

# Endosome to Golgi Transport of Ricin Is Independent of Clathrin and of the Rab9- and Rab11-GTPases

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The plant toxin ricin is transported to the Golgi and the endoplasmic reticulum before translocation to the cytosol where it inhibits protein synthesis. The toxin can therefore be used to investigate pathways leading to the Golgi apparatus. Except for the Rab9-mediated transport of mannose 6-phosphate receptors from endosomes to the *trans*-Golgi network (TGN), transport routes between endosomes and the Golgi apparatus are still poorly characterized. To investigate endosome to Golgi transport, we have used here a modified ricin molecule containing a tyrosine sulfation site and quantified incorporation of radioactive sulfate, a TGN modification. A tetracycline-inducible mutant Rab9S21N HeLa cell line was constructed and characterized to study whether Rab9 was involved in transport of ricin to the TGN and, if not, to further investigate the route used by ricin. Induced expression of Rab9S21N inhibited Golgi transport of mannose 6-phosphate receptors but did not affect the sulfation of ricin, suggesting that ricin is transported to the TGN via a Rab9-independent pathway. Moreover, because Rab11 is present in the endosomal recycling compartment and the TGN, studies of transient transfections with mutant Rab11 were performed. The results indicated that routing of ricin from endosomes to the TGN occurs by a Rab11-independent pathway. Finally, because clathrin has been implicated in early endosome to TGN transport, ricin transport was investigated in cells with inducible expression of antisense to clathrin heavy chain. Importantly, endosome to TGN transport (sulfation of endocytosed ricin) was unchanged when clathrin function was abolished. In conclusion, ricin is transported from endosomes to the Golgi apparatus by a Rab9-, Rab11-, and clathrin-independent pathway.

## INTRODUCTION

Intoxication of cells with the plant toxin ricin involves endocytosis and retrograde transport of the toxin to the *trans*-Golgi network (TGN) and the endoplasmic reticulum (ER) before translocation of the enzymatically active subunit (the A fragment) to the cytosol takes place (Sandvig and van Deurs, 1996; 1999; Wesche *et al.*, 1999). The precise site at

which ricin leaves the endocytic pathway to target the TGN is still unknown, and ricin has been localized in endosomes as well as in lysosomes (van Deurs *et al.*, 1988).

One well-characterized transport route from late endosomes (LE) to the TGN is utilized by mannose 6-phosphate receptors (M6PRs), binding mannose 6-phosphate-tagged proteins at the TGN and delivering them to LE (Goda and Pfeffer, 1988; Munier-Lehmann *et al.*, 1996). At steady state, the receptors accumulate in these two compartments although they are also found to a lesser extent on the plasma membrane and in early endosomes (EE) (Kornfeld and Mellman, 1989). Ligand dissociation occurs at the low pH of LE, from where the uncharged receptors recycle back to the TGN by a pathway dependent on the small GTPase Rab9 (Lombardi *et al.*, 1993; Riederer *et al.*, 1994).

Several lines of evidence suggest that there is more than one transport route from endosomes to the Golgi apparatus. Different transport routes between endosomes and the TGN have been suggested based on identification of proteins common to both endosomes and the TGN (Gruenberg and

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Abbreviations used: anti-CHC, antisense to clathrin heavy chain; CHO, Chinese hamster ovary; EE, early endosomes; ER, endoplasmic reticulum; ERC, endosomal recycling compartment; HMY, poly-histidine, a c-myc epitope, and a tyrosine sulfation site; M6PR, mannose 6-phosphate receptor; LE, late endosomes; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; TGN, *trans*-Golgi network.

Maxfield, 1995; Mukherjee *et al.*, 1997). Also, in polarized MDCK cells transport of ricin from endosomes to the Golgi apparatus is independently regulated at the two poles (Llorente *et al.*, 1996, 1998b). The existence of pathways mediating transport of TGN38 either directly from EE or via the perinuclear endosomal recycling compartment (ERC) to the TGN has been proposed (Ghosh *et al.*, 1998; Mallet and Maxfield, 1999). Furthermore, the Shiga toxin B-fragment has been observed in clathrin-coated structures in ERC and suggested to be transported to the TGN from this destination (Mallard *et al.*, 1998; Johannes and Goud, 2000).

In the case of ricin it has not previously been investigated whether clathrin is required for endosome to TGN transport. Earlier studies revealed that ricin endocytosed by clathrin-independent mechanisms is able to intoxicate cells and therefore probably enters the TGN. However, it is important to note that clathrin function was restored before intoxication was measured. These studies were performed by subjecting cells to low cytosolic pH (Sandvig *et al.*, 1987) or to hypotonic shock and potassium depletion (Moya *et al.*, 1985) to block clathrin-dependent endocytosis. However, in both cases further transport of ricin to the Golgi apparatus (intoxication) was studied after transfer of the cells to normal medium and continued incubation. It should be noted that, even though toxicity experiments might indicate whether or not transport to the TGN functions at a normal rate, additional changes in the retrograde transport pathway might occur and lead to misleading results. Therefore, a more direct way of monitoring endosome to TGN transport will be the quantification of a modified ricin that can be sulfated in the Golgi apparatus (Rapak *et al.*, 1997).

To investigate the routing of a ligand it is important to interfere specifically with transport steps that might be involved. Even when a given protein is not visualized in a compartment such as LE, the possibility still remains that it is passing rapidly through that compartment on its way to the TGN (Straley and Green, 2000). To exclude such a scenario, transport routes running to or through LE can be blocked, for instance by expressing dominant negative mutant Rab proteins.

