FEBS Letters 561 (2004) 44–50 FEBS 28113

Regulation of a COPII component by cytosolic *O*-glycosylation during mitosis

Pierrick Dudognon*, Christine Maeder-Garavaglia, Jean-Louis Carpentier, Jean-Pierre Paccaud

Department of Morphology, University Medical Center, Geneva University, CH-1211 Geneva, Switzerland

Received 7 November 2003; revised 9 January 2004; accepted 11 January 2004

First published online 4 February 2004

Edited by Felix Wieland

Abstract Endoplasmic reticulum (ER)-to-Golgi transport is blocked in mammalian cells during mitosis; however, the mechanism underlying this blockade remains unknown. Since COPII proteins are involved in this transport pathway, we investigated at the biochemical level post-translational modifications of COPII components during the course of mitosis that could be linked to inhibition of ER-to-Golgi transport. By comparing biochemical properties of cytosolic COPII components during interphase and mitosis, we found that Sec24p isoforms underwent post-translational modifications resulting in an increase in their apparent molecular weight. No such modification was observed for the other COPII components Sec23p, Sec13p, Sec31p or Sar1p. Analyzing in more details Sec24p isoforms in interphase and mitotic conditions, we found that the interphase form of Sec24p was *O*-*N*-acetylglucosamine modified, a feature lost upon entering into mitosis. This mitotic deglycosylation was coupled to Sec24p phosphorylation, a feature likely responsible for the increase in apparent molecular weight of these molecules. These modifications correlated with an alteration in the membrane binding properties of Sec24p. These data suggest that when entering into mitosis, the COPII component Sec24p is simultaneously deglycosylated and phosphorylated, a process which may contribute to the observed mitotic ER-to-Golgi traffic block.

Key words: Endoplasmic reticulum-to-Golgi; COPII; Sec24p; Post-translational modification; *O*-*N*-Acetylglucosamine; *O*-Glycosylation; Phosphorylation

1. Introduction

The secretory pathway is responsible for the intracellular transport of newly synthesized molecules. Each transport step in the secretory pathway is mediated by vesicular intermediates that recruit and concentrate cargo molecules from the donor compartment and deliver it to the acceptor compartment. Newly synthesized proteins destined for delivery to the cell surface are inserted cotranslationally into the endoplasmic reticulum (ER) and, once correctly folded, are transported out of the ER. ER-derived vesicle formation is mediated by a set of cytosolic proteins termed COPII. The COPII coat consists of five soluble components: the small GTPase Sar1p, and two cytosolic coat complexes Sec23p–Sec24p and Sec13p–Sec31p [1–5]. In mammalian cells, the Sec23p protein family comprises two isoforms, Sec23Ap and Sec23Bp, while the Sec24p protein family is composed of four isoforms, Sec24Ap, Sec24Bp, Sec24Cp and Sec24Dp [2,3]. Coat assembly is activated by the recruitment of Sar1p-GTP to the membrane via Sec12p, a factor promoting nucleotide exchange on Sar1pGDP. This initial step allows the binding of the Sec23p–Sec24p complex and the subsequent incorporation of cargo into the forming bud. Finally, the recruitment of the Sec13p–Sec31p complex leads to membrane deformation and pinching-off of the vesicle [1,6–8]. The mechanism of the recruitment of COPII components on the ER membranes has been extensively described, but the regulation of this process remains unclear. The first remarkable point of control is the spatial and temporal organization of nucleotide exchange and GTP hydrolysis, which coordinates coat assembly with cargo selection and coat disassembly with vesicle fission. COPII recruitment is also modulated by the lipid composition of membranes [9,10]. Moreover, alterations in the recruitment of the COPII machinery in the presence of several drugs like H89 or okadaic acid underline the potential involvement of kinases or phosphatases for the recruitment of COPII [11–13]. However, the precise target of these phosphorylation events has not yet been identified. Finally, recent reports describing inhibition of ER-to-Golgi traffic by viral proteins suggest the existence of regulatory mechanisms of coat formation which are used by viruses for their replication [14,15]. Beside this fragmentary information, the regulation of COPII recruitment at the molecular level remains mostly an open question.

ER-to-Golgi transport is transiently blocked in mammalian cells during mitosis [16]. Recent quantitative analyses using electron microscopy have shown an increase in the cytosolic pool of COPII components and a parallel decrease in the number of exit sites on ER membranes. These observations suggest the existence of a mechanism of regulation of COPII activity leading to a functional alteration of the structures specialized in the export from the ER compartment [17,18]. The molecular mechanisms responsible for this block of ER-to-Golgi transport are not yet understood. The present report describes that the COPII component Sec24p undergoes post-translational modifications upon entering mitosis: a de-*O*-glycosylation accompanied by phosphorylation. We propose that these alterations are directly responsible for the block in ER-

*Corresponding author. Fax: (41)-22-7025260.
E-mail address: pierrick.dudognon@medecine.unige.ch (P. Dudognon).

Abbreviations: DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TfR, transferrin receptor

0014-5793/04/$30.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(04)00109-7

to-Golgi trafficking and contribute to the Golgi fragmentation observed during mitosis.

2. Materials and methods

2.1. General materials and reagents

Rabbit polyclonal antibodies against hSec23Ap and hSec24Cp proteins have been described previously [3]. Monoclonal antibodies against transferrin receptor (TfR) (clone H68.4), actin or *N*-acetyl glucosamine (GlcNAc, clone RL2) were purchased from Zymed Laboratories. Polyclonal rabbit anti-phosphoserine (Z-PS1), polyclonal rabbit anti-phosphothreonine (Z-PT1), polyclonal rabbit anti-PS/PT/PY and monoclonal anti-phosphotyrosine (clone PY20) were purchased from Bioreagent Company.

