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Spatial separation of Golgi and ER during mitosis protects SREBP from unregulated activation

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Sterol regulatory element-binding proteins (SREBPs) are membrane-bound transcription factors that reside as inactive precursors in the endoplasmic reticulum (ER) membrane. After sterol depletion, the proteins are transported to the Golgi apparatus, where they are cleaved by site-1 protease (S1P). Cleavage releases the active transcription factors, which then enter the nucleus to induce genes that regulate cellular levels of cholesterol and phospholipids. This regulation depends on the spatial separation of the Golgi and the ER, as mixing of the compartments induces unregulated activation of SREBPs. Here, we show that S1P is localized to the Golgi, but cycles continuously through the ER and becomes trapped when ER exit is inhibited. During mitosis, S1P is associated with mitotic Golgi clusters, which remain distinct from the ER. In mitotic cells, S1P is active, but SREBP is not cleaved as S1P and SREBP reside in different compartments. Together, these results indicate that the spatial separation of the Golgi and the ER is maintained during mitosis, which is essential to protect the S1P substrate SREBP from unregulated activation during mitosis.

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Introduction

Proliferation of animal cells requires the growth and partitioning of all cellular components during the cell cycle. The growth and integrity of organelles rely on the coordinated synthesis of lipids and proteins and must be tightly regulated. Sterol regulatory element-binding proteins (SREBPs) are key regulators of lipid and membrane biogenesis. The proteins are membrane-bound transcription factors that reside as silent precursors in the membrane of the endoplasmic reticulum (ER) (Espenshade and Hughes, 2007). The activation of SREBP is achieved by regulated intramembrane proteolysis after transport from the ER to the Golgi apparatus (Brown *et al*, 2000; Rawson, 2002). This process is highly regulated and crucially depends on the integrity and the spatial separation of the ER and the Golgi apparatus. After stimulation, SREBP is transported from the ER to the Golgi apparatus, where the proteins are sequentially cleaved by site-1 and site-2 proteases (S1P and S2P, respectively). Cleavage leads to the release of the active transcription factors from the membrane into the cytosol, which then enter the nucleus to initiate transcription. In the nucleus, SREBP binds to sterol regulatory element sequences in promoter regions to induce genes that regulate cellular levels of cholesterol and phospholipids.

SREBP activation is regulated by cellular levels of sterols. SREBP is bound to its escort protein Scap (SREBP cleavage activation protein), and in the presence of high sterol levels in the ER membrane the complex is retained by binding to the ER-resident protein Insig (Radhakrishnan *et al*, 2007; Sun *et al*, 2007). As sterol levels drop, Scap dissociates from Insig, causing a conformational change in Scap that exposes the binding site for the COPII coat proteins. The SREBP–Scap complex then leaves the ER in COPII vesicles and moves to the Golgi apparatus, where SREBP is cleaved by S1P and S2P to release the active transcription factor (Sun *et al*, 2005).

Previous experiments showed that the presence of S1P and SREBP in the same membrane compartment can induce cleavage and SREBP activation independent of sterol levels in the membrane (DeBose-Boyd *et al*, 1999; Hampton, 2000). Instead of transporting SREBP to the Golgi apparatus, the relocation of S1P into the ER is sufficient for cleavage. This has been shown by two different approaches. Treatment of cells with brefeldin A (BFA) disrupts the Golgi structure and induces parts of the Golgi apparatus to merge with the ER. This causes SREBP to be released from the ER membrane even in the presence of high cellular cholesterol levels, which normally regulate and inhibit the transport and processing of SREBP. BFA-induced activation of SREBP is dependent on S1P, because SREBP remained intact in a mutant cell line lacking active S1P. This suggests that BFA causes S1P to be translocated from the Golgi into the ER to induce SREBP cleavage (DeBose-Boyd *et al*, 1999). In a second approach, S1P was tagged with a KDEL sequence and expressed in cells. ER-resident proteins with a KDEL sequence (e.g. BiP) that escape the ER are bound in the Golgi apparatus by the KDEL receptor and subsequently transported back to the ER (Munro and Pelham, 1987). Expression of S1P–KDEL induces the processing and activation of SREBP, suggesting that active S1P in the ER is sufficient to activate SREBP even if S2P is unaltered (DeBose-Boyd *et al*, 1999; Hampton, 2000).

By mechanisms that resemble BFA treatment, the structural integrity of the Golgi apparatus is also compromised during mitosis as part of its normal inheritance process (Shorter and Warren, 2002). We wanted to explore this possibility, as it is currently unknown whether this process leads to the activation of SREBP, which would be independent of the well-characterized sterol-regulated activation. At the beginning of

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mitosis, the Golgi apparatus breaks down and, after successful cell division, the fragments fuse to re-form a functional Golgi in both daughter cells (Shorter and Warren, 2002). The underlying mechanisms that organize the division of the Golgi during mitosis are still not well understood. Similar to the nuclear envelope, which fragments in prophase and then fuses with the ER, components of the Golgi apparatus (e.g. Golgi enzymes) have also been described to merge with the ER during mitosis to facilitate Golgi partitioning (Zaal *et al*, 1999; Altan-Bonnet *et al*, 2006). However, other works using transiently expressed chimaeric Golgi enzymes found no evidence that Golgi and ER membranes did combine during mitosis (Pecot and Malhotra, 2004).