In the present work we investigated whether pathways regulated by the small GTPases Rab9 and Rab11 are implicated in intracellular transport of ricin to the TGN. We have furthermore studied the importance of clathrin, because so far only the Rab9-dependent pathway to the TGN has been shown to function in a clathrin-independent way and clathrin is found not only on the cell surface and in the TGN but also on endosomes and even on lysosomes (Stoorvogel *et al.*, 1996; Traub *et al.*, 1996; Johannes and Goud, 2000). For this purpose we have established and characterized a stably transfected HeLa-TetOn/Rab9S21N cell line inducibly overexpressing sufficient mutant Rab9 to inhibit recycling of M6PRs to the TGN. Furthermore, we have transiently overexpressed dominant negative mutant and wild-type Rab11 to investigate the role of this small GTPase, which is found both in ERC and on the Golgi apparatus. Finally, we used a stable cell line in which overexpression of antisense to clathrin heavy chain (anti-CHC) can be induced by removal of tetracycline to investigate the role of clathrin in routing of ricin to the TGN. The data indicate that the Rab9- and Rab11-independent TGN transport of ricin functions with-

out clathrin, and they suggest that there may be more than one route from EE to the Golgi compartment.

## MATERIALS AND METHODS

### *Reagents and Antibodies*

The mouse anti-Rab9 antibody (Soldati *et al.*, 1993) was a generous gift from Dr. Suzanne R. Pfeffer (Stanford University, Stanford, CA). Anti-myc tag antibody (mouse ascites, 9E10) used for immunoblotting and immunoprecipitations was obtained from Dr. Harald Stenmark (Institute for Cancer Research, The Norwegian Radium Hospital, Oslo). Rabbit anti-human CI-M6PR antibody was a gift from Dr. K. von Figura, Göttingen, Germany. Anti-myc (9E10) agarose conjugate was from Santa Cruz Biotechnology, (Santa Cruz, CA). Nickel-agarose beads were from Qiagen (Chatsworth, CA) and protein A-Sepharose was from Pharmacia (Piscataway, NJ). The antibiotic geneticin was from Dufeca (Haarlem, Holland) and zeocin was from Cayla (Toulouse, France). [<sup>3</sup>H]Leucine and Na<sub>2</sub><sup>35</sup>SO<sub>4</sub> were from Amersham Pharmacia Biotech (Buckinghamshire, UK). Unless otherwise stated, the reagents were from Sigma Chemical (St. Louis, MO). Protein concentration was determined by the Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA) with the use of bovine serum albumin as the standard.

### *Cell Lines*

Chinese hamster ovary (CHO), Rab9S21N cells (N21), and mock-transfected CHO cells (CVN) were kindly provided by Dr. Suzanne Pfeffer (Riederer *et al.*, 1994) and grown in complete Ham's F-12 (7.5% fetal calf serum [FCS], glutamine, and antibiotics) containing 400 µg/ml geneticin. The stable HeLa-TetOn cell line (Clontech, Palo Alto, CA) was maintained in complete DMEM (7.5% FCS, glutamine, and antibiotics) containing 200 µg/ml geneticin. The stable HeLa-TetOn/Rab9S21N cell line (isolation described below) was cultured in complete DMEM containing 200 µg/ml geneticin and 0.5 µg/ml puromycin (select DMEM). The stable HeLa-TetOn/Rab9S21N/M6PR46HMY cell line (see below) was cultured in select DMEM containing 200 µg/ml zeocin. The HeLa cells were grown in complete DMEM (7.5% FCS, glutamine, and antibiotics). BHK21-tTA/anti-CHC cells that were stably transfected with anti-CHC (Skretting, Iversen, Stahlhut, van Deurs, and Sandvig, unpublished data) were grown in complete DMEM (7.5% FCS, glutamine, and antibiotics) containing 100 µg/ml puromycin, 0.2 mg/ml geneticin, and 2 µg/ml tetracycline. Two days before the experiments the cells were seeded out with and without tetracycline at a cell density of 1 × 10<sup>6</sup> cells/10-cm dish. All tissue culture media and reagents were purchased from Bio-Whittaker (Walkersville, MD).

### *Vectors and Constructs*

The entire coding region of wild-type Rab9 and the mutant Rab9S21N (Dirac Svejstrup *et al.*, 1994) were cloned into the *Sall*/*EcoRV* sites of the tetracycline-inducible expression plasmid pTetSplice (GIBCO/BRL, Grand Island, NY). The plasmid construct M6PR46 HMY-pME18S, encoding the 46-kDa cation-dependent M6PR tagged with poly-histidine, a c-myc epitope, and a tyrosine sulfation site (HMY), was a gift from Dr. Suzanne R. Pfeffer. Restriction fragments containing the entire coding region of wild-type Rab11 and mutant Rab11S25N (gift from Dr. Harald Stenmark) were amplified with the use of polymerase chain reaction, thereby introducing flanking *EcoRI*/*XhoI* sites. Subsequently, N-terminally myc-tagged Rab11 constructs were generated by subcloning the coding region into the *EcoRI*/*XhoI* sites of the pGEM1-myc3 vector (gift from H. Stenmark).

### *Generation of Stable Cell Lines*

The HeLa-TetOn/Rab9S21N cell line was generated by DOTAP (Boehringer Mannheim, Indianapolis, IN) cotransfection of sub-

confluent HeLa-TetOn cells expressing the transactivator-tetracycline chimera (Gossen *et al.*, 1995), with 12  $\mu\text{g}$  Rab9S21N-pTetSplice (containing the mutant Rab9S21N cDNA) and 2  $\mu\text{g}$  of plasmid pPur, according to the manufacturer's instructions (Boehringer Mannheim). After recovery in complete DMEM containing 400  $\mu\text{g}$  geneticin for 24 h, the cells were split in 1:2, 1:5, and 1:10 and cultivated in complete DMEM containing 400  $\mu\text{g}/\text{ml}$  geneticin and 1  $\mu\text{g}/\text{ml}$  puromycin (select DMEM). Individual HeLa-TetOn/Rab9S21N clones were isolated, and expression of the mutant Rab9 protein in a doxycycline-regulated manner was monitored by Western blotting. Selected HeLa-TetOn/Rab9S21N clones with no detectable background expression of mutant Rab9 were subcloned by limiting dilution. Maximal expression was reached after 18 h of induction with doxycycline (2  $\mu\text{g}/\text{ml}$ ) in subconfluent cell cultures.

