Endosome-to-Golgi Transport Is Regulated by Protein Kinase A Type IIα*

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Studies of RIIα-deficient B lymphoid cells and stable transfectants expressing the type IIα regulatory subunit (RIIA) of cAMP-dependent protein kinase (PKA), which is targeted to the Golgi-centrosomal area, reveal that the presence of a Golgi-associated pool of PKA type IIα mediates a change in intracellular transport of the plant toxin ricin. The transport of ricin from endosomes to the Golgi apparatus, measured as sulfation of a modified ricin (ricin sulf-1), increases in RIIα-expressing cells when PKA was activated. However, not only endosome-to-Golgi transport, but also retrograde ricin transport to the endoplasmic reticulum (ER), measured as sulfation and N-glycosylation of another modified ricin (ricin sulf-2), seemed to be increased in cells expressing RIIα in the presence of a cAMP analog, 8-(4-chlorophenylthio)-cAMP. Thus, PKA type IIα seems to be involved in both endosome-to-Golgi and Golgi-to-ER transport. Because ricin, after being retrogradely transported to the ER, is translocated to the cytosol, where it inhibits protein synthesis, we also investigated the influence of RIIα expression on ricin toxicity. In agreement with the other data obtained, 8-(4-chlorophenylthio)-cAMP and RIIα were found to sensitize cells to ricin, indicating an increased transport of ricin to the cytosol. In conclusion, our results demonstrate that transport of ricin from endosomes to the Golgi apparatus and further to the ER is regulated by PKA type IIα isozyme.

The mechanism by which different extracellular ligands that mediate their signals through the same second messenger might give rise to a specific intracellular response has been the subject of intensive research for several years (1). In the case of cAMP signal transduction, it has been demonstrated that the subcellular localization of protein kinase A (PKA)1 is important for the specificity (1).

PKA is composed of two catalytic (C) subunits and one regulatory dimer (Rn) that in the absence of cAMP form an inactive heterotetramer (RnC2). Upon binding of cAMP to the R subunits, the enzyme dissociates and releases two free, active C subunits (2). The Rn dimer is also implicated in the targeting of different PKA isoforms to various intracellular locations and to specific substrates through interactions with protein kinase A-anchoring proteins (3). Four different isoforms of the R subunit have been identified as products of separate genes in mammalian cells, and they have been termed RIA, RIB, RICα, and RICβ (1, 2, 4). They all contain two C-terminal cAMP binding sites, a hinge region that interacts with and inhibits the catalytic subunit, and a dimerization domain responsible for the interaction between the two regulatory subunits that make up the regulatory dimer of PKA (2). Whereas RI subunits are known to be mainly soluble, RII subunits are primarily associated with cytoskeletal elements and membranes (1, 3).

Studies of several human cell lines have revealed that the PKA type IIα isozyme (containing RIIαs and Cα) is concentrated in centrosomes and in Golgi-associated compartments (5, 6). In contrast, PKA type IIβ (containing RICβ) is associated more selectively with the centrosomal region and not with Golgi structures (5). Based on the important role of the Golgi apparatus in intracellular transport and protein sorting and the localization of PKA, which previously has been implicated in vesicle-mediated protein transport processes (7–10), the possibility existed that a distinct Golgi-associated pool of PKA type IIα isozyme was involved in regulation of transport through this organelle. To investigate whether PKA type IIα is involved in the regulation of retrograde transport, we studied the transport of the plant toxin ricin.

Ricin belongs to a family of plant and bacterial toxins that enter cells via the endocytic pathway. The toxin is transported retrogradely through the Golgi to the endoplasmic reticulum (ER) before it enters the cytosol, where it inhibits protein synthesis (11, 12). Ricin consists of an A-chain and a B-chain that are linked by a disulfide bridge. The B-chain binds to terminal galactose in both glycolipids and glycoproteins at the plasma membrane, whereas the A-chain enzymatically inhibits the protein synthesis after entry into the cytosol (12). Because ricin binds to both glycolipids and glycoproteins at the plasma membrane, it will be endocytosed by any vesicle that pinches off. Once ricin is endocytosed, it can be transported through the endosomal compartments, recycled back to the plasma membrane, delivered to the lysosomes, or transported retrogradely to the TGN and to the ER (11, 12).

