

# Selective regulation of the Rab9-independent transport of ricin to the Golgi apparatus by calcium

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## Summary

Transport of ricin from endosomes to the Golgi apparatus occurs, in contrast to the transport of the mannose 6-phosphate receptor, by a Rab9-independent process. To characterize the pathway of ricin transport to the Golgi apparatus, we investigated whether it was regulated by calcium. As shown here, our data indicate that calcium is selectively involved in the regulation of ricin transport to the Golgi apparatus. Thapsigargin, which inhibits calcium transport into the ER, and the calcium ionophore A23187 both increased the transport of ricin to the Golgi apparatus by a factor of 20. By contrast, transport of the mannose 6-phosphate receptor to the Golgi apparatus was unaffected.

Ricin and mannose 6-phosphate receptor transport were measured by quantifying the sulfation of modified forms of ricin and the mannose 6-phosphate receptor. The increased transport of ricin was reduced by wortmannin and LY294002, suggesting that phosphoinositide 3-kinase might be involved in transport of ricin to the Golgi apparatus. Together, these findings indicate that the different pathways to the Golgi apparatus utilized by ricin and the mannose 6-phosphate receptor are regulated by different mechanisms.

Key words: Ricin, Golgi apparatus, Calcium, Rab9

## Introduction

The plant toxin ricin is a heterodimer consisting of an enzymatically active A-chain and a B-chain responsible for binding to glycoproteins and glycolipids at the cell surface and the subsequent endocytosis of the toxin. After being endocytosed, most of the ricin molecules are either recycled or transported to lysosomes for degradation (van Deurs et al., 1986). However, a minor fraction of ricin (~5%) is transported after endocytosis to the Golgi apparatus and then retrogradely to the ER, where toxin translocation to the cytosol occurs (van Deurs et al., 1988; Wales et al., 1993; Rapak et al., 1997; Wesche et al., 1999). In the cytosol, the A-chain of ricin exerts its toxic effect by inactivating ribosomes and thereby blocking protein synthesis. Since ricin is endocytosed by different mechanisms and transported to the Golgi apparatus and the ER, ricin has proven to be a valuable tool for studying intracellular transport (reviewed in Sandvig and Van Deurs, 2000; Sandvig et al., 2002). To monitor transport of ricin to the Golgi apparatus, and also transport from the Golgi to the ER, one can use a modified ricin molecule, termed ricin sulf-2, to which a sulfation site and glycosylation sites have been added (Rapak et al., 1997). This molecule will be subjected to sulfation in the Golgi apparatus and glycosylation in the ER – modifications that can be quantified.

Several lines of evidence suggest the existence of different transport routes to the Golgi apparatus, one being the well characterized, Rab9-dependent route from late endosomes that is utilized by the mannose 6-phosphate receptor (M6PR) (Lombardi et al., 1993; Riederer et al., 1994; Itin et al., 1997; Itin et al., 1999; Nicoziani et al., 2000; Miwako et al., 2001). An alternative route seems to be used by both TGN38 and Shiga toxin B-chain – transport to the TGN occurs from early

endosomes, possibly via the endocytic recycling compartment (Ghosh et al., 1998; Mallard et al., 1998). In the case of Shiga B, this transport may occur through a clathrin- and Rab11-dependent mechanism (Mallard et al., 1998; Wilcke et al., 2000). Ricin also seems to be transported to the Golgi apparatus using a mechanism that differs from that of the M6PR. We have recently found that ricin transport to the Golgi apparatus is independent of the small GTPase Rab9 (Iversen et al., 2001). Interestingly, this transport is also independent of functional clathrin and Rab11, suggesting that it differs from that of Shiga B-chain (Iversen et al., 2001). However, the transport mechanism of ricin to the Golgi apparatus has not yet been characterized. In the present work we describe the study of whether this transport is regulated by calcium.

Calcium plays an important role in intracellular transport. It is involved in both ER to Golgi transport (Schwaninger et al., 1991), intra Golgi transport (Porat and Elazar, 2000) and fusion between late endosomes and lysosomes (Colombo et al., 1997; Peters and Mayer, 1998; Holroyd et al., 1999). To investigate the effect of calcium on the transport of ricin and M6PR to the Golgi apparatus, we treated cells with thapsigargin, which specifically inhibits the ER Ca<sup>2+</sup>-ATPase (Thastrup et al., 1990). This inhibition prevents calcium reuptake into the ER, resulting in calcium depletion of the ER and an increased concentration of calcium in the cytosol. The effect of the calcium ionophore A23187 was also investigated. Both thapsigargin and A23187 increased the transport of ricin to the Golgi apparatus, whereas transport of the M6PR was not affected by such treatment. Interestingly, the increased transport of ricin seen upon thapsigargin-treatment was strongly inhibited by wortmannin or LY294002, suggesting that phosphoinositide 3-kinase (PI3-kinase) might be involved in this mechanism.

## Materials and Methods

### Materials

Ricin, ricin B-chain, wortmannin, Hepes, lactose and geneticin were obtained from Sigma Chemical Co. (St Louis, MO). Thapsigargin, LY294002, PD169316 and SB203580 were from Calbiochem (Nottingham, UK), whereas A23187 was from Biomol (Plymouth Meeting, PA). Fluo-3 AM-ester, Fura Red AM-ester, Calcium Calibration Buffer Kit #2 and Pluronic F-127 were obtained from Molecular Probes (Leiden, The Netherlands).  $\text{Na}_2^{35}\text{SO}_4$  was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Ricin B-chain was labeled with indocarbocyanine (CY3) from Amersham Life Science (Inc., IL) according to the procedure given by the company. Rabbit anti-ricin antibodies and rabbit anti-Shiga toxin antibodies were obtained by standard immunization.