Secondary antibodies conjugated to horseradish peroxidase were obtained from Bio-Rad. Secondary rhodamine-conjugated antibodies were from Jackson Company. Monoclonal antibody against tubulin (clone B512), monoclonal antibodies against phosphoserine (clone PSR-45, ref. P3430) and phosphothreonine (ref. P3555), nocodazole, thymidine, 2-deoxycytidine, 4,6-diamidino-2-phenylindole, NaF, sodium orthovanadate and propidium iodide were purchased from Sigma.

2.2. Cell culture and synchronization methods

HeLa cells were grown in modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin. HeLa cells were synchronized using either nocodazole or thymidine block methods. For nocodazole treatment, cells were incubated between 12 and 16 h in the presence of nocodazole 0.1 μg/ml, and cells blocked in metaphase were harvested in the culture medium by centrifugation. The thymidine block was performed as follows: cells were pre-synchronized by serum starvation for 12 h and then incubated for 16 h in complete medium containing 2 mM thymidine. Cells were then washed in culture medium and the G1-S block was released by incubation with 24 μM deoxycytidine for 9 h. The efficiency of synchronization was verified by FACS analysis (FACSCalibur, Becton Dickinson) by measuring the DNA content of the cellular population using propidium iodide as a DNA marker.

2.3. Immunofluorescence analysis and image processing

HeLa cells were transiently transfected with a plasmid encoding Sec24Cp in fusion with green fluorescent protein (GFP) [19]. In order to preserve the microtubule network, HeLa cells were fixed for 15 min in 4% paraformaldehyde in phosphate-buffered saline (PBS). Cells were permeabilized using Triton X-100 0.01% diluted in PBS. After 30 min incubation in PBS containing 1% bovine serum albumin, the cells were incubated with monoclonal antibodies against β-tubulin for 25 min at room temperature, followed by extensive washing with PBS. Goat anti-mouse rhodamine-conjugated antibodies were added for 25 min. The observation and imaging were performed within the Bioimaging Core Facility of the Geneva University Medical Centre: cells were imaged using a confocal Zeiss LSM 510 microscope and a 63×1.4 numerical aperture oil immersion lens. Pictures were processed with LSM® proprietary software.

2.4. Cell extracts, fractionation, immunoprecipitation

Cell extracts were prepared as either Triton X-100 lysates or cytosol. For Triton X-100 extract, 2×10^6 cells were lysed in 300 μl of lysis buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, Triton X-100 0.1%, 20 mM EDTA, 20 mM EGTA, with the addition of Complete® protease inhibitor from Boehringer Mannheim). Each condition was loaded in duplicate in order to ensure more accurate determination of molecular weight modifications.

To obtain native cytosol and microsomal membranes, cells were resuspended in the appropriate buffer and homogenized using 25 strokes of a tight-fitting glass Potter homogenizer. Post-nuclear supernatant was prepared by centrifuging at $2000\times g$ for 5 min at 4°C. This post-nuclear supernatant was centrifuged at $100\,000\times g$ for 40 min at 4°C, snap-frozen in liquid nitrogen, and stored at −80°C. Buffer P, defined as the 'permissive' buffer, is a simplified buffer used previously for membrane preparations [26,28] and contains 20 mM HEPES pH 7.4 and 37 mM sorbitol. Buffer NP, defined as the 'non-permissive buffer' and previously used for fractionation experiments [2], contains 20 mM HEPES pH 6.8, 100 mM CH_3COOK, 5 mM $MgCl_2$, 8% sucrose, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 20 mM EDTA, 20 mM EGTA and Complete™ protease inhibitor mixture.

For fractionation experiments, microsomal membranes were prepared in NP buffer. The pellet and the supernatant fractions were brought to an equal volume with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer and equal volumes were analyzed by SDS–PAGE and immunoblotting.

Immunoprecipitations were performed at 4°C on a range of proteins from 100 μg to 700 μg of Triton X-100 cell extracts using 20–30 μl of antiserum against Sec24C in a total volume of 500 μl. The precipitate was collected by adding 10 μl of protein A-agarose beads (1:1 slurry; Amersham Pharmacia Biotech, Sweden), washed three times in 1 ml of lysis buffer containing 1% of Triton X-100. The beads were resuspended in reducing SDS–PAGE sample buffer and run on 7.5% polyacrylamide denaturing gels before being transferred to nitrocellulose and analyzed by Western blotting.

In order to monitor the phosphorylation level of interphase and mitotic Sec24Cp, the immunoprecipitation of phosphorylated proteins was done with a mix of monoclonal antibodies to phosphoserine and phosphothreonine amino acids. The immunoprecipitated proteins were collected by adding 10 μl of protein G-agarose beads (1:1 slurry; Amersham Pharmacia Biotech). The beads were then washed three times in 1 ml of lysis buffer containing 0.1% of Triton X-100. The analysis by Western blotting used a serum against Sec24Cp.

2.5. Assays to monitor the stability of the mitotic form of Sec24C

30 μg of cytosol prepared in P buffer were incubated at 37°C for 10–30 min. In order to screen effects of different drugs, we added dimethyl sulfoxide (DMSO) at a final concentration of 3%, or a mixture of phosphatase inhibitors consisting of 1 mM NaF and 3 mM sodium orthovanadate. The molecular weight of Sec24Cp was then assessed by SDS–PAGE and Western blotting. Each condition was loaded in duplicate in order to ensure accurate determination of the post-translational modifications.