In this study, we took advantage of a RIIα-deficient B lymphoid cell line, Reh (13), and reintroduced RIIα by making stable transfectants with a Golgi-associated pool of PKA type IIα to examine the effect on intracellular transport. We used two different ricin constructs to monitor the retrograde toxin trans-

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1 The abbreviations used are: PKA, protein kinase A; TGN, trans Golgi network; ER, endoplasmic reticulum; ricin sulf-1, recombinant ricin with a tyrosine sulfation site; ricin sulf-2, recombinant ricin construct with a sulfation site and three overlapping N-glycosylation sites; FITC, fluorescein isothiocyanate; FCS, fetal calf serum; 8CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; MESNA, 2-mercaptoethanesulfonic acid; mAb, monoclonal antibody; PBS, phosphate-buffered saline.

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port. Within the Golgi apparatus, recombinant ricin with a tyrosine sulfation site (ricin sulf-1) becomes radiolabeled in the presence of radioactive sulfate (14). This has made ricin sulf-1 a valuable tool to study intracellular transport to the TGN. A recombinant ricin construct with a sulfation site and three overlapping N-glycosylation sites (ricin sulf-2) is also modified in the TGN and N-glycosylated in the ER (14). The N-glycosylation of the toxin results in a molecular shift that can be observed on SDS-PAGE. This construct has therefore been used to study intracellular transport to the ER. As shown with the present study, the expression of PKA type IIα (RIIα) on a negative background in a lymphoid cell line leads to modulation of the retrograde transport of ricin, indicating a regulatory role for Golgi-associated PKA type IIα on these transport steps.

EXPERIMENTAL PROCEDURES

Materials—[3H]Leucine and Na,[35S]SO4 were purchased from Amer sham Biosciences. Na125I was purchased from Du Pont. Hygromycin B was bought from Roche Molecular Biochemicals (Mannheim, Germany) and Mowiol was obtained from Calbiochem. C3, goat anti-rabbit, FITC-labeled goat anti-mouse, and FITC-labeled goat anti-rabbit IgG were purchased from Jackson ImmunoResearch (West Grove, PA). Fetal calf serum (FCS), RPMI 1640 medium, RPMI 1640 medium without out sulfate, and streptomycin were bought from Invitrogen and protein and Mowiol was obtained from Calbiochem. Cy3-labeled goat anti-human RIIα and RIIβ polyclonal antibodies and mouse anti-RIIα monoclonal antibodies have been described elsewhere (5). Medial-Golgi (mAb CTR 433) and centrosomal (mAb CTR 453) markers were kindly provided by Dr. Michel Bornens (Institute Curie, Paris, France). The cis Golgi marker TGN46 was obtained from Serotec (Oxford, UK).

Cells and Cell Culture—A human B-lymphoid cell line (Reh) stably transfected with pMe4 vector (clone pMe4) or RIIα under direction of the human metallothionein IIA promoter (clone RIIα) (15) was maintained under standard conditions (5% CO2 in RPMI 1640 medium containing 5% [v/v] FCS, 2 mM L-glutamine, and 100 μg/ml streptomycin) (15). Each third month, the cells expressing RIIα were incubated under standard conditions in the presence of 200 μg/ml hygromycin B. On the day that the experiments were performed, the cells were seeded into Eppendorf tubes at a density of 8 × 104 cells/tube.

Measurement of Protein Synthesis—The cells were washed twice with HEPES medium (bicarbonate-free Eagle’s minimum essential medium buffer with 20 mM HEPES to pH 7.4), and then incubated for the same medium for 30 min at 37 °C. The samples were then incubated in the presence or absence of 350 μM 8CPT-cAMP for 30 min before 1, 10, and 100 ng/ml of ricin were added to the cells, which were further incubated for 30 min at 37 °C. The cells were incubated thereafter with HEPES medium containing 1 μCi/ml [3H]leucine for 20 min at 37 °C, extracted with 5% (v/v) trichloroacetic acid for 10 min followed by a brief wash with the same solution. Subsequently, cells were dissolved in 0.1 M KOH, and the acid-precipitable radioactivity was measured. The results are presented as percentage of radioactivity incorporated in cells incubated without toxin. The concentration of ricin required to inhibit the protein synthesis by 50% was chosen as a measure of the sensitivity of cells to ricin. Variation between duplicate measurements was less than 15%.