### Cells

MDCK II cells [from W. Hunziker (Garred et al., 2001)] were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS), 0.25 mg/ml geneticin, 100 units/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin and 2 mM L-glutamine. The cells were seeded out into 5 cm Petri dishes at densities of  $0.6\text{--}1.0 \times 10^6$  cells per dish two days before the sulfation experiments in the same medium but with 10% FCS. For transfection studies, MDCK II cells were seeded out into 5 cm Petri dishes at a density of  $4.5 \times 10^5$  cells per dish. The next day cells were transfected using FuGENE(TM)-6 (Boehringer Mannheim) according to the procedure given by the company. For confocal studies MDCK II cells were seeded on coverslips two days before the experiments. When polarized MDCK II cells were used, cells were seeded out on polycarbonate filters (Costar Transwell, Sigma; pore size 0.4  $\mu\text{m}$ , diameter 24.5 mm) at a density of  $8 \times 10^5$  per filter and used for experiments 4 days later.

### Measurements of cytosolic calcium concentrations

MDCK II cells grown on coverslips were washed twice with buffer (14 mM NaCl, 2 mM  $\text{CaCl}_2$ , 20 mM Hepes) and incubated with or without 1  $\mu\text{g}/\text{ml}$  thapsigargin or 10  $\mu\text{M}$  A23187 for 30 minutes at 37°C in the same buffer. The cells were then loaded with fluorescent calcium indicators by incubation with buffer containing Fluo-3 AM-ester (5  $\mu\text{M}$ ) and Fura Red AM-ester (50  $\mu\text{M}$ ) for 1 hour at room temperature in the absence or presence of thapsigargin or A23187 (a ten times lower concentration of Fluo-3 was used since the fluorescence of Fluo-3 is around 10 times brighter than Fura Red when excited at 488 nm). To improve loading, the Fluo-3/Fura Red mixture was first mixed 1:1 with the polyol surfactant Pluronic F-127 (20% in DMSO) before addition of buffer. The cells were then washed twice with buffer before incubation for a further 30 minutes in indicator-free buffer with or without thapsigargin or A23187 to allow complete de-esterification of the intracellular AM-esters. Confocal imaging was performed using a Leica TCS NT confocal microscope equipped with a 63 $\times$  objective and a Kr/Ar laser. The 488 nm line of the laser was used for excitation, and the emitted fluorescence was acquired at 530 nm (Fluo-3) and >590 nm (Fura Red) using separate photomultipliers. Images of 1024 $\times$ 1024 pixels were taken, and the Fluo-3/Fura Red fluorescence ratio of defined areas of the cytosol was determined after calculating the mean fluorescence level (0-255) of both channels (probes) using Adobe Photoshop 4.0 imaging software. The Fluo-3/Fura Red fluorescence ratios were then converted to calcium concentration values by in vitro calcium calibration using the Calcium Calibration Buffer Kit #2 (Molecular Probes), which contains buffers with known calcium concentrations. After de-esterification of the AM-esters by chemical hydrolysis (and pH adjustment to 7.0), the Fluo-3/Fura Red mixture (1:10, as this was the ratio used for the indicator loading) was added to small aliquots of the different calcium buffers in the kit. The solutions were then placed on coverslips, and

the mean fluorescence levels at the two emission wavelength were recorded for each solution. The Fluo-3/Fura Red fluorescence ratios were determined, and the calibration points were fitted with Grynkiewicz's equation (Grynkiewicz et al., 1985). The Fluo-3/Fura Red fluorescence ratio obtained this way was 0.13 in the control situation, which corresponds to a calcium concentration of ~80 nM. In the thapsigargin- and A23187-treated cells, the cytosolic calcium levels were elevated, and the ratios were found to be 0.89 and 1.48, respectively, corresponding to calcium concentrations of ~350 nM and ~530 nM, respectively.

### Vectors and constructs

The coding region of the mutant Rab9S21N (Lombardi et al., 1993) was cloned into the *Bam*HI/*Eco*RV sites of the expression vector pCDNA3. The plasmid construct of the 46 kDa cation-dependent M6PR tagged with polyhistidine, a c-myc epitope and a tyrosine sulfation site (M6PR46-HMY) was a gift from Suzanne R. Pfeffer.

### Endocytosis of ricin

Endocytosis of ricin was measured using the ORIGEN analyzer (IGEN, Rockville, MD), which uses electrochemiluminescence detection. Ricin was labeled with N-hydroxysuccinimide ester-activated tris(bipyridine) chelated ruthenium(II) TAG (IGEN) according to the procedure given by the company and simultaneously biotinylated with the reducible ImmunoPure NHS-SS-Biotin (Pierce, Rockford, IL). MDCK II cells were washed with Hepes medium and preincubated with thapsigargin for 30 minutes at 37°C before TAG- and biotin-labeled ricin (30 ng/ml) was added to the medium. The incubation was continued for either 20 minutes or 2 hours. The cells were subsequently washed twice (5 minutes) with 0.1 M lactose in Hepes medium at 37°C to remove surface-bound ricin, then once with cold PBS before they were lysed (lysis buffer: 0.1 M NaCl, 5 mM  $\text{MgCl}_2$ , 50 mM Hepes and 1% Triton X-100). The cells were centrifuged for 5 minutes at 16,000 *g* in an Eppendorf centrifuge to remove the nuclei, and the amount of ricin in the cleared lysates was measured using streptavidin beads (Dynal, Oslo, Norway) and ORIGEN Analyzer (IGEN).