Previous approaches have not addressed the physiological relevance of why some Golgi components may be divided either independently or together with the ER. Here, we show that SREBP is not activated during mitosis, suggesting that the Golgi-resident enzyme S1P remains separated from the ER. This suggests that the distinct boundaries between the Golgi and the ER are preserved not only during interphase, but also during mitosis, to prevent unspecific activation of the transcription factor SREBP.

Results

S1P is a Golgi-resident enzyme

We first determined the distribution and properties of mammalian S1P. Western blot analysis with affinity-purified antibodies against S1P demonstrated that the enzyme is highly enriched in Golgi membranes when compared to rat liver homogenate, membrane-enriched fraction and cytosol (Figure 1A) (Wang *et al*, 2006). The protein was highly enriched in the Golgi fraction and co-purified with the Golgi matrix protein GM130, a well-established Golgi marker protein (Nakamura *et al*, 1995; Seemann *et al*, 2000). The luminal ER-resident chaperone GRP78/BiP was not detectable in the Golgi fraction, but was present in both total lysate fraction and membrane homogenate (lanes 1 and 3).

The Golgi apparatus is composed of at least two different parts: Golgi matrix proteins including peripheral Golgi membrane proteins of the GRASP and golgin protein families and Golgi-resident proteins that associate with the Golgi matrix, such as glycosylation enzymes (Seemann *et al*, 2002; Gillingham and Munro, 2003; Short *et al*, 2005). To characterize the association of S1P with the Golgi apparatus, we tested if the protein can be extracted from intact Golgi membranes with detergent. Golgi membranes were extracted with Triton X-100 and the insoluble Golgi matrix was recovered by centrifugation (Slusarewicz *et al*, 1994). Immunoblotting revealed that S1P was Triton X-100 resistant (Figure 1B). The Golgi glycosylation enzyme mannosidase II (ManII, right panel), which has been shown to bind the Golgi matrix, and also GM130 and the Golgi SNARE GOS-28 (middle panel) were also detergent resistant. In contrast, the transmembrane proteins p24 and Golgin84 that are incorporated into COPI transport vesicles were solubilized and did not associate with the Golgi matrix fraction (left panel).

S1P cycles between the Golgi apparatus and the ER

In intact cells, the treatment of BFA leads to the separation of Golgi matrix proteins from the Golgi enzymes. In the presence of BFA, the Golgi enzymes move back to the ER, leaving behind Golgi remnants, which are tubular-vesicular structures that contain Golgi matrix proteins (Seemann *et al*, 2000). Immunofluorescence analysis showed that S1P colocalized with GM130 in the perinuclear Golgi ribbon of NRK (normal rat kidney) cells (Figure 1C, upper panel). After treatment with BFA for 45 min, the characteristic Golgi structure was dissolved and GM130 was present in dispersed Golgi remnants. However, S1P did not colocalize with GM130, suggesting that it was transported along with Golgi-resident glycosylation enzymes into the ER (compare upper panel with lower panel).

In contrast to Golgi matrix proteins, Golgi enzymes slowly but continuously cycle during interphase between the Golgi complex and the ER (Cole *et al*, 1996; Storrie *et al*, 1998). To test whether S1P behaves like other Golgi enzymes, we inhibited proteins from leaving the ER by microinjection of a dominant-negative form of Sar1 protein ($Sar1^{DN}$; Figure 1D). Under these conditions, the enzymes still recycle back into the ER but over time become trapped. After 4 h, staining for GM130 showed an intact Golgi ribbon that was depleted of ManII (Figure 1D). The staining pattern of S1P was comparable to that of ManII, demonstrating that it is relocalized into the ER when cycling back to the Golgi is inhibited (lower panel).

Figure 1E shows an experiment designed to determine whether S1P is required for Golgi re-formation after BFA treatment. For this purpose, BFA incubation was combined with $Sar1^{DN}$ injection: first, the Golgi was disrupted with BFA and enzymes were relocated into the ER. The cells were then injected with $Sar1^{DN}$ and BFA was washed out for 2 h. The Golgi remnants containing GM130 re-formed the characteristic Golgi ribbon in the perinuclear region of the cells independent of the Golgi enzyme ManII (lower panel) and S1P (upper panel), which were retained in the ER. Together, the results suggest that S1P behaves like a Golgi-resident enzyme that cycles between the Golgi apparatus and the ER.

S1P colocalizes with Golgi matrix proteins during mitosis

Similar to BFA, the structural integrity of the Golgi apparatus is also compromised during mitosis as part of its normal inheritance process. Glycosylation enzymes have been described to relocate into the ER during mitosis (Zaal *et al*, 1999; Altan-Bonnet *et al*, 2006), but Golgi matrix proteins remain distinct from the ER (Seemann *et al*, 2000). Therefore, we analysed the localization of S1P during mitosis to determine in which compartment it resides (Figure 2). Exponentially growing NRK cells were fixed and stained with the DNA dye Hoechst and with an antibody against tubulin to identify cells at different stages of mitosis. They were also stained with antibodies against S1P (Figure 2A) or with anti-S1P and anti-GM130 antibodies together (Figure 2B) to test whether the proteins colocalize. During interphase, GM130 and S1P were both located on the Golgi ribbon in the perinuclear region of the cell. During prometaphase, the Golgi cisternae unstacked and fragmented into clusters of tubules and vesicles. In the course of M-phase, the Golgi membranes (Figure 2B) and S1P (Figure 2A and B) were seen to be separated into similar amounts on each side of the equatorial plate. The majority of Golgi membranes were found concentrated around astral microtubules and the two spindle poles

