Functional Protein Prenylation Is Required for the Brefeldin A-dependent Retrograde Transport from the Golgi Apparatus to the Endoplasmic Reticulum

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In cells exposed to brefeldin A (BFA), enzymes of the Golgi apparatus are redistributed to the endoplasmic reticulum (ER) by retrograde membrane flow, where they may cause modifications on resident ER proteins. We have used a truncated form of the rough ER-specific type I transmembrane glycoprotein ribophorin I as a probe to detect Golgi glycosyltransferases relocated to the ER in a BFA-dependent fashion. This polypeptide (RI332) comprises the 332 amino-terminal amino acids of ribophorin I and behaves like a luminal ER protein when expressed in HeLa cells. Upon treatment of the cells with BFA, RI332 becomes quantitatively O-glycosylated by Golgi glycosyltransferases that are transported back to the ER. Here we demonstrate that pretreatment of the cells with lovastatin, an inhibitor of HMG-CoA reductase, abrogates this modification and that mevalonate, the product formed in the step inhibited by the drug, is able to counteract the effect of lovastatin. We also show by immunofluorescence using mannosidase II as a Golgi marker that the BFA-induced retrograde transport of Golgi enzymes is blocked by lovastatin, although electron microscopy indicates that BFA causes disassembly of the Golgi apparatus into swollen vesicles and tubules. Our observations support the role of a prenylated protein, such as the geranylgeranylated small G protein Rab6, in the retrograde transport from the Golgi apparatus to the ER, since lovastatin acts by inhibiting its prenylation.

A number of cytosolic proteins that are known to be involved in vesicular transport carry isoprenoid modifications required for their membrane attachment and biological activity. These include the small GTP-binding proteins of the Rab family, which play a regulatory role at various steps of vesicular transport (1, 2). Furthermore, the γ-subunit of the heterotrimeric G proteins has been demonstrated to carry an isoprenoid modification (3, 4). The key enzyme involved in the biosynthesis of the isoprenoid moieties such as geranylgeranyl or farnesyl is 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, an enzyme that can be blocked with the competitive inhibitor lovastatin (5). The requirement of isoprenoids for anterograde transport has been demonstrated for the murine leukemia virus envelope protein (6) and the cystic fibrosis transmembrane conductance regulator (Ref. 7).

The fungal metabolite brefeldin A (BFA) has been shown to interfere with various steps of the intracellular vesicular transport pathways. With respect to ER to Golgi transport, BFA treatment leads to an inhibition of anterograde vesicular transport, while the retrograde pathway seems to remain unaffected (8, 9). At the morphological level, this results in an almost complete disappearance of the Golgi apparatus and relocation of Golgi constituents to the ER. These effects are caused by preventing binding of cytosolic coat components to organelar surfaces due to the suppressed activation by BFA of ADP-ribosylation factor proteins, which are key components for the recruitment of coatomer (10).

We have previously shown that a truncated form of ribophorin I comprised of its 332 N-terminal amino acids (RI332) facing the luminal of the ER becomes a substrate for O-glycosylating enzymes that were redistributed to the ER through the action of BFA (11). Using this assay for retrograde Golgi to ER transport as well as immunofluorescence microscopy with Golgi markers, we could demonstrate that the back-transport of Golgi components to the ER requires calcium ions sequestered to intracellular stores (12). The molecular machinery that supports forward transport from the ER through the secretory pathway involves the activity of isoprenylated proteins, such as Rab proteins (10). In fact, anterograde transport is inhibited in the absence of functional protein prenylation (6, 7). We therefore wanted to investigate whether BFA-mediated retrograde protein transport from the Golgi apparatus to the ER is also compromised when the synthesis of isoprenoids is suppressed.

MATERIALS AND METHODS

Reagents—BFA was obtained from Epicentre Technologies (Madison, WI) and kept as a stock solution in methanol (5 mg/ml). dl-Mevalonic acid lactone was from Sigma. Lovastatin (mevinolin) was a generous gift from Merck, Sharp and Dohme Co. and was dissolved in ethanol. All stock solutions were kept at −20 °C. Cell culture components were from Life Technologies, Inc. All other reagents were as described previously (11, 12).