Sulfation of Ricin Sulf-1 and Sulf-2—Recombinant ricin A-sulf-1 and ricin A-sulf-2, modified to contain a tyrosine sulfation site and both a tyrosine sulfation site and three overlapping N-glycosylation sites, respectively, were expressed, purified, and reconstituted with ricin B chain (ricin sulf-1 and ricin sulf-2, respectively) according to the procedure described previously (14). The cells were washed twice in sulfate-free RPMI 1640 medium that contained 2 mM L-glutamine, and then incubated with 0.1 mM/ml Na[35S]SO4 in the same medium for 3 h. The cells were then incubated in the presence or absence of 350 μM 8CPT-cAMP and/or 20 μg/ml cycloheximide for 30 min at 37 °C, before ricin sulf-1 (−300 ng/ml) was added. The incubation was continued for 2 h at 37 °C. The cells were then washed twice for 5 min at 37 °C with HEPES medium that contained 0.1 mM lactose followed by cold PBS (140 mM NaCl and 10 mM NaH2PO4, pH 7.2). The cells were then lysed (lysis buffer, 0.1 mM NaCl, 10 mM NaH2PO4, 1 mM EDTA, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1 mM aminatin, pH 7.4), and centrifuged at 5000 rpm for 10 min at 4 °C. The supernatant was immunoprecipitated overnight at 4 °C with rabbit anti-ricin antibodies immobilized on protein A-Sepharose. The beads were then washed twice with PBS containing 0.5% (v/v) Triton X-100, and the immunoprecipitated material was analyzed by SDS-PAGE (12%) under reducing conditions.

SDS-PAGE—SDS-PAGE was carried out in the presence of β-mercaptoethanol as described previously (16). The gels were fixed in 4% acetic acid (v/v) and 27% (v/v) methanol for 30 min and then incubated with 1 mM sodium salicylate, pH 5.8, in 2% (v/v) glycerol for 30 min. The dried gels were then exposed to Kodak XR-5 films (Eastman Kodak Co.) at −80 °C for autoradiography.

Immunofluorescence Microscopy—For analysis of ricin distribution, ricin was labeled with Cy3 fluorochrome-conjugated secondary antibodies according to the manufacturer’s instructions. The coverslips were coated with poly(d-lysine) (Mw = 150,000) as described previously (17). The cells were washed twice with HEPES medium before addition of Cy-5-labeled ricin (~1000 ng/ml). After incubating the cells for 30 min at 37 °C, they were washed with cold PBS and further incubated with 3% (w/v) paraformaldehyde in PBS for 15 min at room temperature. The cells were then washed 3 times with PBS before incubation with 0.1% (v/v) Triton X-100 dissolved in PBS for 5 min at room temperature. Subsequently, the cells were washed in PBS, and incubated with PBS containing 5% (v/v) FCS for 30 min. The permeabilized cells were incubated with rabbit anti-human RIIα (1:1000) or with mouse mAb CTR 433 (1:10) to label the medial Golgi complex, with mouse mAb CTR 433 (1:1000) to label the cis Golgi, or with sheep anti-human TGN46 (1:100) to label the trans Golgi in PBS containing 5% (v/v) FCS for 30 min at room temperature. The cells were then washed three times for 5 min with PBS containing 5% (v/v) FCS followed by incubation with FITC-labeled goat anti-mouse antibody (1:100) to detect CTR 433 and GM130, with Cy3-labeled goat anti-rabbit antibody (1:500) to detect RIIα, or with FITC-labeled donkey anti-sheep/goat antibody (1:100) to detect TGN46 in PBS containing 5% (v/v) FCS. After staining, the cells were washed three times for 5 min with PBS at room temperature, and the coverslips were mounted in Mowiol. Immunofluorescence microscopy was performed using a Leica (Wetzlar, Germany) confocal microscope. Images were analyzed and processed with a resolution of 1024 × 768 pixels/0.18 μm. Multiple scans were captured with the use of Adobe Photoshop 4.0 (Adobe Systems, Mountain View, CA).