2.6. Preparation of the membranes and recruitment assay

Recruitment properties of Sec24Cp were analyzed as previously described by Aridor et al. [20] with minor modifications. COPII-depleted membranes were prepared in three steps. Microsomal membranes were obtained by 15 min centrifugation ($15\,000\times g$, 4°C) of the post-nuclear supernatant fraction. The membrane pellet was then resuspended in NP buffer in the presence of 1 mM GDP for 10 min at 37°C. The reaction was centrifuged and the pellet successively subjected to a urea wash (0.375 M sorbitol, 20 mM HEPES, 2 M urea and the Complete™ protease inhibitor mixture) and a salt wash (0.375 M sorbitol, 20 mM HEPES, 1 M KCl and Complete™ protease inhibitor mixture) to remove any remaining Sec24C bound to the membranes. The washes were performed at 4°C and followed immediately by a centrifugation at $15\,000\times g$ for 5 min. The membrane pellet was finally resuspended in an appropriate volume of NP buffer at a protein concentration of about 2 μg/μl.

For the recruitment reaction, 75 μg of a mixture consisting of 1 part of interphase cytosol and 3 parts of mitotic cytosol prepared in NP buffer were added to 8 μg of salt-washed interphase membranes in a final volume of 80 μl containing 100 μM GTP-γS, an ATP regenerating system (10×, 20 mM ATP, 4.4 mM GTP, 300 U/ml creatinine phosphate kinase, 10 mM creatine phosphate in 250 mM HEPES pH 6.8) and recruitment buffer (10×, 18 mM $CaCl_2$ and 25 mM MgOAc). Following incubation, the reactions were layered on a 15% sucrose cushion (180 μl) prepared with NP buffer. The reactions were centrifuged for 15 min at $15\,000\times g$ at 4°C, and the membrane pellets solubilized in SDS–PAGE sample buffer were analyzed by Western blotting.

3. Results

3.1. The intracellular localization of Sec24p is altered in mitotic cells

The molecular mechanisms underlying the block in ER-to-Golgi trafficking occurring during the course of mitosis are unknown [16]. A partial redistribution of Sec13p/Sec31p COPII subcomplexes to the cytosol during mitosis has been de-

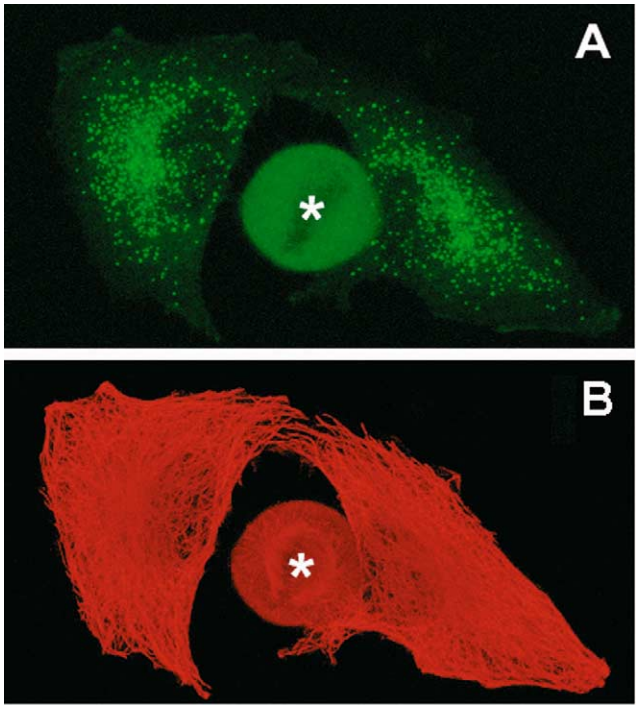

Fig. 1. Steady-state localization of COPII Sec23p–Sec24p subcomplex in interphase and mitotic HeLa cells. HeLa cells were grown on coverslips. Cells were transiently transfected with GFP-Sec24C then fixed and processed for immunofluorescence using a mouse monoclonal anti-tubulin antibody, followed by a rhodamine-conjugated goat anti-mouse antibody. The mitotic cell is indicated with a star on the metaphase plate.

scribed previously [18]. In order to extend these observations, we monitored the distribution of the Sec23p–24p complex during mitosis by confocal microscopy. The comparison in the same field of interphase and mitotic cells showed that the distribution of Sec24p was significantly altered in mitotic cells. Sec24Cp-labeled structures present in interphase cells are replaced by a diffuse staining in mitotic cells (at least 10 mitotic cells were observed by qualitative fluorescence microscopy) (Fig. 1A), confirming what was described for Sec13p, whose recruitment is dependent upon Sec23–24 [18]. Double labeling against the tubulin network allows a more objective identification of both interphase and mitotic cells (Fig. 1B). These results confirmed that membrane recruitment of the Sec23–24p and Sec13–31p complexes is altered within the short time window of mitosis.