Antibodies—A polyclonal rabbit anti-ribophorin I antibody has been described previously (13–15). The monoclonal mouse anti-mannosidase II antibody 53F3C3, a marker for the medial Golgi cisternae (16), was obtained from Berkeley Antibody Co. (Richmond, CA). Texas red-conjugated affinity-purified goat anti-mouse F(ab′)2-IgG was purchased from Accurate Chemical and Scientific Corp. (Westbury, NY), reconstituted with 50% glycerol, and kept at −20 °C. For Western blotting, a guanosine 5′-3-O-(thio)triphosphate; RI332, a truncated form of ribophorin I containing its 332 NH2-terminal amino acids; NRK, normal rat kidney; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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rabbit anti-Rab6 antibody was used (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Affinity-purified goat anti-rabbit IgG conjugated to alkaline phosphatase was obtained from Cappell (Durham, NC), and the phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium was obtained from Kirkegaard and Perry (Gaithersburg, MD).

Cell Culture and Immunoprecipitation—Normal rat kidney (NRK) cells were grown in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal calf serum, d-glucose (4.5 g/liter), L-glutamine (4 mM), penicillin (100 IU/ml), streptomycin sulfate (100 μg/ml), and amphotericin B (25 ng/ml). The permanent HeLa cell transformant (HeLa-RI332) that expresses a truncated form of ribophorin I, comprising 332 N-terminal amino acids of the luminal domain of this rough ER-specific type I transmembrane glycoprotein, has been described elsewhere (15). The methodology for cell culture, radioactive labeling, immunoprecipitation, and SDS-polyacrylamide gel electrophoresis was reported previously (15). In incubations with pharmacological agents, the equivalent amount of solvent(s) was included in control cultures. SDS-polyacrylamide gradient gels (6–11%) were used to obtain an optimal resolution of the native RI332 band and the slowly migrating O-glycosylated forms, observed after BFA treatment. For immunoblotting with the anti-Rab6 antibody, HeLa-RI332 cells were rinsed twice with cold phosphate-buffered saline and then with Tris-HCl (40 mM), pH 7.5, NaCl (75 mM), EDTA (1 mM), dithioretil (1 mM), containing a mixture of proteinase inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin, 2.5 μg/ml pepstatin, 2.5 μg/ml aprotinin, 17 μg/ml benzamidine-HCl). After disruption of the cells by sonication (two 10-s bursts), the cytosolic and total membrane fractions were obtained by centrifugation (150,000 × g for 1 h). To distinguish between unmodified and prenylated forms of Rab6, the proteins were separated on a 12% Tricine gel followed by transfer of the protein bands onto nitrocellulose.

The filters were incubated with rabbit antiserum directed against Tricine gel followed by transfer of the protein bands onto nitrocellulose. and prenylated forms of Rab6, the proteins were separated on a 12% Tricine gel followed by transfer of the protein bands onto nitrocellulose.


to quantitate the change the occurrence and extent of the BFA-dependent modification on RI332 (not shown). To ascertain that the effect of lovastatin on Golgi apparatus to the ER due to the drug-induced relocation of the enzyme to the ER (Fig. 2D). As demonstrated by the perinuclear appearance of the staining for mannosidase II, the BFA-mediated back-transport of the Golgi enzyme was essentially completely prevented, when the cells had been preincubated with lovastatin.