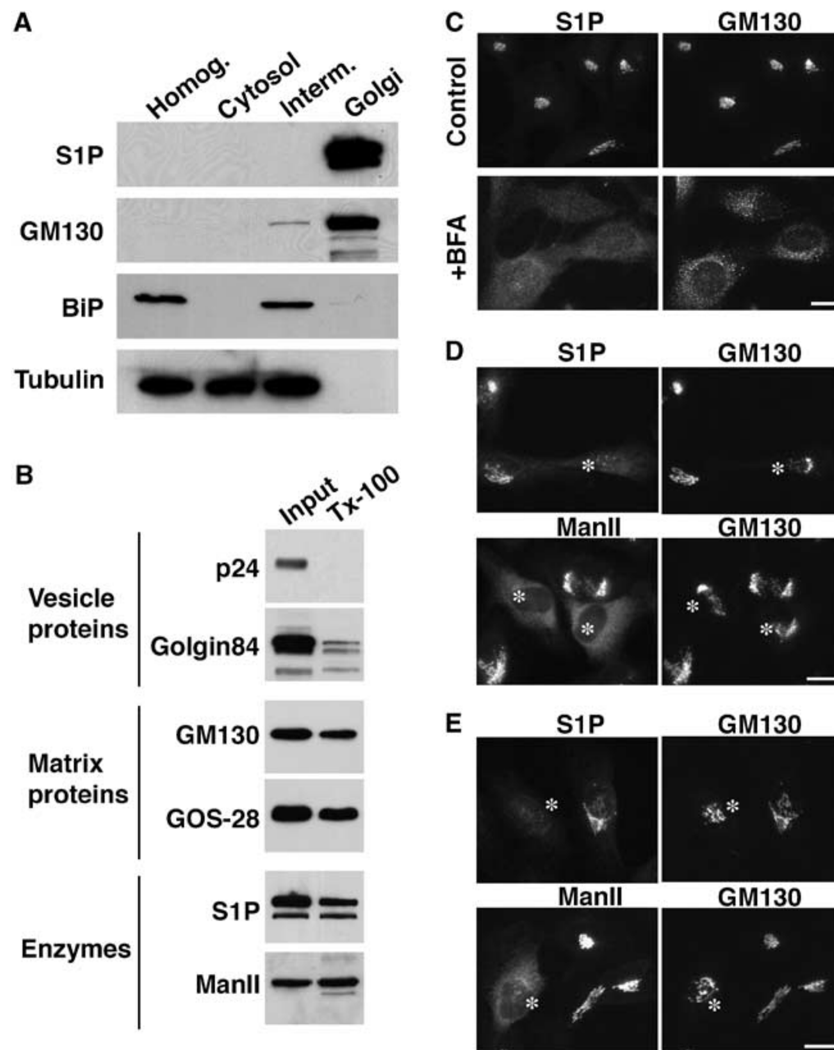

Figure 1 S1P associates with Golgi membranes and recycles through the ER. (**A**) S1P is enriched on Golgi membranes. Rat liver homogenate, cytosol, intermediate membrane fraction and Golgi membranes were separated by 10% SDS–PAGE (40 μg protein for homogenate (homog.), cytosol and intermediate (interm.); 10 μg for Golgi fraction) and analysed by immunoblotting with antibodies against S1P, GM130, Grp78/BiP and tubulin. (**B**) S1P is found in the Triton X-100-insoluble Golgi matrix fraction. Golgi membranes (300 μg) were extracted with Triton X-100 and pelleted by centrifugation. Equal amounts of protein were subjected to 10% SDS–PAGE and immunoblotted with the indicated antibodies. (**C**) BFA separates S1P from GM130. NRK cells were incubated for 45 min with 5 μg/ml BFA or carrier control. Cells were then fixed, permeabilized and stained with mouse anti-GM130 and rabbit anti-S1P antibodies followed by fluorescently conjugated secondary antibodies (anti-mouse AlexaFluor 594 and anti-rabbit AlexaFluor 488). (**D**) S1P is retained in the ER after $Sar1^{DN}$ microinjection. NRK cells were injected with 1 mg/ml purified $Sar1^{DN}$ protein, fixed after 3 h incubation, permeabilized and stained with an antibody against GM130 together with polyclonal antibodies against S1P or ManII. Injected cells were identified by coinjected cascade-blue-conjugated BSA (asterisks). (**E**) The Golgi apparatus re-forms without S1P. NRK cells were incubated for 45 min with 5 μg/ml BFA and injected with recombinant $Sar1^{DN}$ protein as in (D). BFA was washed out and after 2 h the cells were fixed, permeabilized and stained with an antibody against GM130 together with antibodies against S1P and ManII. Scale bars, 15 μm.

and a smaller pool was dispersed throughout the cytoplasm. Finally, the Golgi apparatus re-formed in each of the daughter cells (telophase II). During the entire M-phase, GM130 and S1P showed a high degree of colocalization, suggesting that S1P remained associated with Golgi membranes throughout the cell cycle and was not present in the ER (Figure 2B).

