra et al., 1992b).

We reasoned that as BFA was able to redistribute the fraction previously attached to membranes to the cytosol, maybe its presence would, conversely, impair attachment of cytosolic rab6p to membranes. In order to test that hypothesis, we pulsed NIH-3T3 cells (control and pretreated for 30 minutes with 5 μg/ml BFA) with [^{35}S]methionine plus [^{35}S]cysteine, and either directly processed them for membrane and cytosol fractionation, or chased them 1 hour before fractionation (in the continuous presence of BFA for the treated cells). Rab6p was then immunoprecipitated in each case. The results are presented in Fig. 7: newly synthesized rab6p (pulse) was mainly present in the cytosolic fraction of control cells. A doublet of bands at molecular masses 24 and 23 kDa were detected, the major one being the 24 kDa one, which corresponds to the

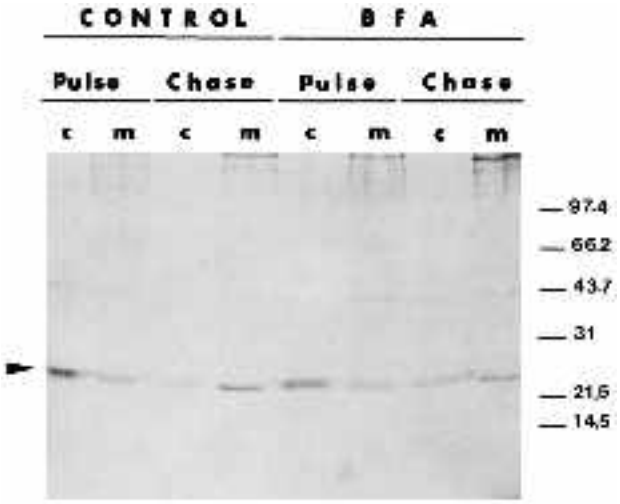

Fig. 7. Neosynthesized rab6p still attaches to membranes in the presence of BFA. NIH-3T3 cells (control and pretreated for 30 minutes with 5 μg/ml BFA) were pulsed for 5 minutes with a mixture of [^{35}S]methionine and [^{35}S]cysteine and either directly processed for cytosol (c) and membrane (m) fractionation, or chased for 1 hour in the continuous presence of BFA (for the treated sample), before fractionation. The immunoprecipitated rab6p, present in each fraction, was run on an SDS-polyacrylamide gel specially designed for resolution of modified and unmodified rab6p (see Materials and Methods for details), and the gel was fluorographed. The newly synthesized rab6p is mainly present in the cytosol after 5 minutes of pulse, in an unmodified 24 kDa form, both in control and drug-treated cells. After 1 hour of chase, it migrates slightly faster (apparent molecular mass 23 kDa), as the prenylated form, and is mostly membrane-bound. Thus, BFA does not seem to impair attachment of newly synthesized rab6p to membranes.

unprocessed form of the protein (Yang et al., 1992). After 1 hour in chase medium, rab6p was found essentially in the membrane fraction, in a form migrating slightly faster (apparent molecular mass 23 kDa), which represents the geranyl-geranylated form of rab6p (Yang et al., 1992). A small amount of the 23 kDa form was also present in the cytosol. The same pattern of migration was observed in the case of BFA-treated cells, suggesting that the drug does not impair attachment of newly synthesized rab6p to membranes. These results also favor the idea that post-translational modifications of rab6p are not prevented by the presence of BFA.

DISCUSSION

First described as a fungal product that inhibits vesicular transport from the ER (Misumi et al., 1986), brefeldin A is a unique drug affecting molecular mechanisms that regulate membrane traffic and organelle structure. Its effects have been best documented on the Golgi apparatus, causing Golgi content and membrane to redistribute into the ER (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989; Doms et al., 1989), but more recently it has been shown to affect most of the organelles involved in intracellular transport, such as TGN, endosomes and lysosomes (Wood et al., 1991). In this article, we present evidence that BFA has a dramatic effect on the localization of the ras-like small GTP-binding protein, rab6p, thought to play a key role in the regulation of membrane traffic at the Golgi and/or post-Golgi level(s). We show by indirect immunofluorescence and biochemical techniques that rab6p is redistributed to a cytosolic pool.