FIG. 1. The BFA-induced retrograde Golgi to ER transport is inhibited by treatment of cells with lovastatin. HeLa-RI332 cells were plated in 35-mm wells of 6-well plates to about 2 × 10⁶ cells/well. After 24 h, the cell cultures were left untreated (lanes a–d) or incubated with lovastatin in the concentration and for the time indicated (lanes e–m). Then the cells were incubated in serum- and methionine-free medium for 30 min and pulse-labeled with the same medium containing [35S]methionine (125 μCi/ml) for 10 min in the continued absence or presence of lovastatin. One sample (lane a) was placed on ice, and the other cells were chased in medium containing 7% fetal calf serum and 5 mM unlabeled methionine for up to 60 min in the presence of BFA alone (5 μg/ml; lanes b–d) or in the presence of both lovastatin and BFA (lanes e–m). Cells were lysed and processed for immunoprecipitation with a polyclonal rabbit anti-ribophorin I antibody. The samples were analyzed by SDS-polyacrylamide gel electrophoresis (6–11% gradient gel) and fluorography (24-h exposure of Kodak X-Omat AR film). RI indicates the position of the native ribophorin I.

RESULTS

To investigate the consequence of impaired isoprenoid synthesis on the BFA-induced retrograde transport of Golgi enzymes to the ER, HeLa cell transformants that permanently express the truncated ribophorin I, RI332, were used. Cells were pretreated with lovastatin at different concentrations and for different time periods, pulse-labeled with [35S]methionine, and then incubated in chase medium containing lovastatin and BFA (5 μg/ml) for up to 1 h (Fig. 1). As described previously (11), BFA treatment causes the complete conversion of RI332 to more slowly migrating species (RI332m), within 1 h, resulting from the acquisition of O-linked sugars after the redistribution of Golgi glycosyltransferases to the ER (Fig. 1, lanes b–d). Prenylation of cells with lovastatin at a low dose (20 μM) for 20 h leads to a significant inhibition of this posttranslational modification (lane c–g), and after a longer preincubation time (32 h; lanes h–j) or at a higher dose of lovastatin for 20 h (80 μM; lanes k–m) O-glycosylation of RI332 is completely abolished. This inhibition is due to a long time effect of lovastatin, since pretreatment of cells with this drug for only 30 min does not change the occurrence and extent of the BFA-dependent modification on RI332 (not shown). To ascertain that the effect of lovastatin is caused by the inhibitory action of this drug on HMG-CoA reductase, cell cultures were also pretreated with lovastatin together with an excess of mevalonate, the product normally formed in the step that is catalyzed by the enzyme, before performing pulse labeling and the chase incubations in the presence of BFA. As expected in this case, O-glycosylation of RI332 was observed as in cells treated with BFA alone (not shown).

From these data it appears unlikely that the inhibition of the BFA-dependent O-glycosylation of RI332 seen in lovastatin-pretreated cells is caused by a direct inhibitory effect of the drug on Golgi glycosyltransferases, since it would be difficult to understand why a relatively long time period would be required before lovastatin exerts its full inhibition. It appears more likely that a metabolite of mevalonate, which requires HMG-CoA reductase for its synthesis, plays a crucial role in retrograde transport from the Golgi apparatus to the ER. We therefore attempted to determine directly whether retrograde flow of Golgi enzymes to the ER is indeed prevented by lovastatin treatment. An immunofluorescence study using a monoclonal antibody directed against mannosidase II, a marker enzyme for the medial Golgi cis-tubular (16), was performed on cells treated with lovastatin and BFA. For these experiments, NRK cells were employed, since the antibody that specifically recognizes the rat protein does not cross-react with the protein from other species and, in addition, the Golgi apparatus in NRK cells apparatus has a more discrete and compact appearance than in HeLa cells (12, 17). Since NRK cells appeared to be less affected by lovastatin than HeLa cells by morphological criteria (see also Ref. 18), a higher dose of the drug was used (60 μM) than in experiments with HeLa cells. In control NRK cells, a perinuclear staining pattern typical for the Golgi apparatus can be observed, with the anti-mannosidase II antibody (Fig. 2A), whereas a reticular ER-like fluorescence staining that extends over the whole cytoplasm is discernible after a 30-min treatment of the cells with BFA due to the drug-induced relocation of the enzyme to the ER (Fig. 2D). As demonstrated by the perinuclear appearance of the staining for mannosidase II, the BFA-mediated back-transport of the Golgi enzyme was essentially completely prevented, when the cells had been preincubated with lovastatin.
bated with lovastatin for 28 h before BFA was added for another 30-min incubation period (Fig. 2E). Treatment of NRK cells with lovastatin alone did not affect the distribution of the Golgi marker within the cells, although a slight swelling of the Golgi apparatus may be recognized (Fig. 2B). When the pre-treatment of cells with lovastatin was carried out in the presence of an excess of mevalonate, the anti-mannosidase II antibody yielded a perinuclear staining (Fig. 2C) as in untreated control cells, while addition of BFA changed the labeling pattern to a reticular one, thus demonstrating the redistribution of the Golgi enzyme to the ER (Fig. 2F).