For analysis of PKA distribution versus centrosomal marker, cells were fixed in 4% paraformaldehyde in PBS for 20 min at 37 °C, rinsed twice in PBS, and incubated for 10 min with 50 mM ammonium chloride in PBS. Subsequently, cells were permeabilized with 0.1% Triton X-100 in PBS with 0.2% BSA. Primary antibodies were diluted in PBS containing 3% BSA to concentrations of 140 ng/ml for mAb CTR 453 (a centrosomal marker), 100 ng/ml for rabbit anti-human RIIα, 500 ng/ml for rabbit anti-human RIIα, and 1 μg/ml for mouse anti-RIIα mAb and incubated for 1 h at room temperature. Cells were then washed three times in PBS (PBS with 1% Tween 20) to remove unbound antibodies followed by incubation with fluorochrome-conjugated secondary anti bodies (FITC and Texas Red) for 1 h at room temperature. Finally, the cells were mounted in CITIFLUOR (Citifluor, London, UK). Confocal microscopy was performed on a Sarastro 2000 confocal microscope (Amersham Biosciences), equipped with an argon laser (458 to 514 nm wavelength). Ten sections of 0.25 μm (averaging five full frames of the same section) were scanned, and stacks of optical sections for each data set were compiled with Voxel View software on an IRIS 4D-70 GT graphics work station (SGI, Mountain View, CA).

Intracellular Accumulation of Ricin—Ricin was 125I-labeled according to the procedure described by Fraker and Speck (18) to a specific activity of 5 × 108 cpm/ng. The intracellular accumulation of 125I-labeled ricin was measured using the ORIGEN analyzer (IGEN Inc., Rockville, MD). Ricin was labeled with Na[125I]iodoacetic acid (19), followed by addition of TAG-labeled ricin (25 ng/ml) to allow intracellular uptake of the toxin for 30 min at 37 °C, and the other half of the samples was washed in cold PBS. The cells were then lysed (lysis buffer, 100 mM NaCl, 5 mM MgCl2, 50 mM HEPES, and 1% (v/v) Triton X-100) for 10 min on ice. Ricin that is both TAG-labeled and

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RESULTS

Expression of PKA-RIIα in a RIIα-deficient, B Lymphoid Cell Line, Reh—To study effects related specifically to expression of RIIα, stably transfected cell lines expressing RIIα under control of the zinc-inducible type IIα metallothionein promoter were made together with control clones transfected with empty plasmid by selection on hygromycin B. Fig. 1 shows characterization of one clone expressing RIIα compared with a control clone. The RIIα transfected clone displayed high levels of RIIα mRNA (A) as well as a CAMP-binding protein, immunoreactive with RIIα antibodies and present in both the soluble S200 and detergent-soluble Tx-100 fractions (B). In contrast, a control-transfected clone did not have any detectable RIIα mRNA or protein. Both cell clones had equal levels of RIIβ mRNA. We conclude that the RIIα-transfected clone expresses RIIα at quite high levels even in the absence of zinc.

PKA-RIIα Expressed in Reh Cells Is Targeted to the Golgi-Centrosomal Region—It has previously been shown that RIIα is localized to the Golgi-centrosomal area in SaOS2 osteosarcoma cells and in COS-7 cells (5, 6). We have examined, by immunofluorescence and confocal microscopy, whether this was also the case when RIIα was expressed in Reh cells. Double staining of the centrosomal marker CTR 453 (Figs. 2, A, C, and E) and RIIα (Figs. 2, B, D, and F) demonstrated that RIIα was absent in wild-type cells (Fig. 2F versus C and E). Furthermore, the distribution of RIIα was wider than that of the centrosomal protein CTR 453 (Fig. 2F versus E). Because RIIβ is targeted to centrosomes and present in Reh cells, as in most cancer cell lines (Fig. 1; Ref. 5), we next examined the distribution of RIIα versus that of RIIβ by dual staining and image overlays (Fig. 2G). Again, RIIα (red) was found in a wider area than RIIβ (green, overlap seems yellow), which in separate experiments showed a distribution overlapping well with that of the centrosomal marker, CTR 453 (not shown). In addition, we performed immunofluorescence studies of the localization of RIIα and the Golgi markers GM130 and TGN46. As shown in Fig. 3, RIIα was partially colocalized with both Golgi markers. Together, these experiments demonstrated that clones expressing RIIα acquired a new PKA isoform that localizes in the centrosomal-Golgi region. To explore the function of this particular pool of Golgi-associated
PKA, we next examined the intracellular transport of ricin in Reh cells in the presence and absence of a Golgi-associated pool of PKA type IIα.