### Preparation of ricin sulf-2

The modified ricin A-sulf-2, containing a tyrosine sulfation site and N-glycosylation sites, was produced, purified and reconstituted with ricin B-chain to form the holotoxin ricin sulf-2 as described previously (Rapak et al., 1997).

### Sulfation of ricin sulf-2

MDCK II cells were washed twice with DMEM without sulfate and incubated with 0.2 mCi/ml  $\text{Na}_2^{35}\text{SO}_4$  for 3 hours at 37°C in the same medium but supplemented with 1 mM  $\text{CaCl}_2$ , 2 mM L-glutamine and 1 $\times$ non-essential amino acids (Life Technologies, Paisley, UK). Thapsigargin or A23187 was added to the medium during the last 30 minutes of this incubation, then ricin sulf-2 (~200 ng/ml) was added and the incubation was continued for 2 hours. The cells were washed twice (5 minutes) with 0.1 M lactose in Hepes medium at 37°C to remove surface-bound ricin sulf-2, then once with cold PBS before they were lysed [lysis buffer: 0.1 M NaCl, 10 mM  $\text{Na}_2\text{HPO}_4$ , 1 mM EDTA, 1% Triton X-100 supplemented with a mixture of protease inhibitors (Roche Molecular Biochemicals, Mannheim, Germany), pH 7.4]. The cells were centrifuged for 10 minutes at 2040 *g* in an Eppendorf centrifuge to remove the nuclei, and the cleared lysate was immunoprecipitated with rabbit anti-ricin antibodies immobilized on protein A-Sepharose beads (Pharmacia, Piscataway, NJ) overnight at 4°C. The beads were then washed twice with PBS containing 0.35% Triton X-100 before the

adsorbed material was analyzed by SDS-PAGE under reducing conditions.

#### Sulfation of the mannose 6-phosphate receptor

Sulfation was performed according to the assay described previously (Itin et al., 1997). MDCK II cells transfected with M6PR46-HMY (as described above) were grown in DMEM without sulfate supplemented with 1.8 mM CaCl<sub>2</sub>, 2 mM L-glutamine and 1×non-essential amino acids, 1×MEM amino acids, 1×vitamin solution, 1 mM sodium pyruvate, 10 mM sodium chlorate and 10% FCS. Subsequently, the cells were washed twice with DMEM without sulfate supplemented with 1 mM CaCl<sub>2</sub>, 2 mM L-glutamine and 1×non-essential amino acids and preincubated with thapsigargin for 30 minutes at 37°C before 0.6 mCi/ml Na<sub>2</sub><sup>35</sup>SO<sub>4</sub> was added to the medium. After 3 hours, the cells were washed twice with cold PBS and lysed in lysis buffer [0.1 M NaCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM EDTA, 1% Triton X-100 supplemented with a mixture of protease inhibitors (Roche Molecular Biochemicals), pH 7.4] containing 25 mM imidazole. The cells were then centrifuged for 10 minutes at 2040 *g* in an Eppendorf centrifuge to remove the nuclei. The cleared lysate was immunoprecipitated with nickel agarose beads (Qiagen, Chatsworth, CA) overnight at 4°C, then the beads were washed four times with lysis buffer containing 25 mM imidazole. The adsorbed M6PR46-HMY was eluted with 25 mM EDTA in lysis buffer and 25 mM imidazole and analyzed by SDS-PAGE under reducing conditions.

#### Sulfation of STxB-Sulf<sub>2</sub>

The modified version of Shiga toxin B-chain containing sulfation sites (STxB-Sulf<sub>2</sub>) was a kind gift from L. Johannes, The Curie Institute, Paris. MDCK II cells were washed twice with DMEM without sulfate and incubated with 0.2 mCi/ml Na<sub>2</sub><sup>35</sup>SO<sub>4</sub> for 3 hours at 37°C in the same medium but supplemented with 1 mM CaCl<sub>2</sub>, 2 mM L-glutamine and 1×non-essential amino acids (Life Technologies). Thapsigargin or A23187 was added to the medium during the last 30 minutes of this incubation, then STxB-Sulf<sub>2</sub> (2.8 µg/ml) was added and the incubation was continued for 2 hours. The cells were washed twice with cold PBS and then lysed [lysis buffer: 0.1 M NaCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM EDTA, 1% Triton X-100 supplemented with a mixture of protease inhibitors (Roche Molecular Biochemicals), pH 7.4]. The cells were centrifuged for 10 minutes at 2040 *g* in an Eppendorf centrifuge to remove the nuclei, and the cleared lysate was immunoprecipitated with rabbit anti-Shiga toxin antibodies immobilized on protein A-Sepharose beads (Pharmacia, Piscataway, NJ) overnight at 4°C. The beads were then washed twice with PBS containing 0.35% Triton X-100 before the adsorbed material was analyzed by SDS-PAGE under reducing conditions.

#### SDS-PAGE

SDS-PAGE was performed in 12% gels as previously described (Laemmli, 1970). The gels were fixed in 4% acetic acid and 27% methanol for 30 minutes and then incubated for 20 minutes in 1 M sodium salicylate, pH 5.8, in 2% glycerol. Kodak XAR-5 films were exposed to the dried gels at -80°C for autoradiography. Moreover, signal intensities of the bands were quantified by exposing the gels to PhosphorImager screens and using the ImageQuant 5.0 software (Amersham Biosciences, Sunnyvale, CA).