To examine the effects exerted by lovastatin and BFA on the structure of the Golgi apparatus, thin section electron microscopy of the HeLa cell transformants was performed. In control cells, the Golgi apparatus comprises small stacks of flattened cisternae associated with tubular-vesicular elements that may be engaged in intracellular transport processes (Fig. 3A). As shown previously (8, 17, 19), after BFA treatment, the stacks are disassembled and replaced by clusters of numerous vesicles and short tubes, frequently closely apposed to ER transitional elements (Fig. 3B). Lovastatin treatment causes swelling of the Golgi cisternae that are almost free of electron-dense material; it is notable, however, that the stacking of the cisternae is preserved at least to some extent (Fig. 3C). After exposure of lovastatin-treated cells to BFA, the stacking of the cisternae is no longer detectable for the most part, and further disassembly of the Golgi apparatus resulting in large clusters of dilated vesicles and possibly very short tubules is discernible (Fig. 3D). The vast dislocation of the membrane-bounded Golgi elements in lovastatin-treated cells may be the reason for the increased size of the Golgi apparatus observed by immunofluorescence labeling (see Fig. 2, B and E).

Next, we wished to examine how rapid the inhibitory effect of lovastatin on the BFA-dependent retrograde transport from the Golgi apparatus to the ER would be reversed after removal of lovastatin. For this purpose, HeLa-RI332 cells were incubated with lovastatin for 28 h, and then the drug was removed for different time periods, after which the acquisition of O-linked sugars by RI332 was assessed by pulse labeling and chase incubations in the presence of BFA (Fig. 4). After removal of lovastatin from the cell cultures for only 30 min, the electrophoretic mobility of RI332 remained unchanged during the chase period that was carried out in the presence of BFA (Fig. 4, lanes h and i), as seen in untreated control cells (lanes a–c) or in lovastatin-pretreated cells and with BFA present during the chase incubations (lanes f and g). Since the BFA-dependent modification of RI332 did not occur after short term removal of lovastatin, it may be concluded that the retrograde transport has not been restored within this time. However, when lovastatin was removed for 12 h from the drug-pretreated cell cultures, the kinetics of BFA-dependent O-glycosylation of RI332 was only slightly slower than in control cells treated with BFA (compare lanes f and k with lanes d and c), and 24 h after removal of lovastatin (lanes l and m), the BFA-mediated process was as rapid as in BFA-exposed control cells (lanes d and c). The resumption of BFA-induced retrograde Golgi to ER transport was not accelerated when an excess of mevalonate was included in the medium after removal of lovastatin from the pretreated cells (not shown). Taken together, these data indicate that it takes a significant amount of time, corresponding to the duration of at least half a cell cycle, to restore BFA-dependent retrograde transport after lovastatin treatment.