Intoxication of Lymphocytes with Ricin Is Affected by RIIα Expression—To investigate whether one or more steps along the intracellular route followed by ricin are affected by RIIα, we tested the sensitivity to ricin of clone RIIα cells with and without the stimulation of PKA and compared it with that of cells deficient in PKA type IIα (clone pMep). To activate PKA, we used a cell-permeable cAMP analog, 8CPT-cAMP, with high affinity for the type II regulatory subunits (23, 24). As shown in Fig. 4, cells expressing RIIα were ~2-fold more sensitive to ricin than the RIIα-deficient cells, and although addition of 8CPT-cAMP had a sensitizing effect on the pMep cells, it sensitized the cells expressing RIIα to a much larger extent. Similar experiments with untransfected cells (Reh), as well as with other clones transfected with RIIα with similar targeting, showed the same pattern of sensitivity (results not shown). Thus, RIIα regulates one or several steps on the route of ricin to the cytosol in lymphoid cells, although RIIα is not strictly required for ricin intoxication.

Sulfation of Ricin Sulf-1 in Cells with or without RIIα—To study the transport of ricin from the plasma membrane to the Golgi apparatus, recombinant ricin sulf-1 that contains a tyrosine sulfation site was used. The cells were incubated in the presence or absence of 8CPT-cAMP and ricin sulf-1 (Fig. 5). Because the protein synthesis was somewhat stimulated in cells preincubated with 8CPT-cAMP, and an increased transport of newly synthesized proteins through the TGN could in theory result in an increased competition for sulfation and thus interfere with the assay; some cells were also incubated without cycloheximide to inhibit protein synthesis. Fig. 5C shows that the sulfation of ricin in control cells increased by ~70% in the presence of 8CPT-cAMP, whereas the sulfation increased by ~120% in the cells expressing RIIα. However, in cells treated with both 8CPT-cAMP and cycloheximide, the sulfation of ricin increased by ~80% in the control cells and by ~250% in the RIIα-expressing cells. This result indicates that when PKA is activated, the transport of ricin to the Golgi apparatus is increased to a larger extent in cells expressing RIIα than in control cells.

Sulfation and Glycosylation of Ricin Sulf-2 in Cells with and without RIIα—To further investigate the transport of ricin to the ER, recombinant ricin sulf-2 that contained a tyrosine sulfation site and three overlapping N-glycosylation sites was used. When ricin sulf-2 was added to control cells (clone pMep) or to RIIα-expressing cells (clone RIIα) in the presence of radioactive sulfate and immunoprecipitated from cell lysates, two bands were visible (Figs. 6, A and B). The upper molecular weight band represents ricin that has been both sulfated and glycosylated, and the lower molecular weight band represents ricin that has only been sulfated. As shown in Fig. 6C, the amount of ricin in the ER, measured as sulfated and glycosylated ricin relative to the total amount of sulfated ricin, was significantly increased in cells expressing RIIα (clone RIIα) compared with the control cells (clone pMep) when PKA was activated by 8CPT-cAMP. These observations indicate that not only the transport of ricin to the Golgi apparatus but also the further transport of the toxin to the ER is increased by RIIα in the presence of 8CPT-cAMP.

