Ribophorin I acts as a substrate-specific facilitator of N-glycosylation

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Summary

The mammalian oligosaccharyltransferase (OST) complex is composed of about eight subunits and mediates the N-glycosylation of nascent polypeptide chains entering the endoplasmic reticulum (ER). The conserved STT3 subunit of eukaryotic OST complexes has been identified as its catalytic centre, yet although many other subunits are equally well conserved their functions are unknown. We used RNA interference to investigate the function of ribophorin I, an ER-translocon-associated subunit of the OST complex previously shown to associate with newly synthesised membrane proteins. We show that ribophorin I dramatically enhances the N-glycosylation of selected membrane proteins and provide evidence that it is not essential for N-glycosylation per se. Parallel studies confirm that STT3 is essential for transferase activity of the complex, but reveal that the two mammalian isoforms are not functionally equivalent when modifying bona fide polypeptide substrates. We propose a new model for OST function where ribophorin I acts as a chaperone or escort to promote the N-glycosylation of selected substrates by the catalytic STT3 subunits.

Supplementary material available online at
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Introduction

N-glycosylation typically occurs co-translationally and is the most common form of protein modification in the ER lumen, promoting protein folding, maturation and quality control (Helenius and Aebi, 2004). Defects in the N-glycosylation process are implicated in a number of human congenital disorders (Freeze and Aebi, 2005). In eukaryotes, N-glycosylation is mediated by a large protein complex, the oligosaccharyltransferase (OST), which transfers a high mannose oligosaccharide en bloc from a dolichol donor to a suitable Asn residue in a nascent polypeptide chain (Kelleher and Gilmore, 2006).

Much of our recent knowledge of the OST comes from *Saccharomyces cerevisiae*, where the complex is composed of up to nine subunits, Ost1p, Ost2p, Wbp1, Swp1, Stt3p, Ost3p/Ost6p, Ost4p and Ost5p (Chavan et al., 2006; Kelleher and Gilmore, 2006). Of these, the first five components are essential for viability (Kelleher and Gilmore, 2006), whereas Ost3p and Ost6p are homologous but nonessential subunits that facilitate the glycosylation of specific substrates (Kelleher and Gilmore, 2006). Ost4p and Ost5p are also nonessential, although deletion of Ost4p causes severe under-glycosylation of proteins and disrupts the OST complex by preventing the incorporation of Ost3p/Ost6p (Spirig et al., 2005).

Mammalian equivalents of many yeast OST subunits are known, and to date comprise: ribophorin I (Ost1p), ribophorin II (Swp1p), OST48 (Wbp1p), N33 and IAP (Ost3p and Ost6p), and Dad1 (Ost2p) (Kelleher and Gilmore, 1997; Kelleher et al., 2003; Shibatani et al., 2005). Two isoforms of Stt3, STT3A and STT3B, have been found (Kelleher and Gilmore, 2006), and these are expressed at varying levels in different cells and tissues, and present in distinct subcomplexes with differing in vitro activities (Kelleher et al., 2003). Two other putative subunits of the mammalian OST complex, DC2 and KCP2, have also been identified although their function is unknown (Shibatani et al., 2005).

There is now a broad consensus that the STT3 subunit(s) of the OST complex acts as its catalytic centre (Nilsson et al., 2003; Yan and Lennarz, 2002b). Hence, studies in prokaryotes have shown that a single polypeptide homologous to Stt3p can mediate N-glycosylation (Kowarik et al., 2006; Wacker et al., 2002). Since a single prokaryotic Stt3p-like protein is sufficient to mediate the primary function of the much larger eukaryotic OST, this raises the question of what role the many other subunits of the eukaryotic complex play (Kowarik et al., 2006). Ribophorin I was identified as a subunit of the mammalian OST well before the discovery of STT3 and was initially proposed to recruit the dolichol-bound glycan to the OST (Kelleher et al., 1992). Later studies suggested that ribophorin I might form part or all of the OST active site (Yan et al., 1999), although this now seems unlikely (Yan and Lennarz, 2002a). Nevertheless, some role for ribophorin I in the process of N-glycosylation is supported by its presence in all three mammalian OST isoforms that display catalytic activity (Kelleher et al., 2003), and studies of its *S. cerevisiae* equivalent, Ost1p, where conditional mutants show reduced levels of glycosylation (Silberstein et al., 1995). However, although there are strong indications that ribophorin I facilitates N-glycosylation, the extent to which it is required and the nature of its role are unknown (Chavan and Lennarz, 2006; Kelleher and Gilmore, 2006).