Redistribution of rab6p in BFA-treated cells is a biphasic process

One of the main findings of this study is that the redistribution of rab6p from the Golgi complex is a biphasic process. The first phase of redistribution of rab6p is observed during 0-15 minutes incubation of cells with BFA. During this time, the kinetics of disappearance of rab6p from the Golgi apparatus as well as the morphological aspect of the intermediate structures stained with anti-rab6p antibodies suggest that its redistribution is coupled to the flow of membrane traffic induced by BFA treatment. Indeed, after a short period of cells in BFA, we have detected rab6p in long, 'necklace' structures extending from the Golgi apparatus and double-labelled with the medial Golgi marker CTR 433. These structures are present in the Golgi complex of normal cells, but are prominently observed in brefeldin A-treated cells. They have been shown to correspond to tubular and tubulovesicular processes possibly involved in the retrograde transport of proteins from the Golgi complex back to the intermediate compartment and ER (Lippincott-Schwartz et al., 1990). In that respect, rab6p, found associated with the cytoplasmic face of medial and *trans*-Golgi cisternae (Goud et al., 1990), behaves, after short incubation in BFA, as a Golgi resident protein such as a mannosidase II or galactosyltransferase (Lippincott-Schwartz et al., 1990). We have recently shown that, in addition to the Golgi stacks, part of rab6p is present in the TGN of NIH-3T3 cells (Antony et al., 1992). BFA also affects the morphology of the TGN at least in some cell lines: the formation of tubular structures emerging from this organelle and thought to fuse with the endosomal network has been documented in NRK cells (Wood et al., 1991). Since antibodies against TGN 38 and rab6p that were available for this study were both polyclonal, we have not been able to perform double-immunofluorescence experiments. However, it seems probable that tubular structures stained with anti TGN 38 antibody also contained rab6p.

The second phase of redistribution of rab6p could be visualized in immunofluorescence experiments after 10-15 minutes of incubation with BFA. At that time, rab6p dissociated from membranes and redistributed into the cytosol, as confirmed by cell fractionation experiments. Although both immunofluorescence and cell fractionation studies do not show definite proof of a cytosolic redistribution of rab6p, they constitute a good body of evidence. While rab6p redistributed into the cytosol, CTR 433 acquired a typical ER staining and TGN 38 began to concentrate in structures reminiscent of the microtubule-organizing center (MTOC). We do not know at present whether rab6p can reach these compartments and then dissociates or whether its release into the cytosol occurs when rab6p is still associated with tubulo-vesicular structures. So far, the only proteins that have been shown to be released from membranes after BFA

treatment are coat proteins. One of the earliest and best studied detectable effects of BFA (within 1-2 minutes after the addition of the drug) is the release into the cytosol of β-COP, a component of the non-clathrin coated vesicles thought to be involved in intra-Golgi transport. More recently, γ-adaptin, a component of clathrin-coated vesicles, which mediate vesicular transport between TGN and endosomes, has also been shown to be released in the cytosol of BFA-treated cells (Robinson and Kreis, 1992). Several hypotheses have been put forward to explain the BFA effect on coat proteins. However, it is likely that BFA does not enhance dissociation of coat proteins from membranes, but rather inhibits their binding. This results in the interruption of anterograde transport and, as a consequence, retrograde transport visualized by the formation of tubulovesicular processes, is favoured (Lippincott-Schwartz et al., 1990). From the results presented here, the redistribution of rab6p into the cytosol appears to be a completely different phenomenon from that observed for coat proteins. Indeed, the release of rab6p from membranes takes much longer than was described for coat proteins. Also, it does not seem to occur if the formation of tubulovesicular processes is impaired. This was demonstrated in experiments performed in the presence of nocodazole, which blocks the formation of tubulo-vesicular structures, but does not prevent the redistribution of β-COP in the cytosol. ATP depletion, either alone or combined with BFA treatment, leads to β-COP redistribution in the cytosol while rab6p release from Golgi membranes is prevented. The cytosolic fraction of rab6p is clearly distinct from the coatomer complexes, which include β-COP, as seen by sucrose gradient centrifugation. In addition, rab6p was not released in the cytosol of cells insentitive to BFA action, such as MDCK (data not shown).

The real target of BFA is still a matter of speculation. Evidence indicates that the assembly of coat proteins on membranes is controlled by GTP-binding proteins. Candidates for such proteins are heterotrimeric G proteins (Ercolani et al., 1990; Stow et al., 1991; Leyte et al., 1992; Pimplikar and Simons, 1993; reviewed in Barr et al., 1992) and proteins of the ARF family (Donaldson et al., 1991, 1992a). Since morphological changes induced by BFA can be prevented by GTPγS and aluminium fluoride, it is possible that BFA interferes with the action of G proteins or ARF proteins on coat assembly. Very recently, it has been shown that BFA affects the mechanism of GDP/GTP exchange of ARF proteins, suggesting that its primary target could be exchange factor(s) modulating ARF function (Donaldson et al., 1992b; Helms and Rothman, 1992). In our study, we also show that aluminium fluoride prevents rab6p redistribution in the cytosol. However, as in the case of nocodazole experiments, it is likely that rab6p does not redistribute after pre-incubation of cells with AlF_4 because the formation of tubulo-vesicular processes is impaired.