Subconfluent HeLa-TetOn/Rab9S21N cells were subjected to DOTAP-mediated cotransfection with 12  $\mu\text{g}$  of M6PR46HMY-pME18S and 2  $\mu\text{g}$  of pZeoSV2 (containing the zeocin resistance gene; InVitroGene). After recovery in select DMEM for 24 h the cells were split 1:2 and 1:10 and transferred to select DMEM containing 200  $\mu\text{g}/\text{ml}$  zeocin. Single clones were isolated and constitutive overexpression of the M6PR46-HMY protein was monitored by Western blot analysis and indirect immunofluorescence microscopy with the use of anti-myc (9E10) antibody. Selected HeLa-TetOn/Rab9S21N/M6PR46HMY clones were subcloned by limiting dilution.

### Measuring Recycling of M6PR from Endosomes to the TGN by *In Vivo* Sulfation

The assay was performed by modifying an assay devised by Pfeffer and colleagues (Itin *et al.*, 1997). Accumulation of unsulfated M6PR46HMY was achieved by culturing the HeLa-TetOn/Rab9S21N/M6PR46 cell line for 3 d to subconfluency in 6-cm culture dishes in a modified sulfate-free SMEM medium (added: 1 $\times$  MEM amino acids, 1 $\times$  nonessential amino acids, 1 $\times$  L-Gln, 1 $\times$  antibiotics, 1 $\times$  vitamin solution, 1 mM sodium pyruvate, 200 mg/ml  $\text{CaCl}_2$ ) containing 7.5% FCS and 10 mM sodium chlorate [to reversibly inhibit protein sulfation; Baeuerle and Huttner, 1986],  $-/+$  induction of Rab9S21N by 2  $\mu\text{g}/\text{ml}$  doxycycline. The medium was changed daily. Subsequently, the cells were washed twice with SMEM and labeled for 3 h with 1 mCi/ml [ $^{35}\text{S}$ ]sulfate (Amersham, Piscataway, NJ) in the modified sulfate-free SMEM medium (as above but without sodium chlorate). The cells were then washed once with phosphate-buffered saline (PBS) and solubilized in 500  $\mu\text{l}$  of RIPA (50 mM Tris-HCl, pH 7.8; 150 mM NaCl; 1% sodium deoxycholate; 0.1% SDS; 1.5% Triton X-100) supplemented with 1 mM phenylmethylsulfonyl fluoride, aprotinin (30  $\mu\text{l}/\text{ml}$ ), and 25 mM imidazole. Insoluble material was pelleted in a microcentrifuge at 14,000 rpm for 10 min. The M6PR46-HMY receptors were purified by adding 15  $\mu\text{l}$  of prewashed nickel agarose beads to the cleared lysate, and the suspension was incubated while rotating for 2 h at 4°C. The precipitate was washed four times with RIPA containing 25 mM imidazole and then eluted with 50  $\mu\text{l}$  of 25 mM EDTA in RIPA and 25 mM imidazole. Sample buffer containing mercaptoethanol was added and the proteins were resolved by 10% SDS-PAGE. The gel was then subjected to fluorography.

### Electron Microscopy

After growth with or without doxycycline the HeLa cells were washed with PBS and fixed with 2% formaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. Then the flasks were cut open and the fixed cells scraped off and pelleted. The pellets were embedded in gelatin, cryoprotected, frozen, and used for ultracytosections. Immunolabeling of ultracytosections was performed with the use of rabbit serum against the human M6PR and protein A-gold (10 nm; purchased from Dr. G. Posthuma, Utrecht University) as previously described (van Deurs *et al.*, 1993; Nicoziani *et al.*, 2000).

### SDS-PAGE and Fluorography

SDS-PAGE was done as described by Laemmli (1970). The gels were fixed in 4% acetic acid and 27% methanol for 30 min. In addition, when  $^{35}\text{S}$ -labeled proteins were analyzed, the gels were treated with 1 M sodium salicylate, pH 5.8, in 2% glycerol for 15 min. Dried gels were exposed for fluorography to XAR-5 films (Kodak, Rochester, NY) at  $-80^\circ\text{C}$ .

### Western Blot Analysis

Cells were washed with PBS and harvested in RIPA with 1 mM phenylmethylsulfonyl fluoride and aprotinin (30  $\mu\text{l}/\text{ml}$ ). Insoluble material was pelleted in a microcentrifuge for 10 min at 8000 rpm. Sample buffer with mercaptoethanol was added to the cleared lysate, SDS-PAGE (10%) was performed, and the proteins in the gel were transferred to a poly(vinylidene difluoride) membrane (Immobilon-P, Millipore, Bedford, MA) by semidry blotting. After blocking with 5% nonfat dry milk powder in PBS containing 0.05% Tween-20 for 1 h, the membrane was incubated for 1 h at room temperature with the mouse anti-Rab9 antibody (1:5, in 5% milk), or mouse anti-c-myc antibody (1:1000). The membrane was then washed three times for 10 min with 0.05% Tween-20 and finally incubated with a secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature. A chemiluminescent detection reagent (SuperSignal CL-HRP; Pierce, Rockford, IL) was used for developing signals.

### Measurements of Ricin Cytotoxicity

The cells were incubated for 4 h in HEPES medium lacking leucine and with increasing toxin concentrations. The cells were then incubated in HEPES medium containing 1  $\mu\text{Ci}/\text{ml}$  [ $^3\text{H}$ ]leucine for 20 min at 37°C. The cells were then extracted with 5% trichloroacetic acid (TCA) for 10 min, followed by a brief washing in 5% TCA, and subsequently dissolved in 0.1 M KOH. The cell-associated radioactivity was measured. The kinetics of ricin toxicity was measured by incubating the cells in leucine-free HEPES medium with 1000 ng/ml ricin for various times. Subsequently, the cells were treated as described above, and the radioactivity incorporated was measured. The deviation between duplicates was <5%.