#### Confocal microscopy studies

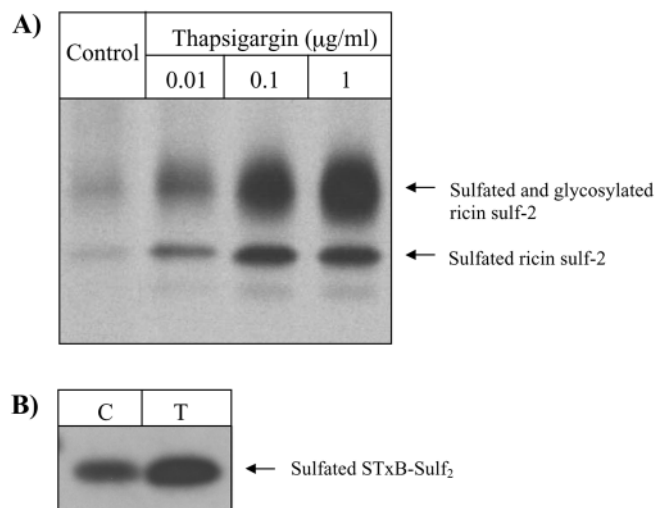
MDCK II cells grown on coverslips were washed twice with Hepes medium and preincubated with thapsigargin for 30 minutes at 37°C before CY3-labeled ricin B-chain (1 µg/ml) was added. After 2 hours of incubation, the cells were fixed with 3% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 and blocked with 5% FCS.

The Golgi apparatus was then labeled with rabbit anti-mannosidase II antibodies (from Kelley Moremen, University of Georgia, Athens, GA) that were visualized by goat anti-rabbit IgG FITC (Jackson Immunoresearch Laboratories, West Grove, PA). Colocalization of the CY3-labeled ricin B-chain and the Golgi apparatus was analyzed by a Leica TCS NT confocal microscope equipped with a 63× objective and a Kr/Ar laser.

## Results

### Thapsigargin and the calcium ionophore A23187 strongly increase the sulfation of ricin sulf-2

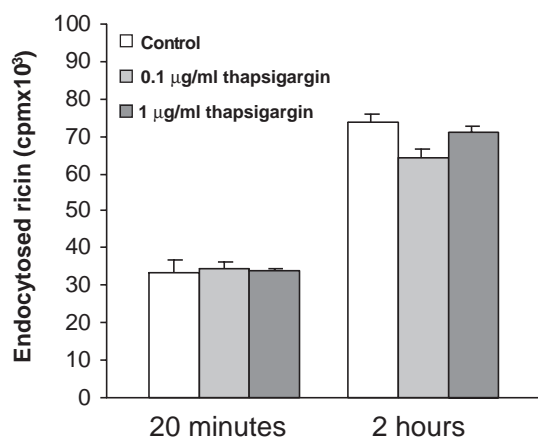
To investigate the effect of calcium on ricin transport to the Golgi apparatus, we first treated MDCK II cells with thapsigargin, which specifically inhibits the ER Ca<sup>2+</sup>-ATPase (Thastrup et al., 1990). This inhibition blocks the reuptake of calcium into the ER, resulting in a depletion of calcium from the ER and an increased cytosolic calcium level. As expected, thapsigargin also increased the cytosolic calcium level in our MDCK II cells as measured by ratiometric confocal microscopy imaging using the fluorescent calcium indicators Fluo-3 and Fura Red (see Materials and Methods). Incubation of MDCK II cells with radioactive sulfate in the presence of thapsigargin increased the sulfation of ricin sulf-2 to a great extent; that is, the amount of sulfated ricin sulf-2 was ~20 times higher in cells treated with 1 µg/ml thapsigargin than in control cells (Fig. 1A; lane 4). As shown in the same figure, the effect of thapsigargin was concentration dependent. To verify that this increase in sulfation of ricin sulf-2 was caused by an increased transport of ricin to the Golgi apparatus, rather than by stimulation of protein sulfation in general in the thapsigargin-treated cells, cell lysates were analyzed by SDS-



**Fig. 1.** Effect of thapsigargin on the sulfation of (A) ricin sulf-2 and (B) STxB-Sulf<sub>2</sub>. MDCK II cells were incubated with radioactive sulfate for 3 hours at 37°C, and during the last 30 minutes thapsigargin was added. Then ricin sulf-2 (~200 ng/ml) (A) or STxB-Sulf<sub>2</sub> (2.8 µg/ml) (B) was added to the medium and the incubation was continued for 2 hours. The cells were subsequently washed, lysed and immunoprecipitated with rabbit anti-ricin antibodies (A) or rabbit anti-Shiga toxin antibodies (B). The adsorbed material was analyzed by SDS-PAGE (12%) before autoradiography (details in Materials and Methods).

PAGE followed by autoradiography. The sulfation of proteins in general was not increased upon thapsigargin treatment, instead it was slightly decreased (data not shown). Furthermore, to exclude the possibility that the increased sulfation of ricin sulf-2 was caused by a higher endocytic uptake of ricin in thapsigargin-treated cells, the effect of thapsigargin on ricin endocytosis was also examined. Fig. 2 shows that the endocytosis of ricin did not increase in the presence of thapsigargin. Together these results indicate that there is an increased transport of ricin from endosomes to the Golgi apparatus upon thapsigargin treatment. We also investigated whether thapsigargin treatment could increase sulfation of Shiga B (STxB-Sulf<sub>2</sub>), since Shiga toxin is also transported to the Golgi apparatus from an early endosomal compartment, but by a mechanism that might differ from that of ricin (Iversen et al., 2001). As shown in Fig. 1B, there was only a two-fold increase in the sulfation of STxB-Sulf<sub>2</sub> in thapsigargin-treated cells. Thus, the stimulation was much smaller than found for ricin. Clearly, when it comes to regulation of transport by calcium, Shiga toxin seems to differ from ricin.