Possible mechanism of BFA-induced rab6p redistribution

Proteins of the rab/Sec4/Ypt1 family are thought to cycle between membranes and cytosol in order to fulfill their function. Although this 'cyclic' model, originally formulated by H. Bourne (1988), has not yet received direct confirmation, several observations support it. At the steady state, a variable amount of rab/Sec4/Ypt1 proteins (up to 50% of the total protein) is found in the cytosol and evidence exists that this pool is in equilibrium with the membrane-bound form (Walworth et al., 1989; Fischer Von Mollard et al., 1991). Cytosolic forms of the proteins are probably kept in their GDP-bound conformation through interaction with GDI, as exemplified in the case of smgp25/rab3A (Sasaki et al., 1990). Since cytosolic proteins seem to be isoprenylated as membrane-bound forms, another function of GDI could be to mask their lipid moiety, thus preventing membrane binding. Conversely, it has been shown that rab proteins can be released from membranes, a process that could be coupled to GTP hydrolysis. Release from membranes might occur through interaction with GDI, as documented by Takai and co-workers for smg p25A/rab3A (reviewed in Takai et al., 1992). In our study, two mechanisms could explain the accumulation of cytosolic rab6p in BFA-treated cells: BFA may induce the dissociation of membrane-bound rab6p from the Golgi complex or, alternatively, inhibit the binding of the pre-existing soluble pool. We cannot rule out for the moment either of these two possibilities. However, we have shown that BFA does not prevent membrane attachment of newly synthesized rab6p in BFA-treated cells. This experiment indicates that the isoprenylation process of rab6p, which is required for stable membrane attachment, is not affected by BFA. In addition, it suggests that the processed protein can be bound to membranes, even in a context where target Golgi membranes have been mixed with others membranes upon BFA action. One cannot exclude the possibility that the membrane attachment process of preexisting soluble rab6p is somewhat different from the one involved in the attachment of newly synthesized rab6p. However, a tentative explanation of the BFA effect on rab6p could be that this drug enhances release of rab6p from membranes by stimulating the action of GDI (or a GDI-like molecule). Such an effect on GDI could also account for inhibition of rebinding of the proteins that have been dissociated from membranes. Alternatively, BFA could reduce the nucleotide exchange reaction on rab6p before attachment to Golgi membranes, similar to what has been described for ARF proteins.

According to the cyclic model, any change to the balance between membrane-bound and cytosolic forms of rab/Sec4/Ypt1 proteins might lead to an alteration in the function of these proteins. So far, no direct evidence exists that modification in the intracellular repartition of any protein of this family affects its function. However, such a link is suggested in the studies that have shown that two rab proteins, rab1A and rab4, are phosphorylated by p34cdc2 kinase in mitotic cells (Bailly et al., 1991). The phosphorylation event, which occurs in the case of rab4 near the C terminus (Van der Sluijs et al., 1992a), modifies the repartition between cytosol and membrane-bound pools. In that case, most of the protein is found in the cytosol of mitotic cells. This event could be correlated with the arrest of endocytosis observed in mitotic cells (Sager et al., 1984; Warren et al., 1984). Accordingly, the redistribution of rab6p observed in BFA-treated cells could be linked to an interruption of the action of this protein.

An interesting observation is that, among rab proteins, only rab6p seems affected by BFA. The three other rab proteins we have studied in this article (rab1A, rab2 and rab4) remained in a membrane-bound versus cytosolic state ratio comparable before and after BFA-treatment of cells. Rab9 protein is also not affected by BFA (Lombardi et al., 1993). Since more than 20 rab proteins have now been identified, it is difficult to draw a general conclusion. However, one can note that BFA perturbs the distribution of the only rab protein that is associated with the Golgi complex. Although BFA is now recognized as having broad action on transport compartments, its most dramatic effect is on the Golgi complex. Future studies should help to unravel this point and to determine whether rab6p function is arrested in BFA-treated cells or if redistibution in the cytosol can only be considered as a 'side effect' of the addition of the drug, caused by the general disruption of the Golgi apparatus.

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