We showed that a subset of newly synthesised membrane proteins remain associated with ribophorin I following their Sec61-mediated integration at the ER (Wilson et al., 2005). Binding to ribophorin I can be observed both in vitro and in vivo, and the interaction does not depend upon the N-glycosylation of the precursor protein (Wilson et al., 2005). Other studies also identified a strong interaction between ribophorin I, and newly synthesised membrane proteins (Lilley and Ploegh, 2004; Santhamma and Sen, 2000), and we speculated that ribophorin I could function to improve the efficiency of N-glycosylation of selected substrates (Wilson et al., 2005). To test this hypothesis, we have developed a novel assay to study the consequences of small interfering RNA (siRNA)-mediated depletion of ribophorin I, STT3A and STT3B upon the N-glycosylation of several different membrane and secretory proteins. We found that the effect of depleting ribophorin I upon N-glycosylation is highly substrate specific, and has either no effect or results in an almost complete inhibition of N-linked glycosylation. Parallel depletions of the two STT3 isoforms reveal that, although most substrates studied require wild-type levels of both to be efficiently N-glycosylated, G-protein-coupled receptors are glycosylated as normal following STT3B depletion. We suggest a new model where ribophorin I acts to enhance the glycosylation of selected substrates by acting as a chaperone or escort for these precursors and facilitating their presentation to the catalytic STT3 subunits of the OST complex.

Results

RNA interference of ribophorin I, STT3A and STT3B expression

We have previously shown that ribophorin I associates with a subset of newly synthesised model membrane proteins after integration at the Sec61 translocon of the ER (Wilson et al., 2005). A simple explanation for this observation is that ribophorin I plays some role during N-glycosylation, but this role is restricted to a subset of OST substrates. If true, this would mean that ribophorin I is not an essential core component of the OST complex. To test this hypothesis, and gain a better understanding of the role of ribophorin I in N-glycosylation, we have developed a system that combines the siRNA-mediated knockdown of specific OST subunits with an in vitro readout for N-glycosylation. In this study we describe the use of this system to address the contributions of ribophorin I, STT3A and STT3B to the N-glycosylation of a variety of different precursor proteins.

In the first instance, HeLa cells were treated with RNA duplexes specific for ribophorin I, STT3A and STT3B mRNAs, and cells were then analysed for levels of these and other OST subunits. Western blotting showed cellular levels of ribophorin I, STT3A and STT3B were specifically reduced to 20% or less of that in control cells after 48 hours (Fig. 1A, compare lanes 1-6 with lane 8 for each product). By contrast, the levels of α-tubulin were largely unaffected (Fig. 1A, lanes 1-8, α-Tub panel), and a non-functional siRNA also had no effect (Fig. 1A, lane 7, see ribophorin I, STT3A and STT3B panels), confirming the losses were not due to pleiotropic effects.

The effect of RNAi-mediated subunit loss on other OST components

It was immediately apparent that knocking down STT3A had a significant effect on STT3B levels (Fig. 1A, cf. lanes 3,4,7 and 8, STT3B panel), whereas a reduction in ribophorin I levels causes a partial loss of STT3B (Fig. 1A, cf. lanes 1,2,7 and 8, STT3B panel). Previous studies have shown that the loss of one OST subunit can destabilise other subunits of the complex (Sanjay et al., 1998) and we found that this is also be true for the mammalian complex(es). We therefore analysed siRNA-treated HeLa cells for three other subunits of the OST complex but found that only Dad1 showed a substantial drop in levels after the knockdown of STT3A expression (Fig. 1B, lanes 2 and 3, Dad1 panel). OST48 and ribophorin II were either unaffected or even increased following the siRNA-mediated depletion of other OST subunits (e.g. Fig. 1B, lane 1, Rib II panel).