It has been shown that the actin cytoskeleton is severely affected by lovastatin treatment of cells (18). It was concluded that a short-lived protein that promotes severing and/or depolymerization of actin cables is normally inhibited by a prenylated protein(s) that is required for actin polymerization and that is only active in its prenylated state. In lovastatin-treated cells the prenylated protein is degraded over time, and no new active protein can be synthesized, since prenylation is inhibited by the drug, resulting in the depolymerization of the actin cytoskeleton. In the presence of the inhibitor of protein synthesis, cycloheximide, in addition to lovastatin, however, the short-lived protein is also depleted, and the lovastatin-induced

**Fig. 2.** The BFA-induced redistribution of Golgi constituents to the ER is inhibited by lovastatin. NRK cells were incubated in the absence (A) or presence of BFA (5 μg/ml) for 30 min (D). Other cultures were treated with lovastatin alone (60 μM) (B) or with lovastatin together with mevalonic acid lactone (25 mM) (C) for 28 h. Another sample was treated with lovastatin for 28 h, followed by a 30-min incubation with this drug together with BFA (E), and one dish was treated with lovastatin and mevalonic acid lactone for 28 h and then for a 30-min period with both drugs and BFA together (F). The cells were fixed, permeabilized, and labeled for immunofluorescence microscopy by incubating the samples sequentially with a monoclonal antibody directed against mannosidase II, a marker of the medial cisternae of the Golgi apparatus, followed by Texas red-conjugated goat anti-mouse F(ab’)2-IgG. Bar, 20 μm.
depolymerization of the actin filaments does not occur (18).

We were interested in determining whether the inhibitory action of lovastatin on BFA-induced retrograde transport could be a consequence of the destabilizing effect of the drug on actin filaments. Therefore, HeLa-RI332 cells were pretreated with lovastatin for 24 h before they were incubated with lovastatin together with cycloheximide for 4.5 h to deplete short lived proteins from the cells. After a short washout of cycloheximide, retrograde transport was examined by pulse labeling followed by chase incubations in the presence of BFA (Fig. 5). It is apparent that the BFA-dependent O-glycosylation of RI332 is inhibited in lovastatin-treated cells also in the presence of cycloheximide (Fig. 5, lanes g and h). As already previously reported (11), cycloheximide does not prevent the BFA-induced modification on RI332 (compare lanes k and l with lanes c and d). To assess the potential importance of the integrity of the actin cytoskeleton for the BFA-induced retrograde transport more directly, cell cultures were treated with cytochalasin D, an inhibitor of microfilament assembly (20), for 1.5 h before pulse labeling and chase incubations in the presence of BFA. It is noticeable that the BFA-dependent O-glycosylation of RI332 occurs with the same kinetics in cytochalasin D-treated cells that have depolymerized microfilaments compared with control cells incubated with BFA during the chase (compare lanes m and n with lanes c and d). It appears, therefore, unlikely that the actin cytoskeleton is involved in retrograde transport from the Golgi apparatus to the ER and in the lovastatin-caused inhibition of this process.

Members of the family of Rab proteins have been implicated in various steps in the vesicular transport that functions between organelles of the endomembrane system (2). Essential for their function is the post-translationally attached isoprene tail. Inhibition of isoprene synthesis by lovastatin is, therefore, expected to lead to the cytosolic accumulation of unprenylated Rab proteins. Since Rab6 localizes to the Golgi apparatus (21) and may in fact be involved in retrograde transport (see “Discussion”), the effect of lovastatin on the prenylation of Rab6

![Figure 3](image1.png) **FIG. 3.** Morphological changes of the Golgi apparatus after treatment of cells with lovastatin and BFA. HeLa-RI332 cell cultures were kept as controls (A) or incubated with BFA (5 μg/ml) for 1 h (B), with lovastatin (40 μM) for 28 h (C), or with lovastatin for 28 h followed by lovastatin together with BFA for 1 h (D). The cells were processed for transmission electron microscopy. Bar, 0.5 μm.