Intracellular Accumulation of Ricin in Cells with and without RIIα—The increased sulfation of ricin sulf-1 and ricin sulf-2 observed in cells expressing RIIα compared with control cells deficient in RIIα could be caused by an increased binding and endocytosis of ricin or by an increased endosome-to-Golgi transport. We therefore investigated the endocytosis of ricin in the presence or absence of 8CPT-cAMP. Fig. 7 demonstrates that the accumulation of ricin after 2 h of incubation was not significantly changed by addition of 8CPT-cAMP or by the expression of RIIα. Similar data were obtained when the cells were incubated with ricin for 30 min (results not shown). In addition, the binding of ricin to the plasma membrane was not significantly altered by 8CPT-cAMP or by expression of RIIα (data not shown). Thus, the increased transport of ricin to the Golgi apparatus cannot be accounted for by a change in the endocytosis of ricin.

Colocalization of Ricin and RIIα in the Golgi Area—As evident from Figs. 2 and 3 and previous reports (5), RIIα mainly exhibits a perinuclear, Golgi-associated localization that is detergent-extractable, indicating membrane-associated localization. To investigate whether RIIα is associated with ricin-containing structures, immunofluorescence studies were performed using antibodies raised against human RIIα (Fig. 8A, visualized by Cy3-labeled secondary antibodies, red), Cy5-labeled ricin (Fig. 8B, blue), and the medial Golgi marker CTR 433 (Fig. 8C, visualized by FITC-labeled secondary antibodies,

FIG. 3. RIIα expressed in an RIIα-deficient, B lymphoid cell line, Reh, colocalizes with Golgi markers. RIIα cells were fixed, permeabilized, double stained with hIIα antibodies (red, A and D) and with the cis Golgi marker GM130 (green, B) or the trans Golgi marker TGN46 (green, E), and analyzed in a confocal microscope. Merged images are shown in C and F.
Both RIIα and ricin seemed colocalized (Fig. 8F) and were also found to partly colocalize with the medial Golgi marker (Fig. 8, D and E, respectively).

It has been reported that both the type IIα and IIβ regulatory subunits are associated with centrosomes (5). Because the previous experiment showed colocalization between ricin and RIIα/H9251, we investigated the distribution and localization of ricin in the centrosomal area. We showed that whereas minor amounts of RIIα/H9251 are present in centrosomes, no ricin was detected in this region (data not shown).

**DISCUSSION**

In the present study, we have investigated the influence of the Golgi-associated type IIα regulatory subunit of PKA on the intracellular transport of ricin. RIIα was expressed on a negative background, and 8CPT-cAMP was used to activate PKA. Because phenotypic differences between clones may arise (e.g., relating to incorporation in the genomic DNA), we investigated several clones expressing RIIα/H9251 with similar results. Different clones transfected with empty vector displayed a phenotype similar to that of wild-type cells.

The first indication that RIIα might be involved in regulation of intracellular transport was found by investigating the entry of the plant toxin ricin into the cytosol, measured as ricin toxicity. Even when PKA was not activated by addition of
external 8CPT-cAMP, the cells expressing RIIα were about 2-fold more sensitive to ricin than the control cells. The most likely explanation seems to be that there is a certain level of endogenous cAMP that partly activates PKA. This explanation was strengthened by the finding that the transfected cells were about 4-fold more sensitive to ricin than the control cells when PKA was activated by addition of external 8CPT-cAMP. Also, the control cells were shown to be slightly more sensitive to ricin when PKA was activated. This might be caused by the activation of other isozymes of PKA that also regulate intracellular transport but, apparently, less efficiently than PKA type IIα. The PKA type IIα might be a better regulator than the other PKA isozymes because it is closer to the vesicular route of ricin transport into the Golgi apparatus. It might be a question of the local concentration of PKA whether it serves as a good regulator.

It has previously been shown that addition of 8-bromo-cAMP to Madin-Darby canine kidney cells gives a selective stimulation of the transport of apically internalized ricin to the Golgi apparatus (25) (Fig. 9). However, in those cells, we cannot ascribe this regulation to a Golgi-located PKA. We here demonstrate that the sulfation of ricin is increased in cells expressing RIIα compared with control cells when PKA was activated by externally added 8CPT-cAMP. This result indicates that RIIα is a strong regulator of the transport of ricin from the plasma membrane to the Golgi apparatus. Earlier studies have demonstrated that calmodulin (26) and calcium (27) can modulate the transport of ricin from endosomes to the Golgi apparatus in other cell lines (Fig. 9). Clearly, different factors are involved in the regulation of retrograde transport.