S1P remains distinct from the ER during mitosis

To biochemically analyse the distribution of S1P during mitosis, synchronized mitotic CHO (Chinese hamster ovary) cells, either control-treated or grown in the presence of BFA (3 h), were harvested by shake-off and post-chromosomal supernatants (PCSs) were separated on a 5–30% glycerol gradient (Jesch and Linstedt, 1998; Jesch *et al*, 2001; Seemann *et al*, 2002). After centrifugation, fractions were collected and membranes were pelleted by centrifugation. In control-treated cells, the majority of S1P was found in the top fractions, revealing a pattern similar to GM130. The ER protein GRP78/BiP, however, was found only in the bottom fractions of the gradient (Figure 3A, right panel). A portion of mitotic Golgi membranes was present in the same fraction as BiP at the bottom of the gradient. Previous work, however, showed that these Golgi membranes consist of larger breakdown products of the Golgi, which remain distinct from the ER (Jesch and Linstedt, 1998; Jesch *et al*, 2001; Seemann *et al*, 2002). In cells incubated with BFA, GM130 continues to be found in the top fractions, comparable to control cells. S1P, however, is shifted to the bottom fractions, consistent

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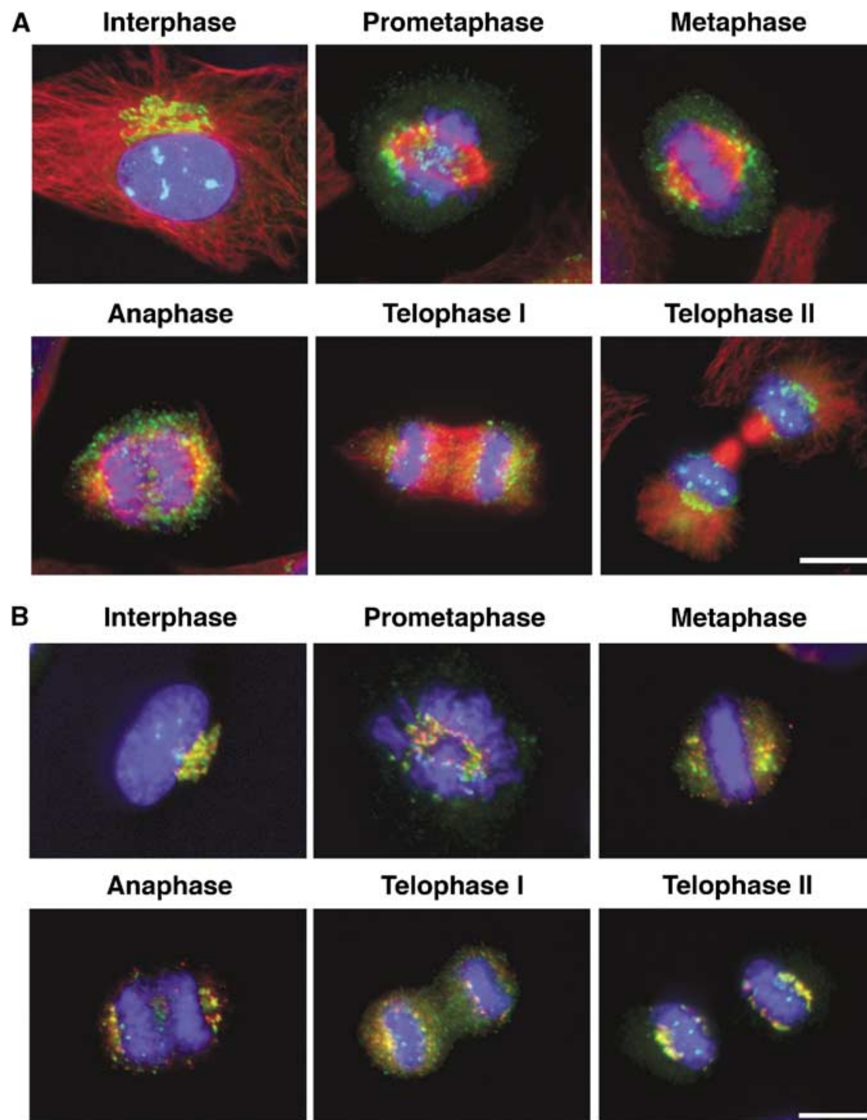

Figure 2 S1P colocalizes with Golgi proteins during mitosis. (**A**) Localization of S1P during mitosis. Exponentially growing NRK cells were fixed, permeabilized and triple stained with antibodies for S1P (green), tubulin (red) and DNA (blue). The different mitotic stages were identified by the organization of the microtubule network and the chromatin. (**B**) GM130 and S1P colocalize during mitosis. Cells were prepared and mitotic cells were identified as in (A) after staining with anti-GM130 and anti-S1P antibodies. Scale bars, 10 μm.

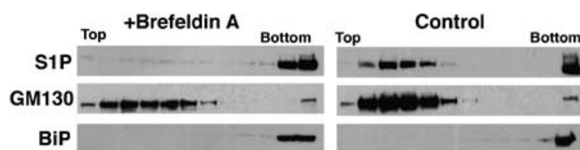

Figure 3 S1P is distinct from the ER during mitosis. Gradient fractionation of mitotic membranes. CHO cells treated with or without 3 μg/ml BFA were mitotically arrested with 2 mM *S*-trityl-L-cysteine. Mitotic cells were collected by shake-off and post-chromosomal supernatants were fractionated on a 5–30% glycerol gradient. Fractions were collected, separated by 12% SDS–PAGE and analysed by immunoblotting for S1P, GM130 and GRP78/BiP.

with ER being shown to contain GRP78/BiP (left panel). These results confirm that during mitosis S1P remains separated from ER membranes, although it was possible to force a translocation of the protease into the ER with BFA treatment.