3.2. *Sec24p proteins support a post-translational modification during mitosis*

These observations led us to hypothesize that post-translational modifications of either Sar1p or Sec23p–Sec24p during mitosis could be responsible for this impaired membrane recruitment. To test this hypothesis, total cell lysates from cells in interphase or metaphase were analyzed by Western blotting. Cells were synchronized in metaphase using three distinct methods. The first one took advantage of the fact that during metaphase, dividing cells have rounded up and lost their attachment to their substrate. They can be collected by vigorous shaking of the culture flask. The main advantage of this method is to avoid any pharmacological agent; but its disadvantage is its very low yield. The second method took advantage of the drug nocodazole, which blocks cells at the initiation of metaphase by interfering with the formation of the mitotic spindle [21]. The third method used an excess of thymidine in the incubation medium, which depletes the pool of deoxycytidine, and leads to cell cycle arrest at the G1/S stage. The cell cycle can be resumed by the addition of an excess of deoxycytidine, resulting in a wave of synchronized cells which are captured when entering metaphase [22]. FACS analysis was performed to verify the synchronization state of cells. As shown in Fig. 2, the apparent molecular weight of most COPII components remained unchanged during metaphase except for Sec24Cp, which exhibited an increase in its apparent molecular weight, while the intensity of its band decreased. This decrease in signal intensity was not due to protein degradation, but rather to a defect of transfer out of the gel and/or a defect of binding of the mitotic Sec24p as efficiently as the interphase Sec24p or to a decrease in antibody avidity for the mitotic form (see below).

The shift in molecular weight of Sec24Cp was independent of the cell lysis method used to prepare cell extracts (mechanical lysis, or detergent-mediated extraction using NP40, CHAPS, RIPA or SDS). The shift was also visible in denaturing SDS–PAGE gels containing 6 M urea, rendering unlikely a conformational change as the only cause of the change in molecular weight (data not shown). Finally, the apparent shift in molecular weight was also observed for the Sec24Ap and Sec24Bp isoforms (Fig. 2B) (no antibodies against Sec24Dp are yet available). Together, these results suggest that a post-translational modification of the entire Sec24 protein family occurs when cells enter metaphase.

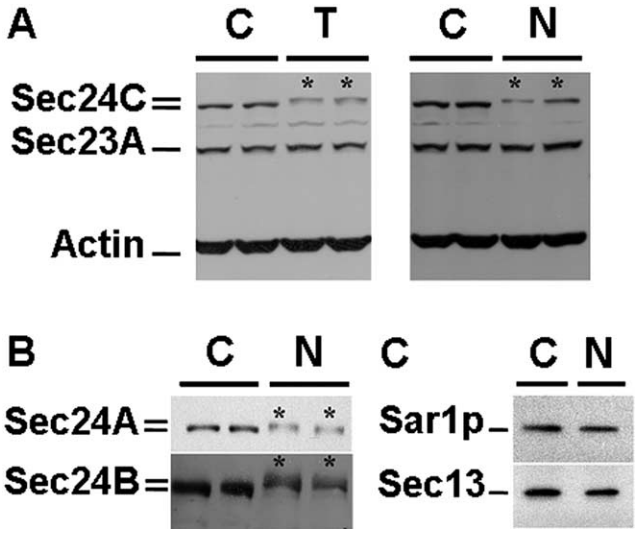

Fig. 2. Sec24p isoforms support post-translational modification during mitosis. A: HeLa cells were grown under appropriate conditions and synchronized either with thymidine block (lane T) or with nocodazole treatment (lane N), or not synchronized (lane C) as described in Section 2. Triton X-100-treated cell extracts were separated on 7.5% SDS–PAGE gels and analyzed by immunoblotting for their content of Sec24Cp, Sec23Ap and actin. Each condition was loaded in duplicate. B,C: Total proteins from interphase (lane C) or mitotic cells (lane N) were probed with polyclonal antibodies either against Sec24Ap or against Sec24Bp, Sec13p and Sar1p proteins. In A and B, stars indicate the mitotic form of Sec24 isoforms.

3.3. Interphase Sec24Cp is de-O-glycosylated on entering mitosis

We proceeded next to the identification of the nature of this modification. To do so, detergent lysates from interphase and mitotic cells were analyzed by Western blotting. Cell extracts were immunoprecipitated with an anti-Sec24Cp antiserum, separated by SDS–PAGE gels, and analyzed by immunoblotting with various antibodies specific for different post-translational modifications such as *O*-glycosylation, ubiquitination and phosphorylation. Ubiquitination was not detected. Surprisingly, we discovered instead that Sec24Cp is *O*-GlcNAc modified during interphase (lane C (control), IP Sec24C, Fig. 3A), and that this glycosylation is lost during mitosis (lanes N (nocodazole), IP Sec24C, Fig. 3A).

To ascertain whether the de-*O*-glycosylation of Sec24Cp was not a side effect of nocodazole, a similar analysis on cells synchronized with thymidine was carried out. Immunoprecipitation was performed with an antibody to *O*-linked GlcNAc antibody and the blot revealed with anti-Sec24Cp antiserum. Under these conditions, Sec24Cp was abundantly precipitated in control cells, whereas only a small amount was precipitated from mitotic cells (lane T (thymidine), Fig. 3B). These results correlated with the incomplete synchronization obtained with thymidine (60–80% of mitotic cells per preparation), which appears on the blot as a double band of Sec24Cp on total extract of mitotic cells (lane Tot.lys.T, Fig. 3B). The final evidence for reversible glycosylation of Sec24Cp entering mitosis was obtained by using wheat germ lectin, which is known