Interestingly, confocal microscopy demonstrated a localization of both ricin and RIIα in the Golgi area. The difference in ricin sulfation was much larger when both cell types were preincubated in the presence of cycloheximide. Such experiments were performed because the protein synthesis was somewhat stimulated in cells incubated with 8CPT-cAMP (data not shown), and an increased transport of newly synthesized proteins through the TGN in theory could result in an increased competition for sulfation. Even though it cannot be excluded that cycloheximide might result in an increased transport of ricin to the Golgi apparatus, the increased sulfation of ricin that was observed in cells expressing RIIα compared with the control cells in the presence of 8CPT-cAMP and cycloheximide strongly supports the notion of a regulatory role of RIIα in intracellular transport from the plasma membrane to the Golgi apparatus.

The increased transport of ricin to the Golgi apparatus in cells expressing RIIα could be caused by increased binding and endocytosis of the toxin. However, no significant stimulation of the binding or the endocytosis of the toxin after 30 min or 2 h of incubation were observed in the RIIα-expressing cells, strongly indicating a selective regulatory role of RIIα in the transport of ricin from the endosomal compartment to the Golgi apparatus and further to the ER.

At the moment, we can only speculate about the molecular mechanism of the regulation of the endosome to Golgi transport of ricin by PKA type IIα. There are several examples of the importance of phosphorylation for transport. For example, it has been shown that the activity of PKA has an effect on the in vitro association of ARF1 to Golgi membranes (28). It is possible that upon stimulation, the Golgi-associated PKA type IIα

![Fig. 7. The effect of 8CPT-cAMP and expression of RIIα on accumulation of ricin in cells deficient in (clone pMep) or expressing RIIα (clone pRIIα). The intracellular accumulation of 125I-labeled ricin was measured in the presence and absence of 8CPT-cAMP after 2 h of incubation at 37 °C as the amount of toxin that could not be removed by lactose treatment. The error bars show half range between duplicates from a representative experiment.](image)

![Fig. 8. Localization of RIIα (A) and ricin (B) in the Golgi area (C). D to F, merged images of A and C, B and C, and A and B, respectively. Lymphoid Reh cells stably transfected with RIIα were incubated with Cy5-labeled ricin (~1000 ng/ml) for 30 min at 37 °C. The cells were then fixed, permeabilized, stained with antibodies against RIIα and a medial Golgi marker (CTR 433), and analyzed in a confocal microscope.](image)

![Fig. 9. Model of ricin endosome to Golgi transport. There is a well-established Rab9-dependent transport from late endosomes (LE) to the Golgi apparatus of furin and the M6PR. TGN38, Shiga toxin B, and ricin seem to be transported to the Golgi apparatus from early/ recycling endosomes (EE/ER). As indicated, several molecules implicated in the transport of ricin from the endosomal compartment to the Golgi apparatus have been identified.](image)
phosphorylates membrane proteins in neighboring compartments that recruit cytosolic proteins involved in the trafficking of ricin between endocytic organelles and the Golgi apparatus. Another possibility is that PKA type IIα is important for the fusion of incoming vesicles with the Golgi apparatus.

Transport of ricin not only to the TGN, where the sulfotransferase is located (29), but also from the Golgi apparatus to the ER, measured as ricin that has been both sulfated and glycosylated, was shown to increase in cells expressing RII type IIα as compared with the total amount of sulfated ricin. However, the fraction of ricin that has been both sulfated and glycosylated could have been a result of the stimulated transport to the Golgi apparatus of this toxin. An increased amount of glycosylated ricin could have been a result of the control cells in the presence of 8CPT-cAMP. An interesting possibility is that PKA type IIα regulates endosome-to-Golgi transport of ricin between endocytic organelles and the Golgi apparatus. Another possibility is that PKA type IIα is important for the fusion of incoming vesicles with the Golgi apparatus in the presence of 8CPT-cAMP, thus indicating an additional regulatory role of RII type IIα in the transport of ricin from the Golgi to the ER.

In conclusion, our results indicate that the Golgi-associated type IIα regulatory subunit of PKA regulates endosome-to-Golgi and Golgi-to-ER transport of ricin in lymphocytes.

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