![Figure 4](image2.png) **FIG. 4.** The lovastatin-caused inhibition of retrograde Golgi to ER transport that is dependent on BFA is only slowly reversed after removal of lovastatin. HeLa-RI332 cells were left untreated (lanes a–e) or treated with lovastatin (40 μM) for 28 h (lanes f–m), after which time the cell cultures (lanes h–m) were washed with and transferred to lovastatin-free medium for the time indicated. Two cell cultures were kept in the medium containing lovastatin (lanes f and g). All cell cultures were then incubated in methionine-free medium for 30 min and pulse-labeled with [35S]methionine for 10 min in the continued absence or presence (lanes f and g) of lovastatin. The cells were chased for up to 40 min in the absence of drugs (lanes a–c), in the presence of BFA alone (5 μg/ml) (lanes d, e, and h–m), or in the presence of both lovastatin and BFA (lanes f and g). The samples were analyzed by fluorography of SDS-polyacrylamide gels that had been loaded with the immunoprecipitates obtained from cell lysates.
was assessed (Fig. 6). To this effect HeLa-R132 cells were treated with lovastatin, and cytosolic (lanes a and b) and membrane fractions (lanes a’ and b’) were obtained. As shown in Fig. 6, in control cells (lanes b and b’) Rab6 found in the cytosol or attached to membranes are prenylated as indicated by the somewhat faster electrophoretic mobility compared with the nonprenylated form. On the other hand, treatment of cells with lovastatin results in the accumulation of a nonprenylated form of Rab6 in the cytosol (lane a), while small amounts of the prenylated form are found associated with membranes (lane a’). By analyzing the distribution of Rab6 between the cytosolic and the membrane fraction, using a quantitative Western blotting assay, it was shown that in control cells 65% of Rab6 is found attached to the membranes, while in lovastatin-treated cells 80% of Rab6 is recovered in the cytosol. The drastic reduction in Rab6 binding to the membranes caused by the lovastatin treatment is a reflection of the effect of this drug on prenylation, and this may account for the inhibition of retrograde transport from the Golgi apparatus to the ER. Since lovastatin treatment is expected to interfere with the prenylation of other proteins, we cannot rule out the involvement of other prenylated proteins in this trafficking step.

DISCUSSION

We have demonstrated that the BFA-mediated retrograde transport from the Golgi apparatus to the ER is inhibited by pretreatment of cells with lovastatin, while the addition of mevalonate counteracts the effect of lovastatin, indicating that mevalonate or downstream metabolites of mevalonate are required for this process. This conclusion is based on the finding that the medial Golgi marker, mannosidase II, does not relocate to the ER upon BFA treatment of lovastatin-pretreated cells; accordingly, the luminal ribosomyl I fragment RIβ2 does not undergo O-glycosylation. The observations that lovastatin treatment of Friend murine erythroleukemia cells abrogates the transport of the murine leukemia virus envelope glycoprotein from the ER to the Golgi apparatus and that the small GTP-binding proteins Rab1p and Rab6p that are associated with the ER and the Golgi apparatus, respectively, remain as nonprenylated forms in the cytosol under these conditions led to the suggestion that the geranylgeranylation of these proteins is a prerequisite for ER to Golgi transport (6). Similar observations were made for the cystic fibrosis transmembrane conductance regulator in primary cultures of human airway epithelia and lung carcinoma cells (7).

For two reasons, it is likely that the effects on protein transport of lovastatin-treated cells are related to isoprenyl lipid function. First, the lovastatin-caused suppression of protein transport could be reversed by the addition of mevalonate, the product normally formed in the step inhibited by the drug (Refs. 6 and 7 and the present study), or of the isoprenoids geranylgeranyl and farnesyl pyrophosphate, but not by the addition of the far downstream metabolite, cholesterol. Second, it has been established that other important metabolites of mevalonate, such as dolichols and cholesterol, are not depleted in lovastatin-treated cells that have been cultured in the presence of fetal calf serum (22, 23).

It has been reported that the regulation of intracellular actin polymerization depends on the function of prenylated proteins (18). Therefore, one could have speculated that the effects of lovastatin treatment on intracellular transport processes may be a consequence of the compromised actin cytoskeleton. Our data indicate, however, that the actions of lovastatin on the microfilaments are distinct from the effect of the drug on BFA-induced retrograde transport, since a short lived protein that is critically involved in the prenylation-dependent regulation of the polymerization state of the actin microfilaments appears to play no role in retrograde protein transport from the Golgi apparatus to the ER. Furthermore, disassembly of the microfilaments by cytochalasin D does not affect BFA-mediated retrograde transport.