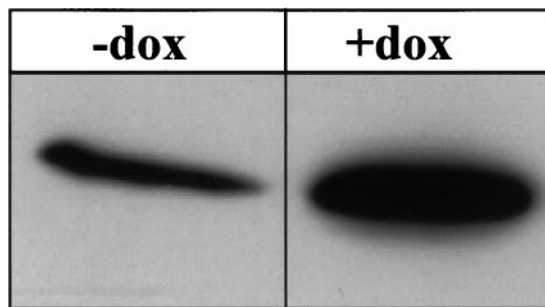
### Sulfation of Ricin sulf-1

Ricin sulf-1, consisting of a modified ricin A-sulf-1 chain containing a tyrosine sulfation site reconstituted with ricin B chain, was produced, purified, and reconstituted as previously described (Rapaport *et al.*, 1997). The cells were washed in DMEM without sulfate and incubated with 100  $\mu\text{Ci}/\text{ml}$   $\text{Na}^{35}\text{SO}_4$  in the same medium. After 4 h, ricin sulf-1 (200 ng/ml) was added, and the incubation was continued for 4 h. The medium was then removed, and the cells were washed (twice for 5 min) with a 0.1 M lactose solution in HEPES medium at 37°C and then with cold PBS, lysed in phosphate buffer (0.1 M NaCl, 10 mM  $\text{Na}_2\text{HPO}_4$ , 1 mM EDTA, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4), and centrifuged to remove the nuclei for 10 min at 5000 rpm in an Eppendorf centrifuge. The supernatant was immunoprecipitated with rabbit anti-ricin antibodies immobilized on protein A-Sepharose for 18 h at 4°C. Finally, the beads were washed twice with cold PBS containing 0.35% Triton X-100, and the adsorbed material was analyzed by SDS-PAGE (12%) under reducing conditions followed by fluorography.

### Ricin Toxicity in HeLa Cells Transiently Expressing Myc-tagged Rab11

HeLa cells ( $1 \times 10^5$  cells) were plated in 12-well trays the day before transfection. After infection for 1 h with a T7 RNA polymerase-recombinant vaccinia virus (vT7; Stenmark *et al.*, 1995), the cells were transfected with 2  $\mu\text{g}$  of the pGEM1, myc-Rab11N25. or wild-





**Figure 1.** Induced expression of the Rab9S21N mutant. The mutant Rab9S21N HeLa-TetOn cell line was grown for 20 h, with (+) or without (–) the tetracycline derivative doxycycline (dox, 2  $\mu$ g/ml). The cells were dissolved, and the lysate was run on SDS-PAGE and analyzed by immunoblotting with the use of anti-Rab9 and ECL.

type myc-Rab11 constructs under control of the T7 promoter and 6  $\mu$ l of the transfection reagent DOTAP in 0.4 ml of HEPES medium, according to the manufacturer's instructions. After 5 h of transfection at 37°C, the transfection medium was removed. Cells were washed once with HEPES and to the wells increasing concentrations of ricin holotoxin were added in leucine-free HEPES medium. Cells were then incubated in the presence of toxin for 4 h at 37°C. After intoxication, the level of myc-tagged Rab11 synthesis was quantitated by a pulse-chase experiment: cells were incubated for 15 min in methionine/cysteine-deficient MEM, radiolabeled for 15 min with 75  $\mu$ Ci of  $^{35}$ S- Easy Tag ExpreSS (NEN Life Science Products, Boston, MA), and then chased for 5 min in DMEM containing 10% serum. Cells were washed once with PBS and solubilized in 500  $\mu$ l of RIPA buffer. Myc-Rab11 proteins were immunoprecipitated from the lysate with the use of anti-myc (9E10) agarose conjugate for 18 h at 4°C. Immunoprecipitations were washed four times with RIPA buffer and subsequently analyzed by SDS-PAGE and autoradiography. Autoradiograms were scanned and the signal intensities were quantitated by densitometry with the use of a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The levels of wild-type or mutant myc-Rab11 synthesis in toxin-treated cells is reported as percentages of that found in control cells not treated with toxin.

## RESULTS

### *Endosome to TGN Transport of M6PRs Is Inhibited in Cells with Inducible Expression of Rab9S21N*

To investigate whether ricin transport to the TGN is dependent on Rab9, a HeLa cell line that could be induced by a tetracycline-derivative (doxycycline) to express Rab9S21N was generated (see MATERIALS AND METHODS). Maximal expression was quantified to be two- to fourfold higher than that of the endogenous protein level (Figure 1).

To verify that the doxycycline-induced Rab9S21N expression was sufficient to inhibit the Rab9-dependent M6PR transport, we performed an assay relying on the TGN localization of the tyrosine sulfotransferase. Tyrosine sulfation of M6PRs engineered to contain the consensus sequence for modification by this enzyme was used as a measure of endosome-to-TGN transport. Sulfur-starved control cells (–doxycycline) or cells induced to express mutant Rab9 (+doxycycline) were labeled with [ $^{35}$ S]sulfate for 90 or 180 min. Then the M6PR46HMYs were purified by cell lysis and nickel agarose binding. Only transfected cells stably expressing the M6PR46HMY construct, as confirmed by Western

analysis (Iversen, Skretting, Llorente, Nicoziani, van Deurs, and Sandvig, unpublished results), displayed a sulfate-labeled polypeptide that binds to the nickel resin (Figure 2A). Furthermore, Figure 2B shows that the sulfation of M6PR46HMYs was significantly reduced in cells expressing Rab9S21N, displaying an ~60% inhibition as compared with the control cells. Similarly, CHO cells that constitutively express mutant Rab9S21N at approximately twofold the endogenous level have been reported to be inhibited in the transport of M6PRs from LE to the TGN in vivo (Riederer *et al.*, 1994).

The steady-state level of the M6PR46HMY was found to be the same both in control cells and in the induced mutant-Rab9 cells as determined by Western blot analysis (Iversen, Skretting, Llorente, Nicoziani, van Deurs, and Sandvig, unpublished results). Quantitative electron microscopy revealed that the same appeared to be true for the steady-state level of the 300-kDa M6PR. Studies of the HeLa-TetOn/Rab9S21N cells with the use of immunogold labeling of M6PRs showed that the endosomes displayed no changes in morphology or degree of M6PR labeling after induction of mutant Rab9S21N expression (Iversen, Skretting, Llorente, Nicoziani, van Deurs, and Sandvig, unpublished results). Furthermore, the majority of the M6PRs (~90%) were localized in endosomes/multivesicular bodies. Very few receptors were observed in the TGN/Golgi, making it impossible to quantify any redistribution of M6PRs in this organelle at steady state.