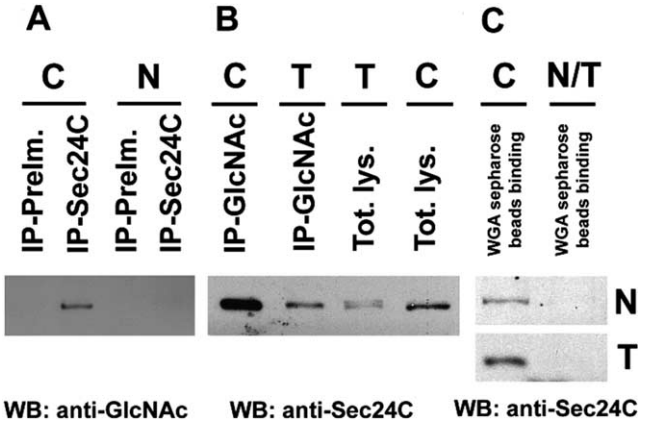

Fig. 3. Modification in the level of *O*-glycosylation of Sec24Cp during mitosis. A: Control (lane C) and nocodazole-mitotic (lane N) HeLa cells were lysed in Triton X-100-containing buffer and immunoprecipitated with either pre-immune serum (lanes IP-PreIm.) or anti-Sec24Cp immune serum (lanes IP-Sec24C). Immunoprecipitates were analyzed by SDS–PAGE followed by Western blotting as described in Section 2. Membranes were probed with a monoclonal anti-*O*-linked GlcNAc antibody. B: Mitotic cells synchronized with the thymidine block as well as control cells were prepared and lysed in Triton X-100-containing buffer. Control and mitotic Sec24Cp forms were immunoprecipitated with monoclonal anti-*O*-linked GlcNAc (respectively lane IP GlcNAc C and lane IP GlcNAc T). The immunoprecipitates were resolved by SDS–PAGE and analyzed by Western blotting using polyclonal antibodies against Sec24Cp protein. Control and mitotic total extracts were loaded as controls of the quality of the cytosol (respectively lane Tot. Lys. C and lane Tot. Lys. T). C: Cytosols prepared from interphase (lane C) and mitotic cells (synchronized with either thymidine block (lane T) or nocodazole (lane N)) were incubated with wheat germ lectin Sepharose beads. Absorbed proteins on beads were resolved and analyzed by SDS–PAGE and immunoblotting against Sec24Cp.

to bind *N*-acetyl-β-glucosaminyl residues. Cell extracts obtained from mitotic cells prepared with two different methods of synchronization (T for thymidine block and N for nocodazole treatment) were applied to wheat germ lectin agarose beads and the bound proteins analyzed by Western blotting. An important quantity of Sec24Cp is captured in interphase conditions while mitotic Sec24Cp is not absorbed on wheat germ lectin agarose beads (Fig. 3C). Again, interphase Sec24Cp was highly *O*-glycosylated, whereas its mitotic counterpart lacked any sign of glycosylation.

As negative controls, we monitored the level of *O*-glycosylation of the other COPII components (Sec23p, Sar1p and Sec13p), none of these proteins showed any sign of cytosolic *O*-glycosylation by immunoprecipitation using antibodies to *O*-GlcNAc residues (data not shown). Thus, de-*O*-glycosylation of Sec24p proteins occurs during mitosis, and it is attractive to postulate that this modification on this COPII component may be implicated in the transport arrest observed during cell division.

3.4. Sec24Cp supports de-O-glycosylation and phosphorylation during mitosis

Nuclear and cytosolic *O*-glycosylations have been described for an increasing number of proteins, and are thought to regulate protein function in a similar manner to phosphorylation [23,24]. Interestingly, *O*-glycosylation and phosphorylation can occur on the same protein in an exclusive manner, the protein switching between its phosphorylated and glycosylated states [25–27]. In our system, de-*O*-glycosylation correlated unexpectedly with an increase in the molecular weight of Sec24p instead of a decrease of its molecular weight, suggesting that other post-translational modification(s) was (were) occurring during mitosis. To monitor the phosphorylated status of Sec24Cp during mitosis, immunoprecipitation with a mix of monoclonal antibodies against phosphoserine and phosphothreonine were done and revealed by Western blotting with serum against Sec24Cp (Fig. 4A). In these conditions, Sec24Cp is immunoabsorbed in mitotic protein extract (lane IP phospho Ser/Thr N) and not in interphase protein extract (lane IP phospho Ser/Thr C). Thus Sec24Cp is phosphorylated during mitosis in mammalian cells. As an alternative way to confirm the nature of the post-translational modification, we took advantage of the following empirical observation: when cell extracts from mitotic cells were incubated under 'permissive' conditions for 10 min at 37°C (see Section 2), an enzymatic activity present in the freshly prepared mitotic cytosol converted the high molecular weight, mitotic form of Sec24p protein back into its interphase lower molecular weight form (lower panel, lane N at 37°C versus lane N at 4°C, Fig. 4B). On the contrary, when incubated in the non-permissive buffer, no change in Sec24Cp molecular weight was observed (upper panel, Fig. 4B).

This assay was used to investigate whether dephosphorylation could be implicated in the return of Sec24Cp to its interphase molecular weight. When a mixture of phosphatase inhibitors containing NaF and sodium orthovanadate was added to the permissive cytosol, the return of Sec24Cp to its interphase molecular weight was blocked (Fig. 4C), suggesting the involvement of phosphatase in the molecular weight change. Note the increase in intensity of the Sec24 band increased to the level of the interphase form in the absence of protein synthesis, suggesting that the decrease in