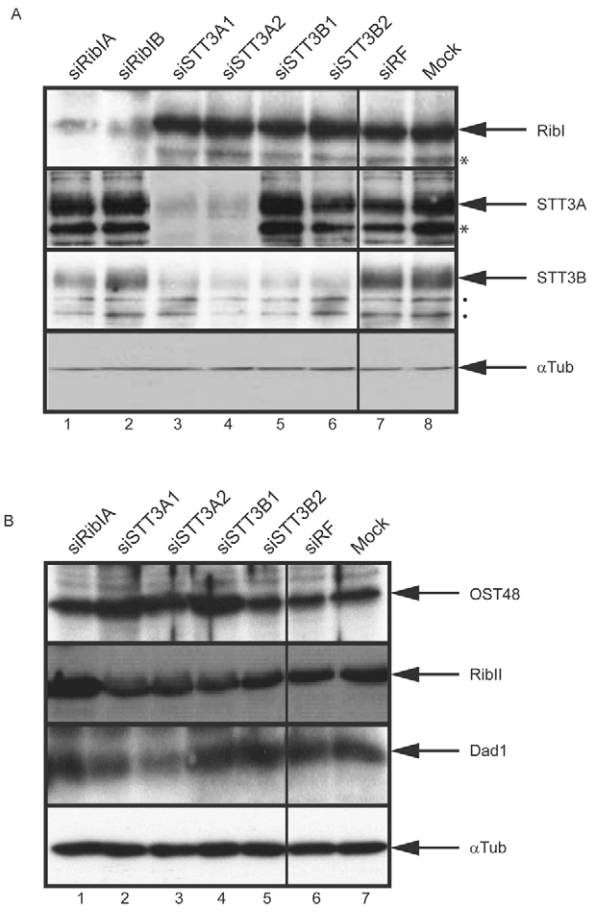


Fig. 1. Consequences of siRNA-mediated knockdown of ribophorin I, STT3A and STT3B. (A) Lysates of cells 48 hours after transfection with ribophorin I, STT3A and STT3B siRNA duplexes (lanes 1-6), a non-functional siRisc-free control (siRF) (lane 7), or mock-transfected cells (lane 8) were probed with antibodies specific for ribophorin I (RibI), STT3A, STT3B or α-tubulin (αTub). Mature forms of these proteins are indicated by arrows and the location of unglycosylated forms of ribophorin I and STT3A are shown by asterisks. For STT3B, the signal is relatively weak, and two non-specific products are also detected (filled circles). (B) Lysates of cells were prepared as in A except the RibIB siRNA duplex was omitted. Samples were probed using antibodies recognising OST48, ribophorin II (RibII), Dad1 or α-tubulin.

Integrity of the OST complex

Mammalian OST subunits associate directly (Fu et al., 1997; Kelleher and Gilmore, 1997) to form large oligomeric complexes that can be observed upon native gel electrophoresis (Shibatani et al., 2005; Wang and Dobberstein, 1999). Similar OST complexes can be recovered with intact enzymatic activity using glycerol gradient fractionation and/or chromatographic purification (Kelleher and Gilmore, 1997; Kelleher et al., 2003). On the basis that the loss of a subunit might disrupt the entire OST complex (Sanjay et al., 1998), and thus cause an 'indirect' loss of function, we analysed the effect of siRNA-mediated depletion upon OST integrity.

Various OST subunits were analysed by centrifugation of digitonin-solubilised HeLa cell extracts using glycerol gradients (Kelleher and Gilmore, 1997; Nikonov et al., 2002). In control cells, a substantial proportion of six OST subunits were recovered in high molecular weight complexes (Fig. 2A and supplementary material Fig. S1A, gradient fractions 9-12). These complexes equate to those previously shown to possess peak OST activity as defined by N-glycosylation of a model tripeptide (Kelleher and Gilmore, 1997), and we conclude that they represent enzymatically active OST isoforms (Kelleher et al., 2003). Where cells were depleted of ribophorin I, we found no major change in the distribution of STT3B, OST48, Dad1 or ribophorin II (see Fig. 2B and supplementary material Fig. S1B), but a proportion of STT3A was released from the larger complexes (see Fig. 2B, gradient fractions 1-6). Nevertheless, the majority of STT3A remained associated with other OST subunits, and we conclude that the loss of ribophorin I does not result in a major disruption of the OST complex. Consistent with our observation that total levels of STT3B are reduced following ribophorin I knockdown (cf. Fig. 1A), the loss of ribophorin I reduces the levels of unassembled, or partially assembled, STT3B (Fig. 2. cf. panels A and B, fractions 1-6, STT3B). Detection of STT3B by western blotting using the only currently available serum is relatively poor (Kelleher et al., 2003), and better reagents will be needed to fully define these changes.