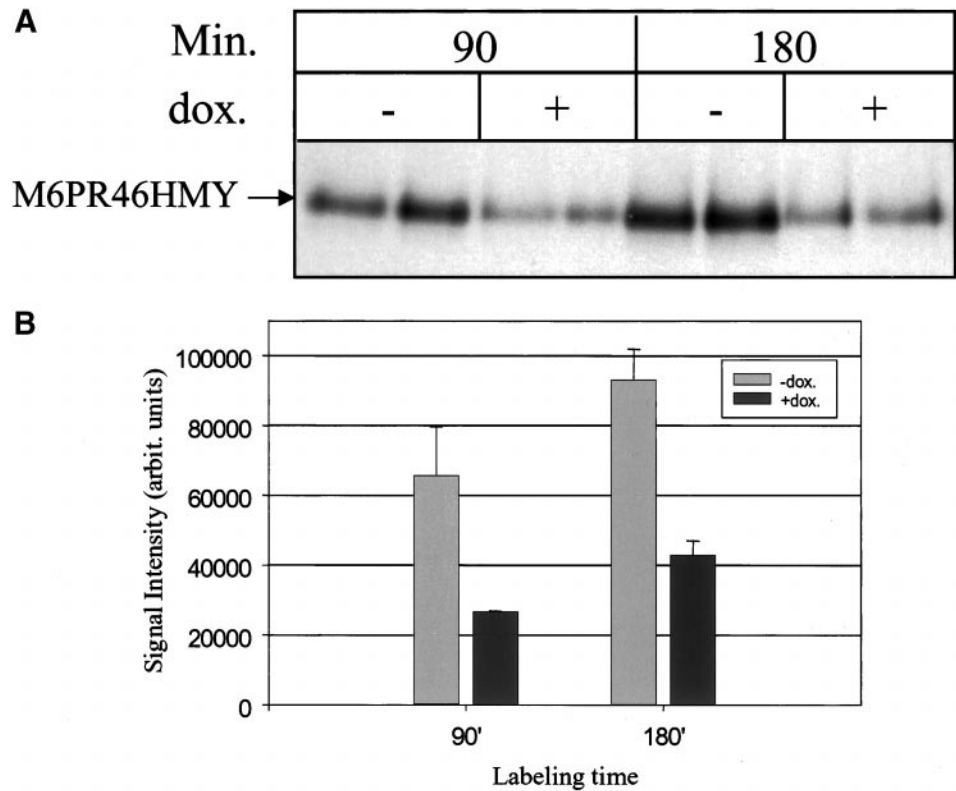
### *Effect of Rab9S21N Overexpression on Transport of Ricin to the TGN*

To investigate whether transport of ricin from endosomes to the TGN was affected by inhibition of the Rab9-mediated pathway, we measured sulfation of the modified ricin sulf-1. Figure 3 shows that sulfation of the modified ricin sulf-1 by the tyrosine sulfotransferase localized in the TGN was not affected by the doxycycline-induced expression of mutant Rab9. A control experiment showed that expression of mutant Rab9 did not affect endocytosis of ricin (Iversen, Skretting, Llorente, Nicoziani, van Deurs, and Sandvig, unpublished results). Thus, inducible expression of mutant Rab9 does not affect the transport of ricin from endosomes to the Golgi apparatus. Furthermore, CHO cells that constitutively express Rab9S21N (Riederer *et al.*, 1994) displayed a degree of sulfation similar to its control (Iversen, Skretting, Llorente, Nicoziani, van Deurs, and Sandvig, unpublished results), indicating an unchanged rate of ricin uptake to the TGN also in this cell line.

If ricin passes through a Rab9-dependent step on its way to the TGN, the expression of mutant Rab9 could possibly affect the ability of ricin to inhibit protein synthesis in the cells. However, in agreement with the lack of effect of mutant Rab9S21N on ricin sulfation, mutant Rab9 expression did not affect ricin cytotoxicity (Iversen, Skretting, Llorente, Nicoziani, van Deurs, and Sandvig, unpublished results). The kinetics of ricin toxicity was also the same in both situations.

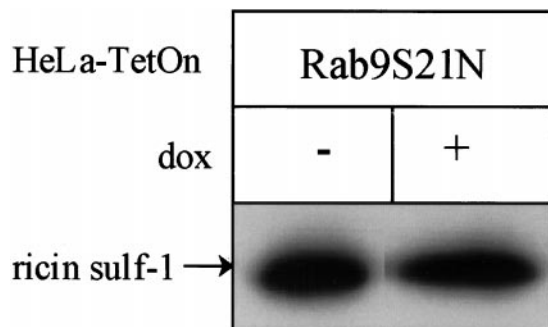
### *Effect of myc-Rab11S25N Overexpression on Transport of Ricin to the TGN*

Because ricin entry to the TGN was independent of Rab9, it might be that the toxin enters the TGN from EE and possibly



**Figure 2.** In vivo sulfation of the M6PR46 HMY. (A) SDS-PAGE displaying sulfation of M6PR46HMY purified from the HeLa-TetOn/Rab9S21N/M6PR46HMY cell line grown with (+) or without (-) Rab9S21N induction by doxycycline (dox) and then  $^{35}\text{SO}_4^{2-}$ -labeled for 90 or 180 min, respectively. (B) Intensities of the respective bands; the presence of dox. (black bar) or absence of dox. (gray bar), were determined by densitometric quantitation. Values shown represent the averages of duplicate determinations from a representative experiment (as in A), whereas the bars represent the deviations between duplicates.

via the ERC. We therefore performed a ricin sulfation experiment on HeLa cells transiently overexpressing myc-tagged wild-type Rab11 or the dominant interfering mutant Rab11N25 (Figure 4). No significant difference in ricin sul-