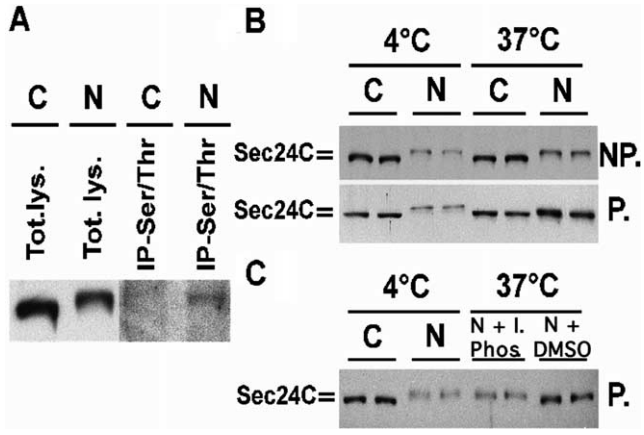

Fig. 4. Evidence of phosphorylation on Sec24Cp isoform during mitosis. A: Mitotic cells, synchronized using the method of thymidine blocking, as well as control cells were prepared and lysed in Triton X-100-containing buffer. Control and mitotic Sec24Cp forms were immunoprecipitated with a mixture of monoclonal antibodies against phosphoserine and against phosphothreonine (respectively lane IP phospho Ser/Thr C and lane IP phospho Ser/Thr N). The immunoprecipitates were analyzed by Western blotting using polyclonal antibodies against Sec24Cp. Control and mitotic total extracts were loaded as controls of the quality of the cytosol (respectively lane Tot. Lys. C and lane Tot. Lys. N). B: Interphase (lane C) or mitotic (lane N) cytosols were prepared in the permissive buffer (P, lower panel) or the non-permissive buffer (NP, upper panel) (see Section 2) and incubated for 10 min at 4°C or 37°C. The molecular weights of the mitotic and interphase forms of Sec24Cp were resolved by SDS–PAGE and analyzed by immunoblotting to detect Sec24Cp isoforms. C: In the same cytosols prepared in permissive buffer, either DMSO (lane N+DMSO) or a mix of phosphatase inhibitors (lane N+I.Phos.) were added (see Section 2). Cytosols were analyzed by Western blotting using Sec24Cp antibodies to monitor the evolution of the molecular weight under these conditions.

protein band intensity observed during mitosis was not caused by degradation of Sec24Cp, but rather by a decreased avidity of the antibody for the phosphorylated mitotic form.

Taken together, these results strongly suggest that the increase in molecular weight of Sec24 proteins observed during mitosis was due to phosphorylation.

3.5. *Membrane recruitment properties of mitotic Sec24Cp are altered*

This oscillation between two states strongly suggested that the function of Sec24 proteins is regulated during mitosis by post-translational modifications. In order to compare the functional properties of the mitotic and interphase forms of Sec24Cp, we monitored the recruitment properties of interphase and mitotic forms of Sec24Cp. This functional analysis was performed using a previously described COPII recruitment assay [13,20,28,29]. Washed ER membranes are incubated with cytosol under conditions in which COPII can be recruited to and stabilized on membranes [20]. To compare the recruitment properties of interphase and mitotic cytosol within the same assay, we mixed interphase and mitotic cytosols in appropriate quantities in non-permissive buffer, and incubated this mixture with purified and washed microsomes. Transferrin receptor (TfR) was used as a membrane marker to control membrane loading. We verified that the incubation conditions did not allow the transformation of mitotic Sec24Cp to its interphase state (data not shown). As shown in Fig. 5, the mitotic form of Sec24Cp could not be recruited to membranes, contrary to the interphase form. These results strongly show that the post-translational modification of Sec24 proteins during mitosis is linked to an impairment in COPII coat formation.

4. Discussion

In mammalian cells, intracellular trafficking is blocked during mitosis [16]. We have previously shown that the COPII subcomponent Sec13–31p complex is delocalized in part to the cytosol during metaphase [18]. In the present work, we demonstrate that post-translational modifications of the COPII component Sec24Cp take place during mitosis: upon entering metaphase, *O*-glycosylated Sec24p is deglycosylated and phosphorylated. These modifications affect the recruitment of the Sec23–24p complex to ER membranes. It is very attractive to hypothesize that these modifications could contribute to the block of protein transport between the ER and the Golgi during mitosis. No apparent post-translational modifications were observed on Sar1p, Sec23p and Sec13p. The molecular weight modification affects not only Sec24Cp, but also Sec24Ap and Sec24Bp, suggesting that this phenomenon is not isoform-specific: all Sec24p isoforms contain between five and eight potential *O*-glycosylation sites [25]. In the absence of antibodies against Sec24Dp, we could not verify its status during mitosis, but we assume that it is also subject to mitotic post-translational processing.

Using three different methods, we show that Sec24 proteins are *O*-GlcNAc modified during interphase, a feature which is reversibly lost upon entering mitosis (Fig. 3). Cytosolic *O*-glycosylation is a common post-translational modification described on a large set of proteins like transduction signal effectors, transcription factors, nuclear pore proteins and cytoskeletal elements [23]. *O*-Glycosylation may alter protein functions. For example, activities of the synaptic terminal marker synapsin I and the transcription factor Sp1 are modified by *O*-GlcNAcylation [30,31]. A similar post-translational modification has also been observed on AP3 and AP180, proteins involved in clathrin-mediated endocytosis [32].