We next wanted to investigate whether it was the elevated cytosolic calcium level or the depletion of calcium in the ER that was responsible for the thapsigargin-induced stimulation of ricin transport to the Golgi apparatus. For this purpose, we investigated the effect of another calcium mobilizing compound, the calcium ionophore A23187. A23187 increases the permeability to calcium of all cellular membranes, thus cells exposed to A23187 in the presence of a normal extracellular calcium concentration will achieve an elevated calcium concentration in the cytosol, as well as in the lumen of the ER. We checked that the calcium level in the cytosol was elevated in our experiments using ratiometric confocal imaging with fluorescent calcium indicators (see Materials and Methods). Only in the absence of extracellular calcium



**Fig. 2.** Effect of thapsigargin on the endocytosis of ricin. MDCK II cells were preincubated with or without the indicated concentrations of thapsigargin for 30 minutes at 37°C before TAG- and biotin-labeled ricin (30 ng/ml) was added to the medium. The incubation was continued for either 20 minutes or 2 hours before the cells were washed with 0.1 M lactose and then lysed. The amount of TAG- and biotin-labeled ricin in the lysates was measured using streptavidin beads and Origen Analyzer. Deviations between duplicates are represented by error bars.

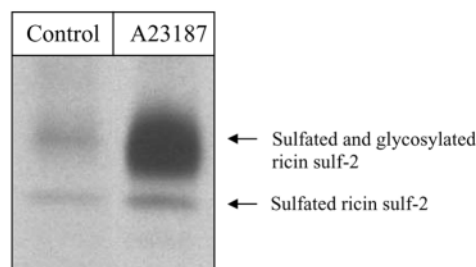
A23187 is able to deplete the ER of calcium. We therefore incubated MDCK II cells with radioactive sulfate in the presence of 10 µM A23187 using medium containing 1 mM calcium. This concentration of A23187 stimulated the transport of ricin to the Golgi apparatus to the same extent as 1 µg/ml thapsigargin (Fig. 3), indicating that it is the elevated cytosolic calcium level that is responsible for the stimulated transport of ricin.

### Thapsigargin increases the Golgi transport of both apically and basolaterally internalized ricin in polarized cells

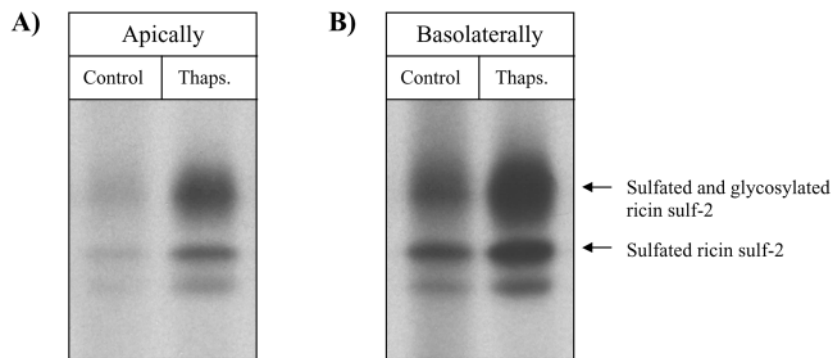
Some intracellular transport routes are regulated in a different manner in non-polarized and polarized cells. Also, pathways leading from either the apical or the basolateral pole of polarized cells are differentially regulated (Llorente et al., 1996; Llorente et al., 1998). We therefore examined whether thapsigargin also affected the transport of ricin in polarized cells. Thus, MDCK II cells grown on polycarbonate filters were preincubated for 30 minutes with thapsigargin (1 µg/ml) before ricin sulf-2 was added either to the apical or to the basolateral pole. Fig. 4 shows that thapsigargin increased the transport of ricin to the Golgi apparatus both when ricin was internalized apically or basolaterally. A23187 exerted a similar effect (data not shown).

### Ricin B-chain has a more perinuclear location in cells treated with thapsigargin than in untreated cells

Consistent with the data obtained by the sulfation experiments, we were also able to visualize by immunofluorescence the increased transport of ricin to the Golgi apparatus upon thapsigargin treatment. MDCK II cells were incubated with CY3-labeled ricin B-chain in the presence of thapsigargin (1 µg/ml). As shown in Fig. 5, ricin B-chain had a more perinuclear location in the cells that were treated with thapsigargin compared with the control cells (the colocalization of ricin with mannosidase II was increased by a factor of three in the thapsigargin-treated cells; from 4.6% (of total internalized ricin) to 14.6%, as quantified by analyzing the respective images using the Adobe Photoshop 4.0



**Fig. 3.** Effect of the calcium ionophore A23187 on the sulfation of ricin sulf-2. MDCK II cells were incubated with radioactive sulfate for 3 hours at 37°C, and 10 µM A23187 was added for the last 30 minutes. Then ricin sulf-2 (~200 ng/ml) was added to the medium, and the incubation was continued for 2 hours. The cells were subsequently washed with 0.1 M lactose, lysed and immunoprecipitated with rabbit anti-ricin antibodies. The adsorbed material was analyzed by SDS-PAGE (12%) before autoradiography.