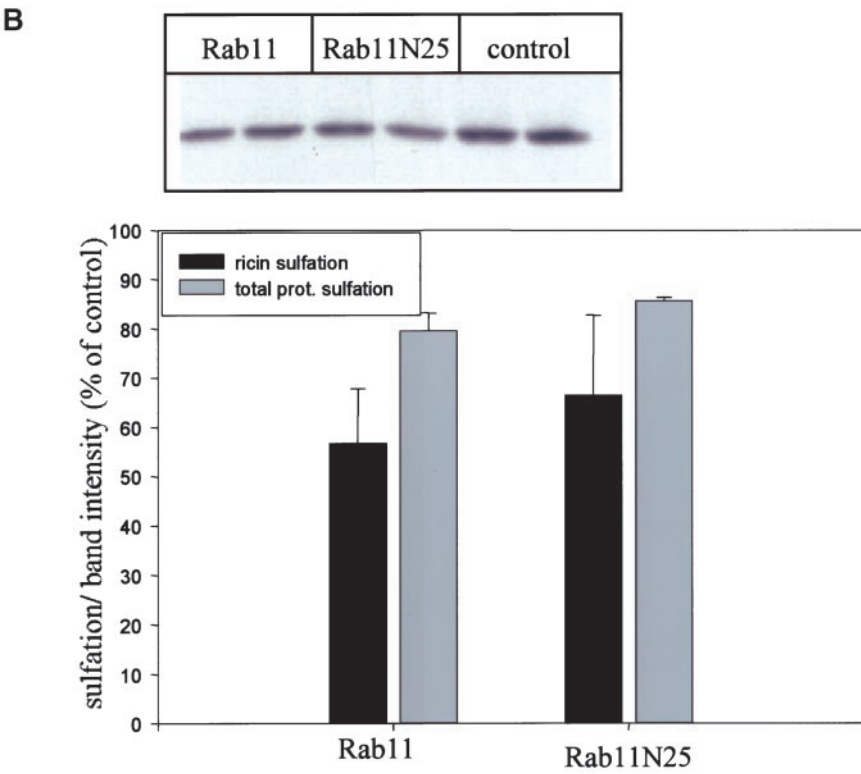
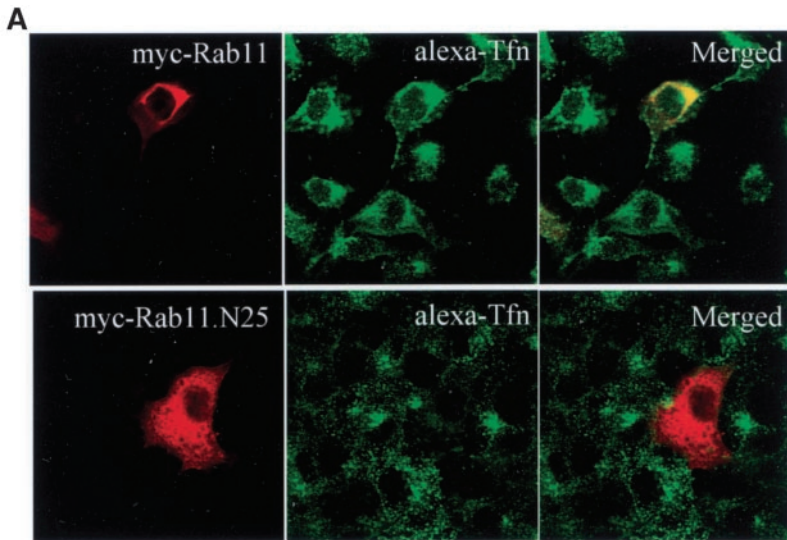
**Figure 3.** Tyrosine sulfation of ricin sulf-1 in the HeLa-TetOn cells expressing mutant Rab9S21N. The cells were grown with (+) or without (-) doxycycline (dox) for 1 d. The cells were then washed with SMEM without sulfate and incubated with  $\text{Na}_2^{35}\text{SO}_4$  (100  $\mu\text{Ci/ml}$ ) for 4 h. Ricin sulf-1 was added, and incubation was continued for 4 h. The cells were then washed with 0.1 M lactose in HEPES-medium at 37°C and with ice-cold PBS before cell lysis. The nuclei were removed by centrifugation, and the supernatant was immunoprecipitated with rabbit anti-ricin antibodies and protein A-Sepharose for 18 h at 4°C. Finally, the immunoprecipitate was analyzed by SDS-PAGE (12%) under reducing conditions followed by fluorography.

fation between cells transfected with wild-type Rab11 or with Rab11N25 was observed (Figure 4B). Immunofluorescence microscopy was performed to reveal that >60% of the cells expressed myc-tagged Rab11 proteins. Furthermore, overexpression of mutant Rab11 resulted in an inhibited transport of alexa-labeled transferrin into the ERC, as expected (Figure 4A).

We also investigated whether the toxicity of ricin was affected by mutant Rab11. The toxicity of ricin was assessed by two different experimental approaches in HeLa cells transiently overexpressing wild-type Rab11 or the mutant Rab11N25: either by a pulse-chase experiment monitoring the decrease in new synthesis of the transiently expressed myc-tagged Rab11 proteins (Figure 5; Simpson *et al.*, 1995) or by monitoring the decrease in protein synthesis in all the cells (Iversen, Skretting, Llorente, Nicoziani, van Deurs, and Sandvig, unpublished results). By both methods, overexpression of wild-type Rab11 or Rab11N25 mutant gave no effect on ricin toxicity.