SREBP remains uncleaved during mitosis

As our results found no evidence for the relocation of S1P into the ER during mitosis, we investigated whether its substrate, SREBP, is cleaved. SREBP is retained in the ER membrane in the presence of high sterol levels by binding to the ER-resident protein Insig (Yang *et al*, 2002). As the level of sterols drops, the SREBP-binding protein Scap dissociates from Insig and the SREBP–Scap complex is transported to the Golgi apparatus. There, SREBP is cleaved by S1P and S2P to release the active transcription factor. Independent of sterol levels in the ER, the treatment of interphase cells with BFA redistributes S1P from the Golgi apparatus into the ER (Figure 1B), leading to cleavage and activation of SREBP (DeBose-Boyd *et al*, 1999). To test whether SREBP is cleaved during mitosis, unsynchronized cells grown in the presence of cholesterol were stained with antibodies against SREBP (Figure 4A, upper panels). If S1P is located in the ER during mitosis, one would expect to detect the cleaved

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transcription factor portion of SREBP in the nucleus once cell division is complete. However, daughter cells that just finished mitosis, apparent by the remnants of the central spindle connecting the daughter cells, showed an ER-like staining pattern similar to interphase cells. In contrast, cells depleted of cholesterol that just finished cell division showed a strong nuclear staining for the cleaved active transcription factor portion of SREBP. The results show that S1P stays distinct from the ER, and SREBP remains unprocessed during mitosis (Figure 4A, lower panels).

To corroborate these results, we analysed whether SREBP is cleaved during mitosis by immunoblotting. Control cells or cells growing in the presence of BFA were enriched for 5 h in mitosis and harvested by shake-off (mitotic cells, M). Lysates from the remaining attached cells (interphase cells, I) and mitotic cells were probed with antibodies against endogenous SREBP (Figure 4B). BFA-treated cells showed the cleaved form of SREBP (left panel, lanes 3 and 4, lower band), as well as a reduced amount of full-length SREBP (upper band). Furthermore, in BFA-treated mitotic cells, an increased amount of SREBP was cleaved (compare lanes 3 and 4). In untreated mitotic and interphase cells, however, cleaved SREBP was not detectable and only unprocessed full-length SREBP was observed (lanes 1 and 2). Blots were reprobed with an antibody against the phosphorylated form of histone 3, which is specifically phosphorylated in mitotic cells (Dai *et al*, 2005). The difference in SREBP cleavage was not due to an altered expression level of S1P during the cell cycle or BFA treatment, as shown by immunoblots with an antibody against S1P (Figure 4B).

S1P is active in mitosis

One possible explanation for the absence of detectable cleavage products of SREBP in post-mitotic cells could be that S1P might be inactivated during mitosis. To address this possibility, we probed for S1P activity by using ATF6–GFP as a reporter. In contrast to SREBP, which is cleaved by the relocation of S1P into the ER alone (Figure 4B), ATF6 remains intact until ER stress is induced (Shen *et al*, 2002; Nadanaka *et al*, 2007). Similar to SREBP, the cleaved transcription factor then enters the nucleus, leading to the activation of ER stress response elements (Ron and Walter, 2007). Therefore, the induction of ER stress by DTT addition to mitotic cells should reveal whether S1P is active in mitosis. We induced the relocation of S1P into the ER before the cells entered mitosis in stably transfected CHO cells that express human ATF6 tagged with GFP under an inducible promoter, as antibodies against endogenous processed ATF6 were not sensitive enough. ATF6–GFP-expressing cells were treated with BFA together with nocodazole to arrest cells in mitosis and monitored by phase-contrast time-lapse microscopy (Figure 5). Cells arrested in mitosis were identified and DTT was added for 45 min to induce ER stress and thereby ATF6–GFP cleavage. The drugs were washed out to allow the cells to exit mitosis (0 min). At the end of the experiments (80 min), the cells were fixed and stained for the nuclear localization of ATF6–GFP. Cells that were treated with DTT during mitosis showed a strong nuclear localization of ATF6–GFP compared to cells that were not exposed to DTT (Figure 5). However, when BFA was omitted and cells were incubated with DTT alone, no nuclear form was observed (middle panel). This suggests that S1P remained active during mitosis. In summary, we conclude that SREBP is protected from unregulated activation because the separation of the ER from the Golgi apparatus is maintained during mitosis.

Discussion

In this study, we explored whether the Golgi-resident protease S1P activates the ER-localized transcription factors SREBPs during cell division. We show that S1P has similar properties as Golgi-resident glycosylation enzymes. Under steady-state interphase conditions, S1P is localized to Golgi membranes, where it binds to the Triton X-100-insoluble Golgi matrix *in vitro*. After BFA treatment or blocking proteins from leaving the ER, glycosylation enzymes are relocated into the ER. Similarly, S1P slowly but continuously cycles between the Golgi complex and the ER, which is in contrast to matrix proteins that remain on the Golgi apparatus.