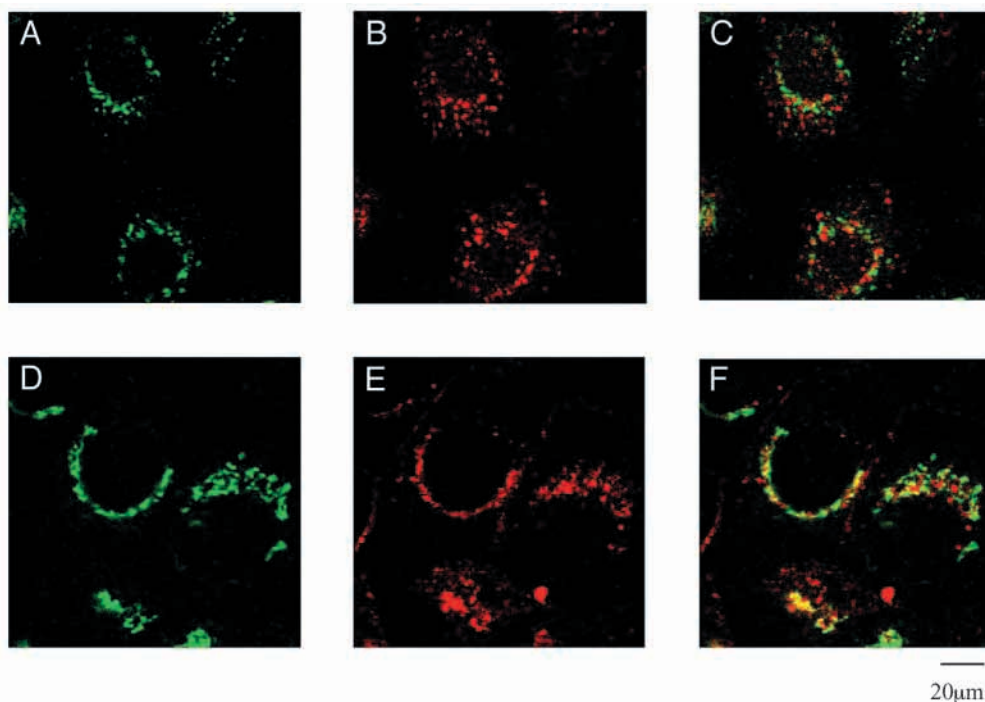
**Fig. 4.** Effect of thapsigargin (Thaps) on the sulfation of apically or basolaterally internalized ricin sulf-2. MDCK II cells grown on polycarbonate filters were incubated with radioactive sulfate for 3 hours at 37°C, and thapsigargin (1 µg/ml) was added for the last 30 minutes. Then ricin sulf-2 (~200 ng/ml) was added either to the apical (A) or basolateral pole (B), and the cells were further treated as described in the legend to Fig. 3.

software). This result supports the idea that a rise in cytosolic calcium levels leads to an increased transport of ricin to the Golgi apparatus. That the increase in colocalization is not larger is probably because of ricin transport to the ER (as seen by an increased glycosylation).

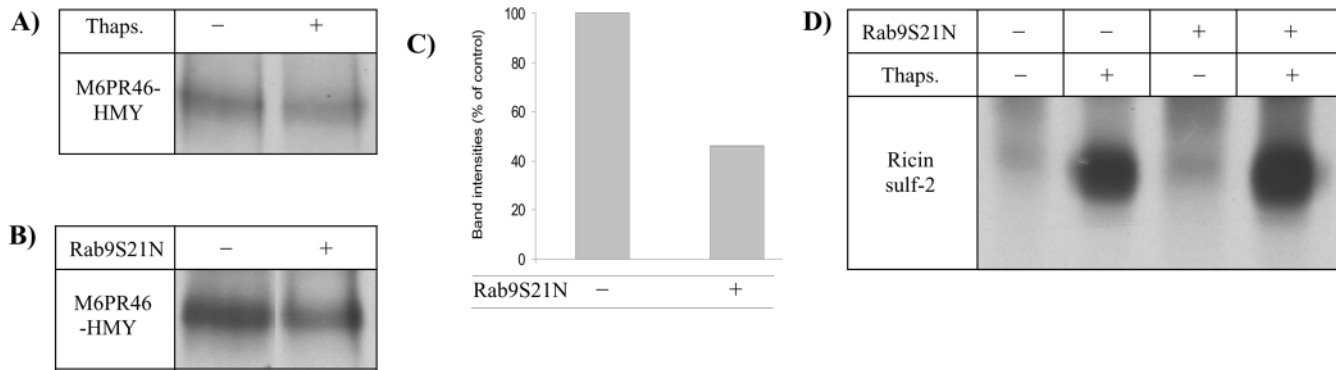
#### Thapsigargin does not increase the Rab9-dependent transport of the M6PR to the Golgi apparatus

We recently found that the transport of ricin to the Golgi apparatus occurs independently of Rab9 in HeLa cells, suggesting that ricin is transported to the Golgi apparatus through another pathway than the one used by M6PR (Iversen et al., 2001). We therefore wanted to investigate whether calcium selectively regulated ricin transport to the Golgi apparatus or whether thapsigargin also increased the transport of the M6PR. Thus, MDCK II cells were transfected with the M6PR46-HMY, a plasmid construct of M6PR tagged with polyhistidine, a c-myc epitope and a tyrosine sulfation site (Fig. 6). To achieve an accumulation of unsulfated M6PR46-HMY in the cells, protein sulfation was reversibly inhibited by incubating the cells with 10 µM sodium chlorate as described

earlier (Itin et al., 1997; Iversen et al., 2001). Two days after transfection, the cells were preincubated with thapsigargin for 30 minutes before radioactive sulfate was added to the medium (now sodium chlorate-free), and the incubation was continued for 3 hours. As shown in Fig. 6A, the amount of sulfated M6PR46-HMY was not increased upon thapsigargin treatment, in contrast to what was observed for ricin sulf-2 (Fig. 6D), suggesting that the transport of ricin and the M6PR are differentially regulated and that they use different pathways to the Golgi. To obtain further support for the view that ricin utilizes a Rab9-independent pathway in MDCK II cells, as observed earlier in HeLa cells, and that the thapsigargin-induced transport of ricin also occurs independently of Rab9, we performed ricin sulfation experiments not only on untransfected MDCK II cells but also on MDCK II cells transfected with the dominant-negative mutant Rab9 (Rab9S21N). As shown in Fig. 6D, ricin transport was stimulated to about the same extent after transfection with Rab9S21N. To test that the mutant Rab9 was expressed at sufficiently high levels to inhibit late endosome to Golgi transport of the M6PR, we also examined the effect of Rab9S21N-transfection in MDCK II cells cotransfected with