### *Anti-CHC Does Not Affect TGN Transport of Endocytosed Ricin*

Clathrin has been observed on endosomes and suggested to be involved in TGN transport of Shiga toxin B-fragment. Therefore, the role of clathrin on intracellular transport of ricin with the use of BHK cells with tetracycline-inducible expression of anti-CHC was investigated. Expression of anti-CHC leads to nonfunctional clathrin-coated pits, thereby selectively inhibiting uptake by clathrin-dependent endocy-

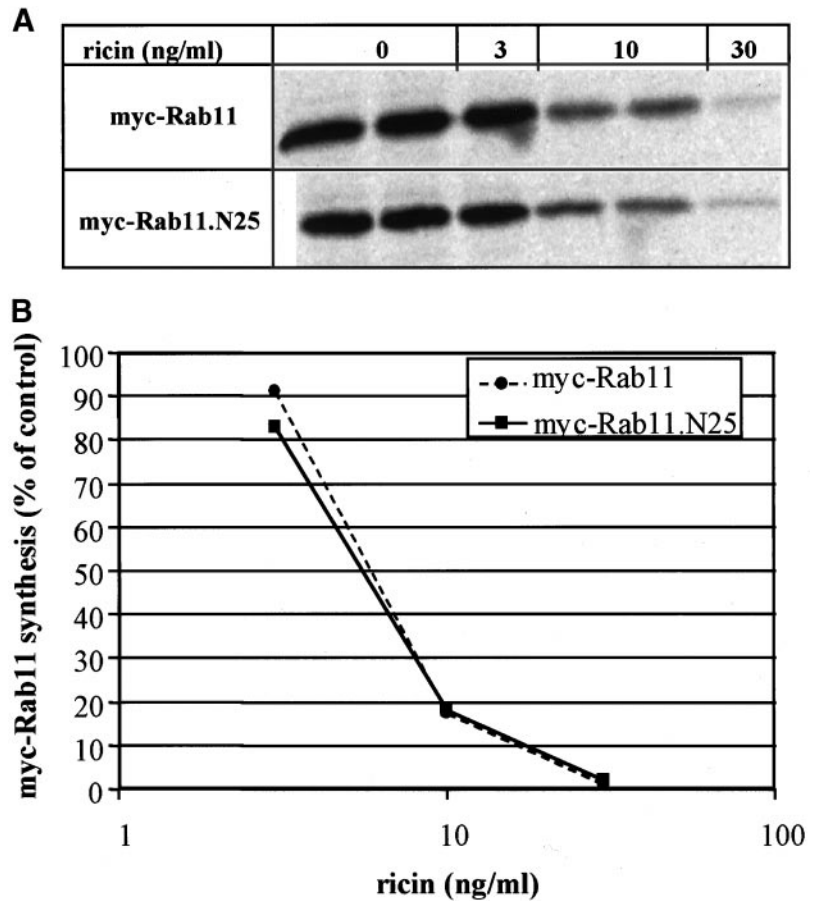


**Figure 4.** Overexpression of mutant Rab11N25 in HeLa cells does not affect ricin entry into the TGN. The cells were transfected either with myc-tagged wild-type Rab11 or myc-tagged mutant Rab11N25. Transient expressions for 5 h were allowed before any further steps. (A) Effect of myc-Rab11 on localization of transferrin. HeLa cells transfected with myc-tagged Rab11 were incubated with alexa-transferrin for 10 min at 37°C, and then the cells were fixed and stained with anti-myc (9E10). (B) Ricin sulfation was performed by preincubating the transfected cells with [<sup>35</sup>S]sulfate for 1 h followed by incubation with ricin sulf-1 for 2.5 h. Labeled ricin sulf-1 was immunoprecipitated and subjected to SDS-PAGE and fluorography (top). Bottom, intensities of the respective bands representing sulfated ricin determined by densitometric quantitation (black bars), and the total sulfation of cell-associated proteins was determined by measuring radioactivity of 5% TCA-precipitated proteins (gray bars). Control values were obtained from untransfected cells. Values shown represent the averages of duplicated determinations from a representative experiment and the error bars show deviations between duplicates.

tos. As a consequence, endocytosis of ricin was reduced by 50% (Skretting, G., Iversen, T.-G., Stahlhut, M., van Deurs, B., and Sandvig, K., manuscript under revision).

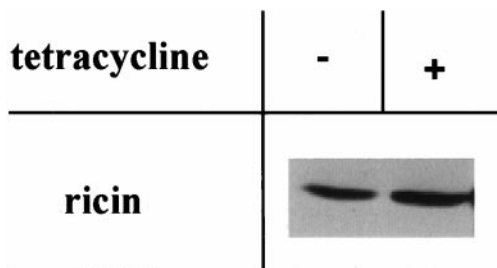
To measure whether anti-CHC expression affected transport of ricin to the Golgi apparatus we used ricin sulf-1, which can be sulfated in the Golgi apparatus. Figure 6 shows that the amount of ricin transported to the Golgi apparatus was reduced twofold in the anti-CHC expressing cells. However, because ricin endocytosis was reduced to a similar extent also, the data suggest that expression of anti-CHC

have no effect on transport of ricin from endosomes to TGN. Furthermore, to investigate whether expression of anti-CHC changes the ability of ricin to intoxicate the cells, increasing concentrations of ricin were added to cells grown in the presence or absence of tetracycline for 2 d, and 4 h later protein synthesis was measured. Cells expressing anti-CHC were somewhat less sensitive to ricin (approximately two-fold; Iversen, Skretting, Llorente, Nicoziani, van Deurs, and Sandvig, unpublished results). Again, the difference in toxicity reflected only the reduced endocytic uptake of toxin.



**Figure 5.** Ricin toxicity is unaffected by overexpression of Rab11N25 in HeLa cells. HeLa cells transiently expressing either mutant or wild-type myc-Rab11 were incubated with increasing concentrations of ricin for 4 h and pulse-labeled with [<sup>35</sup>S]methionine/cysteine for 15 min. Then the cells were solubilized, and myc-tagged Rab11 proteins were purified with anti-myc (9E10) antibody agarose conjugate. Purified myc-Rab11 were run on 12% SDS PAGE followed by fluorography (A). Ricin toxicity was measured by densitometric quantitations of the signal intensities (B).

Also, the kinetics of the ricin toxicity was the same whether or not anti-CHC was expressed (Iversen, Skretting, Llorente, Nicoziani, van Deurs, and Sandvig, unpublished results).