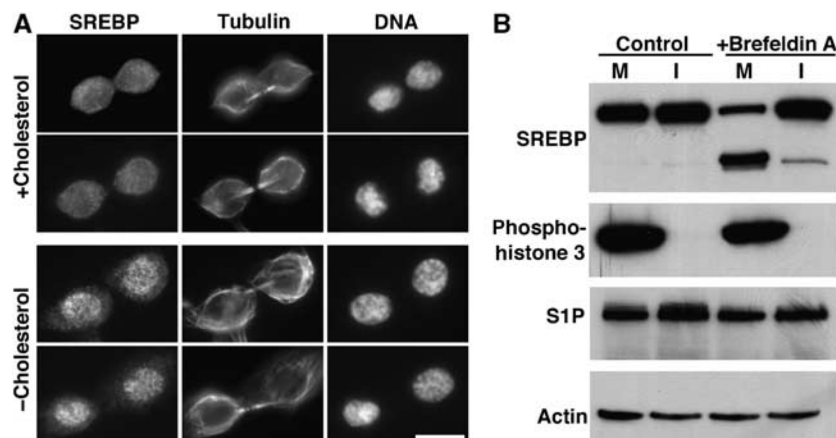

Figure 4 SREBP remains uncleaved in mitosis. (**A**) SREBP is not localized in the nuclei of post-mitotic cells. CHO cells were grown in sterol-depleting medium in the absence or presence of 1 μg/ml 25-hydroxycholesterol for 16 h. Cells were fixed, permeabilized and stained for SREBP and tubulin. DNA was labelled with Hoechst. Scale bar, 10 μm. (**B**) Biochemical analysis reveals SREBP processing in mitotic and interphase cells only after the addition of BFA. CHO cells treated with 3 μg/ml BFA or carrier control were arrested in mitosis for 5 h in the presence of 2 mM *S*-trityl-L-cysteine. Mitotic cells (M) were harvested by shake-off and the remaining cells (interphase, I) were collected by trypsinization. Equal amounts of protein (30 μg) were separated by 10% SDS–PAGE and immunoblotted with the indicated antibodies.

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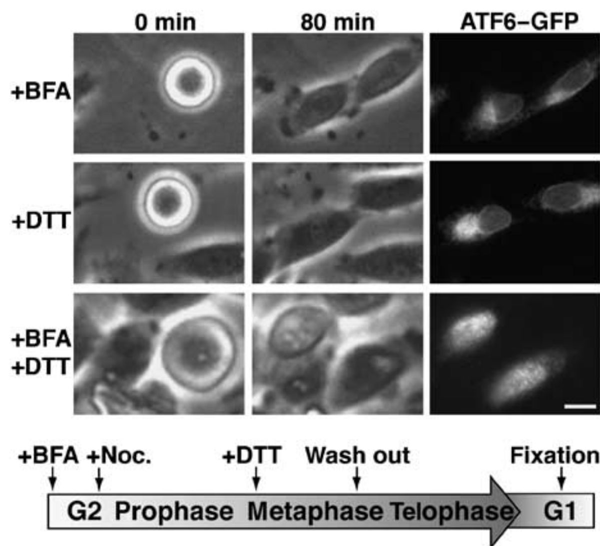

Figure 5 S1P remains active in mitotic cells. Stably transfected CHO cells were induced to express ATF6–GFP by induction with 5 μM ponasterone A for 16 h. Cells were then arrested in mitosis with 0.2 μg/ml nocodazole in the presence or absence of 3 μg/ml BFA. The arrested cells were then treated for 45 min with 2 mM DTT or control. The drugs were removed and the cells were followed by time-lapse microscopy ($t = 0$ min) until mitosis was complete ($t = 80$ min). The cells were fixed and stained with antibodies to GFP to detect ATF6–GFP. Note that only cells that entered mitosis in the presence of BFA that were further treated with DTT in mitosis showed cleaved ATF–GFP in the nucleus (+BFA +DTT), whereas in control cells treated with only BFA (+BFA) or DTT (+DTT), ATF6–GFP was restricted to the ER. Scale bar, 10 μm.

SREBPs are transcription factors that are the key regulators of genes for the biogenesis of cholesterol, lipids and membranes. The proteins reside as silent transmembrane precursors in the ER. After stimulation, the proteins leave the ER and are translocated to the Golgi complex by vesicular transport. In the Golgi, they are sequentially cleaved by two proteases S1P and S2P, leading to the release of the active transcription factor from the membrane into the cytosol and subsequent transport into the nucleus. This regulation of activation requires distinct boundaries between the Golgi complex and the ER. Indeed, it is sufficient to process SREBP to the active, nuclear form by relocating S1P into the ER membrane with a KDEL retrieval signal or by relocating the protease into the ER after BFA treatment (DeBose-Boyd *et al*, 1999).

Previous work analysed the distribution of Golgi-resident glycosylation enzymes by immunofluorescence, showing that under steady-state conditions about 80–90% of the signal was detectable in the Golgi complex and the remaining 10–20% was present in the ER (Rhee *et al*, 2005; Altan-Bonnet *et al*, 2006). As S1P shows similar dynamics as glycosylation enzymes, and therefore a significant percentage of the protein would be in the ER, a constant low level of SREBP activation during interphase would be expected. However, we did not see this small amount of cleaved SREBP. This could be explained by a short half-life of the activated transcription factor. Nevertheless, a potential low level of SREBP activation caused by cycling of S1P through the ER might be desirable for cell growth so that a basal activation level of genes involved in lipid biogenesis induced by SREBP might remain constant. This might be especially true, as the SREBP pathway is essential for growth and cell cycle progression (Goldstein and Brown, 1990; Goldstein *et al*, 2006).