**Fig. 5.** Effect of thapsigargin on the localization of ricin B-chain. MDCK II cells grown on coverslips were incubated for 30 minutes at 37°C in the absence (A-C) or presence of 1 µg/ml thapsigargin (D-F). Then CY3-labeled ricin B-chain (1 µg/ml) (red) was added, and the incubation continued for 2 hours. The Golgi apparatus was labeled with sheep anti-human TGN46 antibodies followed by donkey anti-sheep/goat IgG FITC (green). C and F represent the merged images.



**Fig. 6.** Effect of Rab9S21N and thapsigargin (Thaps) on the sulfation of M6PR46-HMY and ricin sulf-2. (A,B) MDCK II cells transfected with M6PR46-HMY or cotransfected with M6PR46-HMY and Rab9S21N were preincubated for 30 minutes at 37°C with or without 0.1 µg/ml thapsigargin. Then radioactive sulfate was added, and the incubation was continued for 3 hours. The cells were subsequently washed, lysed and immunoprecipitated using Ni-agarose beads. The adsorbed material was eluted with 25 mM EDTA and analyzed by SDS-PAGE (12%) before autoradiography. (C) Graphic illustration of the signal intensities of the bands in B representing sulfated M6PR46-HMY. The band intensities were determined by densitometric quantification using Image-Quant 5.0. (D) MDCK II cells untransfected or transfected with Rab9S21N were incubated with radioactive sulfate for 3 hours at 37°C, and thapsigargin (0.1 µg/ml) was added for the last 30 minutes. Then ricin sulf-2 (~200 ng/ml) was added to the medium, and the cells were further treated as described in the legend to Fig. 3.

the M6PR46-HMY. As shown in Fig. 6, the transport of the M6PR was reduced by 54% in Rab9S21N-transfected cells (Fig. 6B,C), verifying that the expression of Rab9S21N was high enough to inhibit the Rab9-dependent transport route. As discussed in an earlier study (Iversen et al., 2001), the reduction in sulfation will, because of newly synthesized receptors passing through the Golgi apparatus during the incubation with radioactive sulfate, give an underestimation of the inhibition of the Rab9-dependent transport.

#### Wortmannin and LY294002 inhibit thapsigargin-induced stimulation of ricin transport to the Golgi apparatus

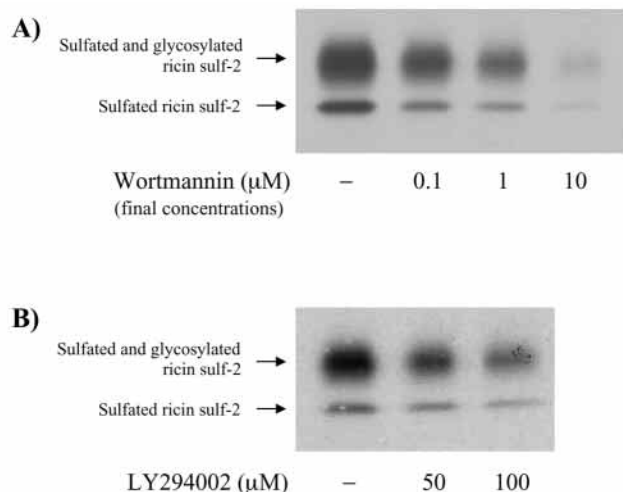
We next tried to identify any calcium sensor(s) that might be involved in regulating ricin transport to the Golgi apparatus. We had earlier found that calmodulin plays a role in the regulation of ricin transport in polarized MDCK I cells (Llorente et al., 1996), thus a possible involvement of this calcium-binding protein in the thapsigargin-induced stimulation of ricin transport was examined. However, we were not able to suppress the thapsigargin-induced stimulation of ricin transport by incubating the cells with the calmodulin antagonist W7 (data not shown), suggesting that Ca<sup>2+</sup> does not increase the transport of ricin to the Golgi apparatus via calmodulin. We further investigated whether PI 3-kinase was involved in the mechanism that regulates ricin transport, since the lipid products of this kinase are involved in vesicle-mediated protein transport (reviewed in Simonsen et al., 2001). MDCK II cells incubated with radioactive sulfate were preincubated with increasing amounts of the PI 3-kinase inhibitor wortmannin before addition of thapsigargin and ricin sulf-2. Because of the instability of wortmannin (Woscholski et al., 1994), it was added repeatedly during the incubation time to the final concentrations of 100 nM, 1 µM or 10 µM, respectively. As shown in Fig. 7A, the thapsigargin-stimulated ricin transport was reduced by 40% in the presence of 100 nM wortmannin (quantified using the ImageQuant 5.0 software). 1 µM wortmannin inhibited the transport even more (60%),

whereas an almost complete block was observed in the presence of 10 µM of the inhibitor. Control experiments showed that the reduction in sulfation of ricin sulf-2 by wortmannin was not due to a reduction in protein sulfation in general or a reduction in the endocytic uptake of ricin (data not shown). The effect of wortmannin on the A23187-stimulated ricin transport was also examined and found to be similar to that of thapsigargin-induced transport (data not shown). Further confirmation of a possible involvement of PI 3-kinase in the mechanism that regulates ricin transport to the Golgi apparatus was obtained by demonstrating a strong inhibition by another PI 3-kinase inhibitor, LY294002, which is structurally different from wortmannin (Fig. 7B). Also ricin transport occurring in the absence of increased cytosolic calcium levels was strongly reduced by wortmannin and LY294002 (data not shown).