**Figure 6.** Ricin sulfation in cells with inducible expression of anti-CHC. BHK21-tTA/anti-CHC cells were grown with (+) or without (-) tetracycline for 2 d. The cells were then washed with SMEM without sulfate and incubated with Na<sub>2</sub><sup>35</sup>SO<sub>4</sub> for 4 h. Then ricin sulf-1 was added, and incubation was continued for 4 h. The cells were washed with 0.1 M lactose in HEPES-medium at 37°C and with ice-cold PBS and then lysed. The supernatant was immunoprecipitated with rabbit anti-ricin antibodies and protein A-Sepharose for 18 h at 4°C. The immunoprecipitate was analyzed by SDS-PAGE under reducing conditions followed by fluorography.

**DISCUSSION**

In the present study we investigated endosome to TGN transport of ricin by expression of mutant Rab-GTPases and anti-CHC. To draw any conclusions about a possible role of Rab9 on ricin transport to the Golgi, it is essential to directly demonstrate that the expression level of mutant Rab9 is high enough to affect intracellular transport. In this study this was performed with the use of a M6PR46HMY construct that can be sulfated. The rate of sulfation of the modified M6PR46HMY was significantly reduced in the doxycycline-induced cells. The mutant Rab9S21N is predicted to interfere with Rab9-nucleotide exchange factor interactions, and because a basal level of wild-type Rab9 is still present a complete block in the recycling could not be expected (Riederer *et al.*, 1994). Also, the observed differences in sulfation most likely underestimate the extent of inhibition of recycling. During the <sup>35</sup>SO<sub>4</sub>-pulse one would expect some newly synthesized M6PR46HMYs to be sulfated in the TGN, thereby contributing to an increased background level.

We specifically found that ricin transport from endosomes to the TGN, monitored as sulfation of a modified ricin, was unaffected by mutant Rab9 expression both after doxycycline-induced expression in HeLa cells and when constitutively expressed in CHO cells. Hence, our results indicate that ricin is transported from endosomes to the Golgi in a



Rab9-independent manner. This agrees with an earlier study by Simpson *et al.* (1995), in which toxicity measurements were performed on HeLa cells transiently expressing the mutant Rab9. However, in that study it was not investigated whether the level of mutant Rab9 expression was high enough to affect any process.

The constitutively expressing Rab9S21N CHO cells have been found to display an increased steady-state level of M6PRs and of lysosomal enzymes like cathepsin D (Riederer *et al.*, 1994). Such a "compensatory" effect was not observed in the doxycycline-inducible Rab9S21N cells, in which both the steady-state level and the expression level of cathepsin D remained unchanged after induction by doxycycline (Iversen, Skretting, Llorente, Nicoziani, van Deurs, and Sandvig, unpublished results). Electron microscopy revealed that the majority (~90%) of the CI-M6PRs were localized in endocytic structures and that the degree of labeling was the same in both the  $-/+$  doxycycline-induced cells. This agrees with previous reports that at steady state endogenous M6PRs are found predominantly in LE, and <5–10% are located at the cell surface (Kornfeld and Mellman, 1989). An exception is the HEP-2 cell line in which the M6PRs rapidly leaves the multivesicular bodies, and at steady state they are found predominantly in the TGN and in vacuolar structures in the peripheral cytoplasm (Hirst *et al.*, 1998).

Recently we found that an inactive mutant of dynamin inhibits transport of ricin from endosomes to the Golgi apparatus (Llorente *et al.*, 1998a). This does, however, not necessarily imply that clathrin is involved in this transport step. There is evidence for the presence and function of dynamin in vesicle formation without colocalization of clathrin (Oh *et al.*, 1998; McNiven *et al.*, 2000; Nicoziani *et al.*, 2000). Expression of mutant dynamin also induced a redistribution of CI-M6PRs to LE/lysosomes (Llorente *et al.*, 1998a; Nicoziani *et al.*, 2000). However, although both ricin transport to the Golgi apparatus and M6PR distribution are affected by mutant dynamin, this does not imply that ricin and M6PRs use the same pathway, because different forms of dynamins associated with various organelles have been identified (Cao *et al.*, 1998).

Intriguingly, it has been reported that transport of TGN38 to the Golgi apparatus is dependent on low pH (Chapman and Munro, 1994), whereas transport of ricin and Shiga toxin is not (Sandvig and van Deurs, 1996), although all three molecules may be entering the TGN directly from EE, possibly via the perinuclear recycling compartment (ERC). Thus, traveling in the same direction does not necessarily imply identical requirements or mechanisms for the transported proteins. Both ricin and Shiga toxin are protein toxins that are transported retrogradely to the ER before translocation to the cytosol. Nevertheless, the routes followed by these toxins on their way to the ER differ: although Shiga toxin binds to a glycolipid on the surface of the cell, it is transported into clathrin-coated pits before being endocytosed. Inhibition of clathrin-dependent endocytosis has been shown to protect against Shiga toxin (Sandvig *et al.*, 1989). In contrast, ricin is endocytosed by more than one mechanism (Sandvig and van Deurs, 2000). Moreover, although Shiga toxin B-fragment has been visualized in clathrin-coated areas of the ERC and may be dependent on functional clathrin for sorting to the TGN (Mallard *et al.*, 1998), the data shown

here indicate that this does not seem to be the case for ricin. A possible explanation could be that ricin binds to both glycoproteins and glycolipids, thus allowing the toxin to be transported by more than one transport mechanism from the EE/ERC to the TGN.

We considered the possibility that Rab11 might be important for TGN transport of ricin. It has been reported that Rab11 regulates transport of the transferrin receptor and its ligand from sorting endosomes to the perinuclear ERC and their recycling back to the plasma membrane (Ullrich *et al.*, 1996; Ren *et al.*, 1998). Moreover, overexpression of the dominant interfering mutant Rab11N25 has been shown to inhibit this pathway. Interestingly, a preliminary result has been reported indicating that Rab11 may regulate the EE-to-Golgi transport of Shiga toxin B-fragment (Johannes and Goud, 2000). In contrast, we found that overexpressing mutant Rab11 did not affect ricin toxicity, although it did inhibit transport of transferrin to the ERC, indicating that transport of ricin to the TGN is not dependent on functional Rab11.

Our data suggest that there exist more transport routes from endosomes to the TGN than previously anticipated. Investigations of different protein toxins are likely to provide information about the various routes to the TGN.

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