At the beginning of mitosis, the Golgi apparatus disassembles into tubular vesicular fragments, which fuse after successful partitioning to re-form a new Golgi apparatus in both daughter cells (Shorter and Warren, 2002). Similar to the nuclear envelope that is reabsorbed into the ER during mitosis, it has been suggested that Golgi-resident enzymes also relocate into the ER. Because Golgi enzymes are mobile and cycle between the ER and the Golgi complex (Zaal *et al*, 1999; Altan-Bonnet *et al*, 2006), and because at the beginning of mitosis exit from the ER is inhibited (Featherstone *et al*, 1985; Collins and Warren, 1992), Golgi enzymes may accumulate in the ER. Although the inhibition of ER exit during interphase caused the relocation of S1P into the ER, we found no evidence for the presence of S1P in the ER membranes during mitosis. Moreover, S1P showed a similar staining pattern as GM130, decorating mitotic Golgi fragments in proximity to the spindle poles during all stages of mitosis and a smaller pool was found dispersed throughout the cytoplasm. The mitotic Golgi signal has been proposed to be generated by dispersed Golgi membranes that are below the optical resolution (Jesch *et al*, 2001; Jokitalo *et al*, 2001; Axelsson and Warren, 2004; Pecot and Malhotra, 2004), whereas other reports suggested that the signal represents Golgi enzymes that are dispersed throughout the ER (Zaal *et al*, 1999; Altan-Bonnet *et al*, 2006). We directly addressed this issue by gradient fractionation of mitotic membranes. The majority of S1P co-migrated with GM130 and remained distinct from the ER-containing fraction. S1P was found in the ER fraction when the Golgi enzymes were shifted into the ER by BFA before the cells entered mitosis. Second, only the intact precursor of SREBP was detected by immunoblotting in interphase and mitotic cells under high sterol conditions, whereas the processed and cleaved form was detected only after incubation with BFA. If cells enter mitosis in the presence of BFA, however, an increased amount of SREBP was cleaved, arguing that S1P remains active during mitosis.

Previous studies to determine whether Golgi enzymes move into the ER during mitosis gave opposite conclusions. The approaches relied on the overexpression of chimaeric proteins (Pecot and Malhotra, 2004) in combination with temperature alterations to trap the molecules in the ER during mitosis (Altan-Bonnet *et al*, 2006). Our approach addressed the physiological relevance of whether the Golgi enzymes remain independent of the ER. In principle, relocation of S1P into the ER membrane could cleave and activate SREBP already during cell division. This would ensure rapid transcriptional activation of genes for cholesterol, lipid and membrane biogenesis to allow the growth of organelles in G1. The fact that S1P remained distinct from the ER and that we did not detect activation of SREBP during mitosis suggest a mechanism that specifically activates the transcription factors during G1 when the cells need to grow in preparation for the next cell division.

From these experiments, we showed that the spatial separation between S1P in the Golgi apparatus and its substrate SREBP remains intact in the ER during mitosis to prevent an inappropriate activation of these transcription factors. This mechanism seems sufficient to block SREBP activation, because mixing of S1P with SREBP alone leads to SREBP cleavage.

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Materials and methods

Reagents and antibodies

The following antibodies were used (kindly provided by those listed below): monoclonal antibodies against actin and α-tubulin (Sigma, St Louis, MO), GM130, Gos28, Golgin84, GRP78/BiP (BD Biosciences, San Jose, CA) and SREBP-2 (7D4; DeBose-Boyd *et al*, 1999). Polyclonal antibodies: affinity-purified S1P (DeBose-Boyd *et al*, 1999), SREBP-2 (raised in rabbits against amino acids 32–250 of hamster SREBP-2), phospho-histone H3 (Ser10) (Millipore, Billerica, MA), GM130 (Martin Lowe), Golgin84 (Ayano Satoh), anti-ManII (Graham Warren), p24 (Tommy Nielsson), GFP (Pelletier *et al*, 2002). AlexaFluor 488-conjugated goat anti-rabbit antibodies, AlexaFluor 594-conjugated goat anti-mouse antibodies, Hoechst 33342, cascade-blue-conjugated BSA and ponasterone A were from Invitrogen (Carlsbad, CA). BFA was from Epicenter Technologies (Madison, WI), and Mowiol 4-88 and protease inhibitor cocktail 3 were obtained from Calbiochem (La Jolla, CA). Fortified cosmic calf serum and fetal bovine serum were from Hyclone (Logan, UT). DTT, nocodazole and cholesterol were obtained from Sigma. All other reagents were from Calbiochem, Invitrogen and Sigma. Recombinant His-tagged $Sar1^{DN}$ protein was expressed in *Escherichia coli* and isolated on Ni-NTA agarose (Qiagen, Valencia, CA) as described (Rowe and Balch, 1995). The buffer was exchanged by dialysis against 25 mM Hepes pH 7.2, 125 mM potassium acetate, 1 mM $MgCl_2$, 1 mM glutathione, 10 μM GDP and 50 μM EGTA.