Concerning COPII proteins, several studies have suggested

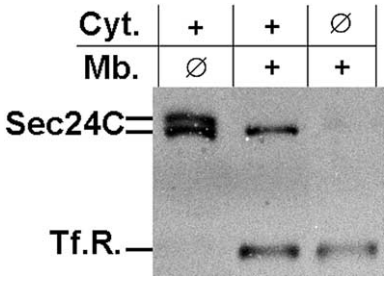

Fig. 5. Mitotic Sec24Cp is not able to bind interphase membranes in an in vitro recruitment assay. Membranes from interphase cells were prepared and washed under mild salt conditions (see Section 2) (third lane from left). In parallel, native cytosol from interphase and mitotic cells (ratio: 3×mitotic proteins:1×interphase proteins; see Section 2) (first lane from left). After the recruitment reaction, the pellet was separated from the supernatant, and each pellet fraction was analyzed by Western blotting, revealing either TfR (as the marker of the total membranes used for the recruitment reaction) or the Sec24Cp bound to the membranes (central lane). The synoptic table indicates the content of each lane. In the table, Cyt and Mb mean respectively cytosol and membranes. ∅ signifies no biological material from the corresponding row.

that phosphorylation might regulate these of protein complexes and affect their trafficking function between the ER and the Golgi apparatus. Beside the direct demonstration that Sec31p is a phosphoprotein [33], most evidence is indirect. Drugs such as the protein kinase inhibitor H89, or phosphatase inhibitors such as okadaic acid or microcystin L, inhibit early transport events such as the recruitment of COPII components on the ER membrane [11–13,34]. But the identity of the proteins targeted by these modifications is still unknown.

We observed that the loss of sugar residues during mitosis is accompanied by another modification, which induces a visible molecular weight change of the protein. We excluded ubiquitination as being responsible for the observed molecular weight change using antibodies to ubiquitin. We provide both direct and indirect evidence suggesting that the mitotic Sec24Cp was phosphorylated. First, using antibodies to phosphoserine and phosphothreonine, we could immuno-isolate Sec24Cp, revealing the phosphorylated status of mitotic Sec24Cp. Second, taking advantage of the fact that mitotic cytosol incubated under appropriate conditions enables the high molecular weight Sec24 protein to return to its original, interphase state, we postulated that the loss of mass could be due to a dephosphorylation event, and as such could be blocked by common phosphatase inhibitors. Indeed, NaF and orthovanadate were able to block the reappearance of the interphase molecular weight of Sec24Cp (Fig. 4C), suggesting that phosphatase activity is necessary for this transformation to occur. Taken together, these results suggest that phosphorylation of Sec24 proteins occurs upon entry of the cell into mitosis, which in turn is coupled to de-*O*-glycosylation and a gain in molecular weight.

Several studies have found a 'yin-yang' relationship between *O*-GlcNAc and phosphorylation. In most cases, this interrelationship was established using drugs like phosphatase inhibitors or kinase activators [25,35,36]. In a few specific examples, this reciprocal relationship between glycosylation and phosphorylation has been mapped to the same residue [27,37–39]. However, for other proteins, the functional role of *O*-glycosylation/*O*-phosphorylation was less clear [37,40].

The sequential recruitment of COPII components to the membrane, leading to the formation of vesicles, is well characterized [41]. If any step in this cascade of events is inhibited, anterograde trafficking is prevented. We postulated that the observed post-translational modifications of Sec24 protein could influence its recruitment to membranes, as hinted at by the partial delocalization of the mitotic complex observed by immunofluorescence. To test this hypothesis, we used a modified recruitment assay previously described [20,28,29], and demonstrated that the mitotic deglycosylated, phosphorylated Sec24Cp is unable to bind to interphase membranes (Fig. 5). Thus, this provides evidence to support the hypothesis that Sec24Cp activity is functionally regulated by post-translational modifications such as *O*-glycosylation and phosphorylation.

Previous studies have reported that during mitosis, the fragmentation of the Golgi apparatus via a COPI-dependent mechanism as well as through phosphorylation of several proteins involved in the maintenance of the Golgi stacks were mainly responsible for the block of ER-to-Golgi transport [42–46]. In this report, we demonstrate that COPII is affected by a new post-translational modification, namely cytosolic de-*O*-glycosylation, coupled to phosphorylation. This modification affects the function of COPII by inhibiting its normal recruitment on ER membranes, leading to a block in anterograde vesicle formation and eventually to the disappearance of the Golgi cisternae during mitosis. The next step in understanding the precise molecular mechanism of the ER-to-Golgi trafficking block is to identify the enzymes responsible for the post-translational modifications. *O*-Glycosylation is mediated by *O*-GlcNAc transferase, recently cloned in mammalian cells [47,48]. However, the mechanisms involved in the control of *O*-GlcNAc transferase activity are unknown. The tools described in this paper open the way to biochemical characterization of the enzymatic activities controlling COPII function during mitosis.

Acknowledgements: We thank Benghezal Mohammed, Roland Le-Borgne, Michael Pepper, Gisou Van der Goot and Pierre Cosson for helpful discussions during the entire project, as well as Wanjin Hong for the anti-Sec13p antiserum. Work in this laboratory is supported by grants of the Swiss National Science Foundation.