Evidence for the localization of GTP-binding proteins to the ER and the Golgi apparatus and for their participation in intracellular transport steps along the secretory pathway has been previously reported (24–26). Moreover, inhibition of anterograde transport from the ER is sensitive to the nonhydrolyzable GTP analog, GTP•S (25, 27). The inhibition by GTP•S of BFA-induced retrograde transport from the Golgi apparatus to the ER, as assayed by the acquisition of resistance of the secretory glycoprotein α, antitransferrin with endoglycosidase H in permeabilized HepG2 cells in the presence of BFA has also been reported (28). These data and our results with lovastatin-pretreated cells are compatible with a role of a GTP-binding protein whose activity requires functional cellular isoprenylation in BFA-mediated retrograde transport. The observation that the effect of lovastatin becomes evident only after long time periods of pretreatment suggests that the endogenous pool of the functional isoprenylated compound that regulates membrane transport has to be depleted to a considerable extent. Since the resumption of retrograde transport occurs only a long time after removal of lovastatin from the cell.

Fig. 5. The inhibition by lovastatin of the BFA-induced Golgi to ER transport is not caused by changes in the actin cytoskeleton. During the pretreatment stage, HeLa-R132 cells were left untreated (lanes a–d) or were incubated with lovastatin (Lov) (40 μM) for 24 h followed by lovastatin together with cycloheximide (CHA) (5 μg/ml) for 4.5 h (lanes e–h). Other cultures were pretreated with cycloheximide alone for 4.5 h (lanes i–l) or with cycloheximide D (Cyt. D) (25 μM) for 1.5 h (lanes m and n). All cell cultures were then incubated in methionine-free medium for 30 min and pulse-labeled with [35S]methionine for 10 min in the absence of drugs (lanes a–d and i–l) or in the presence of lovastatin (lanes e–h) or cycloheximide D (lanes m and n). Finally, the cells were chased for up to 40 min in the absence of drugs (lanes a and b) or in the presence of BFA (5 μg/ml) (lanes c and d), lovastatin and cycloheximide (lanes e and f), lovastatin, cycloheximide, and BFA (lanes g and h), cycloheximide alone (lanes i and j), cycloheximide and BFA (lanes k and l), or cycloheximide D and BFA (lanes m and n). The samples were analyzed by fluorography of SDS-polyacrylamide gels that had been loaded with the immunoprecipitates obtained from cell lysates.

Fig. 6. Lovastatin-treated cells accumulate nonprenylated Rab6 in the cytosol. HeLa-R132 cells were kept as controls (lanes b and b’) or treated with lovastatin (40 μM) (lanes a and a’) for 18 h. Cells were rinsed and lysed, and cytosolic (Cyt) and total membrane (Mb) fractions were processed for immunoblotting with the anti-Rab6 antibody. The filled arrowhead marks the nonprenylated form of Rab6, while the faster migrating prenylated forms are indicated by open arrowheads.
cultures and is not accelerated by the addition of exogenous mevalonate, it may be speculated that the nonisoprenylated form of the compound acts as a dominant-negative inhibitor of the transport step.