Cell culture

NRK cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fortified cosmic calf serum (Hyclone), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a standard tissue culture incubator with 5% CO_2. CHO cells were grown in medium A (1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium supplemented with 100 μg/ml streptomycin sulphate and 100 U/ml penicillin) supplemented with 5% fetal calf serum (Hyclone). Sterol-depleting medium contains medium A supplemented with 5% newborn calf lipoprotein-deficient serum, 50 μM sodium mevalonate and 10 μM compactin (prepared as described by Sun *et al*, 2005).

To establish a CHO cell line that stably expresses GFP–ATF6 gene in an inducible system, we used pIND and pVgRXR vectors, which are components of an ecdysone-inducible mammalian expression system (Invitrogen). pIND-GFP-ATF6 encodes full-length human ATF6 fused with GFP at the N terminus driven by the ecdysone-inducible promoter (G418 resistant) and pVgRXR encodes ecdysone receptor (zeocin resistant). Expression of ATF6–GFP was induced with 5 μM ponasterone A for 14–16 h before the experiments.

Microinjection and immunofluorescence analysis

Capillary microinjection was performed with a Transjector 5246 and a Micromanipulator 5171 (Eppendorf, Westbury, NY). NRK cells grown on glass coverslips were injected into the cytoplasm with 1 mg/ml $Sar1^{DN}$ together with 5 mg/ml cascade-blue-conjugated BSA to mark injected cells. For immunofluorescence analysis, cells were fixed and permeabilized for 10 min in methanol at −20°C or first fixed in 3.7% formaldehyde in PBS followed by permeabilization in methanol at −20°C. The cells were then incubated with the indicated primary antibodies followed by secondary antibodies conjugated to Alexa fluorophores (goat anti-mouse AlexaFluor 594 and goat anti-rabbit AlexaFluor 488). DNA was stained with Hoechst 33342 and the cells were mounted in Mowiol 4-88. Fluorescence analysis was performed with an LD Plan-Neofluar ×40/1.3 DIC or a Plan-Apochromat ×63/1.4 DIC objective (Zeiss, Thornwood, NY). For phase-contrast time-lapse microscopy, cells were observed at 37°C in CO_2-independent medium (Invitrogen) supplemented with 10% fortified cosmic calf serum (Hyclone) with an A-PLAN ×10/0.25 PH1 objective (Zeiss) and an Axiovert 200M microscope equipped with a temperature incubation chamber (Zeiss). Fluorophores were exited with an XBO75 xenon lamp (Zeiss) in combination with the following fluorescence filter sets from Chroma Technology (Rockingham, VT): 31000 for Hoechst and cascade-blue illumination, 41001 for AlexaFluor488 and 41004 for AlexaFluor594. Images were captured with an Orca-285 camera (Hamamatsu, Hamamatsu City, Japan) and the software package Openlab 4.02 (Improvision, Lexington, MA).

Cell fractionation

For preparation of total lysates, cells of one 150 mm dish were washed twice with PBS on ice and then scraped into 5 ml of ice-cold PBS. The cells were pelleted (500 *g* for 5 min at 4°C) and resuspended in 1 ml of buffer A (250 mM sucrose, 20 mM tricine pH 7.8, proteinase inhibitors). After incubation for 20 min on ice, cells were homogenized with a Dounce homogenizer (20 strokes). The protein concentrations of the lysates were determined and proteins were dissolved in SDS sample buffer containing 2% β-mercaptoethanol, separated by SDS–PAGE and processed for immunoblotting. Triton X-100 extraction experiments were performed as described previously (Slusarewicz *et al*, 1994). Golgi membranes were purified from rat liver as described (Wang *et al*, 2006).

Preparation of mitotic cells

Cells were seeded at day 0 in growth medium at a confluence of 70%. At day 1, the medium was changed to growth medium supplemented with either 5 μM *S*-trityl-L-cysteine (Acros Organics, Morris Plains, NJ) or 0.2 μg/ml nocodazole to enrich cells in mitosis. After 4–6 h, the mitotic cells were collected by shake-off and the remaining attached cells were washed three times with growth media and then collected by trypsinization (interphase cells).

Gradient centrifugation

Mitotic cells from two confluent T175 flasks were collected by shake-off, pelleted by centrifugation (5 min at 500 *g*) and resuspended in 12 ml of buffer B (10 mM Hepes pH 7.4, 250 mM sucrose, 1 mM EDTA, protease inhibitors) and pelleted again. The cells were resuspended in 1 ml of buffer C (10 mM Hepes pH 7.4, 50 mM NaCl, 1 mM EDTA) and broken using a ball bearing homogenizer with a clearance of 10 μm (Isobiotec, Heidelberg, Germany). The homogenate was centrifuged for 5 min at 1000 *g* at 4°C and the PCS was recovered. For gradient centrifugation, 250 μl of an 80% sucrose solution was applied to the bottom of an SW50.1 tube. The sucrose was overlaid with 4.3 ml of a 30–5% glycerol solution in buffer D (10 mM Hepes pH 7.4, 1 mM EDTA) and finally topped with PCS (900 μl). The gradients were centrifuged for 20 min in an SW50.1 rotor at 114 562 g_{av} and 4°C. A total of 12 fractions (450 μl each) were collected from the top, diluted in buffer D and membranes were recovered by centrifugation at 71 680 g_{av} for 30 min at 4°C. The membrane pellets were dissolved in 2× SDS sample buffer (2% β-mercaptoethanol) and analysed by immunoblotting.

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