We also investigated whether protein phosphorylation is involved in the mechanism that regulates ricin transport. Since both A23187 and thapsigargin activate p38 MAPK (Chao et al., 1992), we investigated a possible involvement of this kinase. However, none of the MAPK inhibitors used (PD169316 and SB203580) were able to suppress the thapsigargin-stimulated transport of ricin to the Golgi apparatus (data not shown), suggesting that MAPK does not play a role in this mechanism. Also inhibitors of PKC, PKA, MEK and Src-kinase have been tested, but none of these exerted any effect on the thapsigargin-induced stimulation of ricin transport to the Golgi apparatus.

#### Discussion

We here show that an elevated calcium concentration in the cytosol stimulates the transport of ricin to the Golgi apparatus by about 20 times. In these experiments we investigated the effect of both thapsigargin and A23187 since these two compounds increase the cytosolic calcium level by different mechanisms. By comparing their effect on ricin transport, we have demonstrated that it is the elevated cytosolic calcium



**Fig. 7.** Effect of the PI 3-kinase inhibitors wortmannin and LY294002 on the thapsigargin-stimulated transport of ricin to the Golgi apparatus. MDCK II cells were incubated with radioactive sulfate for 3 hours at 37°C, and after 2 hours increasing concentrations of wortmannin (A) or LY294002 (B) were added to the medium. During the last 30 minutes the cells were incubated in the presence of 0.1 μg/ml thapsigargin. Then ricin sulf-2 (~200 ng/ml) was added, and the incubation was continued for 2 hours. The cells were further treated as described in the legend to Fig. 3. Because of the instability of wortmannin, equal amounts were added every 45 minutes during the incubation time (3 hours) so that the final concentrations were 100 nM, 1 μM or 10 μM.

concentration, rather than the depletion of calcium in the ER, that is responsible for the stimulated transport of ricin. Furthermore, control experiments showed that thapsigargin or A23187 did not increase sulfation of proteins in general or affect the endocytic uptake of ricin. Our results from confocal microscopy experiments support the conclusion that calcium seems to play an important role in the regulation of ricin transport from endosomes to the Golgi apparatus.

It now appears that there are several pathways between endosomes and the Golgi apparatus (Goda and Pfeffer, 1988; Ghosh et al., 1998; Mallard et al., 1998; Mallet and Maxfield, 1999; Johannes and Goud, 2000; Iversen et al., 2001; Nichols et al., 2001), although the best characterized route is the Rab9-dependent pathway from late endosomes that is utilized by the M6PR (Lombardi et al., 1993; Riederer et al., 1994; Itin et al., 1997; Itin et al., 1999; Nicoziani et al., 2000; Miwako et al., 2001). In contrast to the results obtained with ricin, sulfation experiments on cells transfected with the M6PR46-HMY revealed that the transport of the M6PR to the Golgi apparatus was not increased upon elevated cytosolic calcium levels. Furthermore, ricin transport to the Golgi apparatus seems to occur, as previously found in HeLa cells (Iversen et al., 2001), independently of Rab9 in MDCK II cells. The thapsigargin-induced transport of ricin also seemed to be independent of Rab9 as sulfation was not reduced by transfection of cells with mutant Rab9. In the same experiment the transport of M6PR in Rab9S21N transfected cells was also analyzed, and the results confirmed that the expression of mutant Rab9 was high enough to inhibit the Rab9-dependent transport route to the Golgi apparatus. Thus, calcium does not seem to regulate

protein trafficking to the Golgi apparatus in general, but seems to selectively stimulate the Rab9-independent endosome to Golgi pathway used by ricin. Interestingly, thapsigargin also stimulated sulfation of Shiga B, but only by a factor of two, suggesting that there are differences in transport mechanisms between these two toxins. Increased knowledge of intracellular routing of protein toxins might in the future provide us with sufficient insight to prepare drugs that prevent intoxication and thereby be used as therapeutics.

In polarized cells, transport of proteins internalized from either the apical or the basolateral pole can be differentially regulated (Llorente et al., 1996; Llorente et al., 1998). However, as shown here, the transport of ricin to the Golgi apparatus was found to be stimulated by the elevated cytosolic calcium level both when ricin was internalized apically or basolaterally. Thus, the calcium-mediated regulation of ricin transport to the Golgi apparatus occurs independently of whether ricin passes through early apical or basolateral endosomes.

The PI 3-kinase inhibitors wortmannin and LY294002 significantly reduced the thapsigargin-induced transport of ricin. A concentration between 1 and 10 μM of wortmannin was required to obtain strong inhibition. However, several recent reports describe PI 3-kinases that are sensitive to micromolar levels of wortmannin only (Jones and Howell, 1997; Warashina, 2000; Hidaka et al., 2001). Thus, the transport of ricin to the Golgi apparatus seems to be dependent on PI 3-kinase, since low concentrations of wortmannin reduced ricin transport by as much as 40%.

From the present work we conclude that ricin transport to the Golgi apparatus is regulated by calcium, possibly via PI 3-kinase, and that this regulation is selective since it does not affect the Rab9-dependent M6PR transport. Thus, different pathways operating between endosomes and the Golgi apparatus are regulated differentially.

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