References

[1] Schekman, R. and Orci, L. (1996) Science 271, 1526–1533.
[2] Paccaud, J.P., Reith, W., Carpentier, J.L., Ravazzola, M., Amherdt, M., Schekman, R. and Orci, L. (1996) Mol. Biol. Cell 7, 1535–1546.
[3] Pagano, A., Letourneur, F., Garcia-Estefania, D., Carpentier, J.L., Orci, L. and Paccaud, J.P. (1999) J. Biol. Chem. 274, 7833–7840.
[4] Shaywitz, D.A., Orci, L., Ravazzola, M., Swaroop, A. and Kaiser, C.A. (1995) J. Cell Biol. 128, 769–777.
[5] Shugrue, C.A., Kolen, E.R., Peters, H., Czernik, A., Kaiser, C., Matovcik, L., Hubbard, A.L. and Gorelick, F. (1999) J. Cell Sci. 112, 4547–4556.
[6] Antonny, B., Madden, D., Hamamoto, S., Orci, L. and Schekman, R. (2001) Nat. Cell Biol. 3, 531–537.
[7] Barlowe, C. and Schekman, R. (1993) Nature 365, 347–349.
[8] Weissman, J.T., Plutner, H. and Balch, W.E. (2001) Traffic 2, 465–475.
[9] Matsuoka, K., Orci, L., Amherdt, M., Bednarek, S.Y., Hamamoto, S., Schekman, R. and Yeung, T. (1998) Cell 93, 263–275.
[10] Spang, A., Matsuoka, K., Hamamoto, S., Schekman, R. and Orci, L. (1998) Proc. Natl. Acad. Sci. USA 95, 11199–11204.
[11] Pryde, J.G., Farmaki, T. and Lucocq, J.M. (1998) Mol. Cell. Biol. 18, 1125–1135.
[12] Lee, T.H. and Linstedt, A.D. (2000) Mol. Biol. Cell 11, 2577–2590.
[13] Aridor, M. and Balch, W.E. (2000) J. Biol. Chem. 275, 35673–35676.
[14] Doedens, J.R., Giddings Jr., T.H. and Kirkegaard, K. (1997) J. Virol. 71, 9054–9064.
[15] Xu, A., Bellamy, A.R. and Taylor, J.A. (2000) EMBO J. 19, 6465–6474.
[16] Warren, G. (1993) Annu. Rev. Biochem. 62, 323–348.
[17] Farmaki, T., Ponnambalam, S., Prescott, A.R., Clausen, H., Tang, B.L., Hong, W. and Lucocq, J.M. (1999) J. Cell Sci. 112, 589–600.
[18] Prescott, A.R. et al. (2001) Traffic 2, 321–335.
[19] Stephens, D.J., Lin-Marq, N., Pagano, A., Pepperkok, R. and Paccaud, J.P. (2000) J. Cell Sci. 113, 2177–2185.
[20] Aridor, M., Weissman, J., Bannykh, S., Nuoffer, C. and Balch, W.E. (1998) J. Cell Biol. 141, 61–70.
[21] Mullins, J.M. (1984) Cell Biol. Int. Rep. 8, 107–115.
[22] Galavazi, G. and Bootsma, D. (1966) Exp. Cell Res. 41, 438–451.
[23] Hart, G.W. (1997) Annu. Rev. Biochem. 66, 315–335.
[24] Comer, F.I. and Hart, G.W. (2000) J. Biol. Chem. 275, 29179–29182.
[25] Wells, L., Vosseller, K. and Hart, G.W. (2001) Science 291, 2376–2378.

[26] Cheng, X., Cole, R.N., Zaia, J. and Hart, G.W. (2000) Biochemistry 39, 11609–11620.
[27] Cheng, X. and Hart, G.W. (2001) J. Biol. Chem. 276, 10570–10575.
[28] Aridor, M., Bannykh, S.I., Rowe, T. and Balch, W.E. (1999) J. Biol. Chem. 274, 4389–4399.
[29] Aridor, M., Fish, K.N., Bannykh, S., Weissman, J., Roberts, T.H., Lippincott-Schwartz, J. and Balch, W.E. (2001) J. Cell Biol. 152, 213–229.
[30] Cole, R.N. and Hart, G.W. (1999) J. Neurochem. 73, 418–428.
[31] Roos, M.D., Su, K., Baker, J.R. and Kudlow, J.E. (1997) Mol. Cell. Biol. 17, 6472–6480.
[32] Murphy, J.E., Hanover, J.A., Froehlich, M., DuBois, G. and Keen, J.H. (1994) J. Biol. Chem. 269, 21346–21352.
[33] Salama, N.R., Chuang, J.S. and Schekman, R.W. (1997) Mol. Biol. Cell 8, 205–217.
[34] Davidson, H.W., McGowan, C.H. and Balch, W.E. (1992) J. Cell Biol. 116, 1343–1355.
[35] Vosseller, K., Wells, L. and Hart, G.W. (2001) Biochimie 83, 575–581.
[36] Griffith, L.S. and Schmitz, B. (1999) Eur. J. Biochem. 262, 824–831.
[37] Chou, T.Y., Hart, G.W. and Dang, C.V. (1995) J. Biol. Chem. 270, 18961–18965.
[38] Kelly, W.G., Dahmus, M.E. and Hart, G.W. (1993) J. Biol. Chem. 268, 10416–10424.
[39] Medina, L., Grove, K. and Haltiwanger, R.S. (1998) Glycobiology 8, 383–391.
[40] Chou, T.Y., Dang, C.V. and Hart, G.W. (1995) Proc. Natl. Acad. Sci. USA 92, 4417–4421.
[41] Kirchhausen, T. (2000) Nat. Rev. Mol. Cell Biol. 1, 187–198.
[42] Nakamura, N., Lowe, M., Levine, T.P., Rabouille, C. and Warren, G. (1997) Cell 89, 445–455.
[43] Lowe, M. et al. (1998) Cell 94, 783–793.
[44] Shorter, J. and Warren, G. (1999) J. Cell Biol. 146, 57–70.
[45] Lowe, M., Gonatas, N.K. and Warren, G. (2000) J. Cell Biol. 149, 341–356.
[46] Dirac-Svejstrup, A.B., Shorter, J., Waters, M.G. and Warren, G. (2000) J. Cell Biol. 150, 475–488.
[47] Kreppel, L.K., Blomberg, M.A. and Hart, G.W. (1997) J. Biol. Chem. 272, 9308–9315.
[48] Lubas, W.A., Frank, D.W., Krause, M. and Hanover, J.A. (1997) J. Biol. Chem. 272, 9316–9324.