The involvement of GTP-binding proteins in intracellular vesicular transport has been pinpointed to several discrete steps (for a review, see Ref. 29). The first step in a cycle of vesicle budding, targeting, and fusion that may be regulated by a GTP-binding protein is the recruitment of ADP-ribosylation factor and coat proteins to the donor membrane. This step appears to be controlled by a heterotrimeric G protein, since enhanced binding of ADP-ribosylation factor and coat proteins to membranes is observed when trimeric G proteins are activated by GTPγS or aluminum fluoride, and their binding is diminished upon addition of Gpγ subunits that would block the activation of Ga (30). A heterotrimeric G protein may also play a role in BFA-mediated retrograde transport, since the BFA-induced release of a coat protein from Golgi membranes is blocked after activation of a G protein with GTPγS, aluminum fluoride, or the peptide mastoparan (30, 31). In lovastatin-treated cells, it may well be that, due to the lack of isoprenylation, the Gpγ subunits accumulate in the cytosol and are no longer capable of interacting with the corresponding α-subunit or recruiting it to the membrane. Thus, the G protein may be compromised in its regulatory function in ADP-ribosylation factor and coat protein assembly, for instance, in that the Gα subunit remains constitutively activated, promotes coat protein binding to the membrane, and abrogates the downstream effect of BFA.

Another step in intracellular transport appears to be controlled by the small GTP-binding proteins of the Rab subfamily (2). These proteins also require isoprenylation to associate with membranes and to exert their function (32). Since a Rab protein in its nonisoprenylated form would be inactive, this would correspond to the state of the protein that has GDP permanently bound to it. In this respect, much has been learned from studies with mutated Rab proteins that have altered guanosine nucleotide binding or hydrolysis capacities (e.g. see Refs. 33 and 34). It has been demonstrated that Rab6, which is found associated with the Golgi apparatus, relocated to the cytosol upon treatment of cells with BFA (35). This relocation, however, is rather a slow process, since for 10–15 min after the addition of BFA Rab6 could still be localized to tubulo-vesicular structures that colocalized with a Golgi marker, thus possibly constituting membranous elements effecting retrograde transport. Rab6 has also been implicated in the intratransport functions related to Golgi trafficking (21). Overexpression of Rab6 or of a mutant variant that is locked in its GTP-bound form and remains constitutively active inhibited anterograde transport and caused dispersion of the Golgi apparatus (21). In fact, the diffuse, reticular immunofluorescence staining of medial Golgi markers observed with cells overexpressing the GDP-bound Rab6 mutant protein would even be compatible with an ER-like staining pattern. In contrast, overexpression of a GDP-bound Rab6 variant resulted in a more prominent appearance of the Golgi apparatus, possibly due to an enlargement of this compartment. An alternate interpretation of the results is, therefore, that the GDP-bound form of Rab6 is a positive regulator of retrograde transport, and its overexpression results in the transport of Golgi markers to the ER. In a recent publication from the same laboratory, it was concluded that Rab6 participates, in fact, in the retrograde transport from the Golgi apparatus to the ER (36). The results with lovastatin-treated cells presented here are in accordance with this view, considering that the nonisoprenylated form of the protein would correspond to its inactive form. In this scenario, retrograde transport from the Golgi apparatus to the ER should be inhibited. Since anterograde transport is suppressed in lovastatin-exposed cells as well (6, 7), other small GTP-binding proteins that function to promote forward flow might also be affected.

The precise role of Rab proteins in the docking and fusion cycle of vesicular carriers and how these proteins relate to the targeting and fusion machinery constituted by different soluble NSF attachment protein receptors (SNAREs) and accessory proteins are still not entirely clear (29). Although it has been proposed that Rab proteins function at a late step in vesicular transport, i.e. targeting and/or fusion (37–39), evidence has been obtained that Rab proteins may also be required for vesicle formation (2) and for the assembly of SNARE complexes (40). Our results with lovastatin-treated cells favor the possibility of an early step in retrograde transport that is affected by the drug, since the typical staining pattern for the Golgi apparatus seen in immunofluorescence labeling experiments using a medial Golgi marker is preserved after BFA treatment of lovastatin-exposed cells. Furthermore, as revealed by transmission electron microscopy, the Golgi apparatus appears to remain rather compact, although disassembly into diluted vesicles and tubules occurs. In conclusion, our results are compatible with a role of an isoprenylated protein, such as the small GTP-binding protein Rab6, in the formation of vesicles that effect retrograde transport from the Golgi apparatus to the ER.

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