The effect of STT3A depletion upon the distribution of the OST subunits was distinct from that of the ribophorin I knockdown. Thence, the distribution of ribophorin I and Ost48 remained similar to control cells (Fig. 2 cf. panels A and C), but a significant proportion of ribophorin II was released into smaller complexes (supplementary material Fig. S1, cf. panels A and C). Clear evidence for a loss of both STT3B and Dad1 was also observed (Fig. 2 and supplementary material Fig. S1, cf. panels A and C), consistent with our analysis of total cell lysates (cf. Fig. 1A,B). Thus, the loss of STT3A results in a substantial reduction in the levels of the STT3B and Dad1 present in the high molecular weight complexes normally associated with functional OST activity. By contrast, a fraction of the ribophorin II subunits and the majority of ribophorin I and OST48 remain part of higher molecular weight complexes.

The effect of STT3B depletion was notable for the fact that STT3A levels and distribution were essentially normal, as were those of ribophorin I and OST48 (Fig. 2, cf. panels A and D). There was an increase in the proportion of ribophorin II found in smaller complexes, as also observed when the loss of STT3A resulted in a reduction in STT3B levels (supplementary material Fig. S1, cf. panels A,C and D). Surprisingly, we also observed some reduction in Dad1 levels across the higher

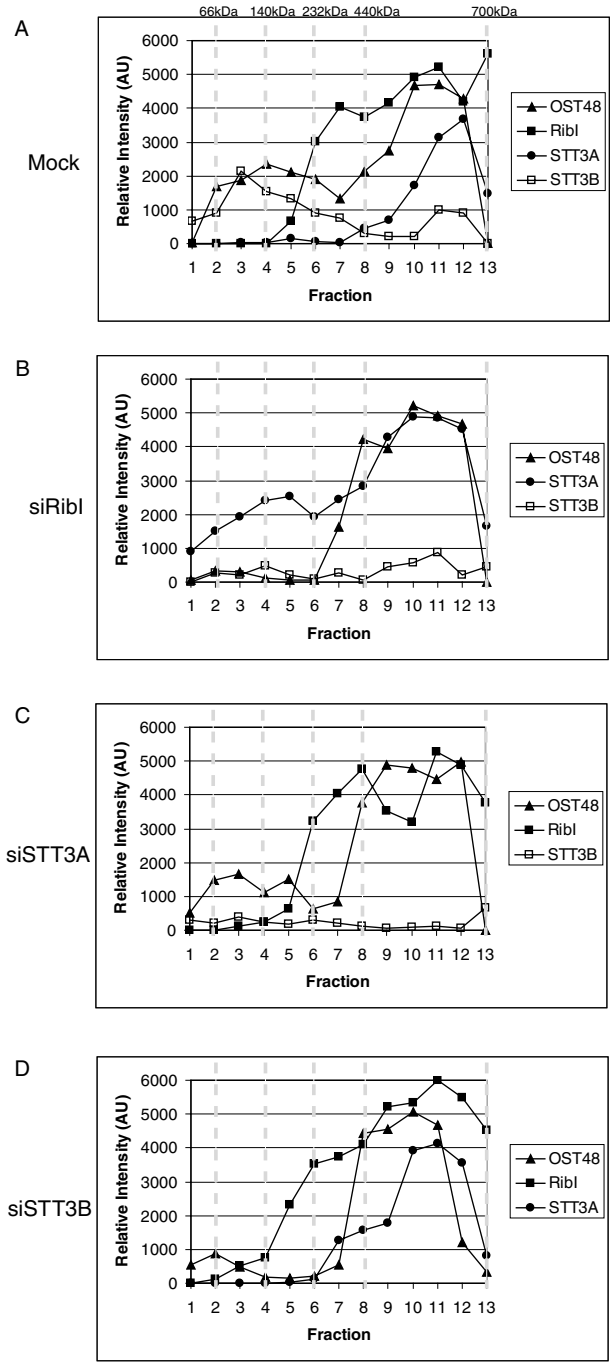


Fig. 2. Glycerol gradient analysis of OST complexes after siRNA treatment. HeLa cells were mock treated (A) or transfected with siRNAs against ribophorin I (B), STT3A (C) or STT3B (D) and subsequently solubilised in homogenisation buffer containing 1.5% digitonin. The resulting supernatant was loaded onto 8-30% glycerol gradients containing 0.125% digitonin and after centrifugation, thirteen 1-ml fractions were collected and analysed by immunoblotting for the presence of ribophorin I (RibI), OST48, STT3A and STT3B. The data are expressed graphically, and represent the relative band intensity for each component detected following correction of any background. Signals were quantified using Aida software (Fuji), and several different exposures times were used to ensure that the film gave a linear response.

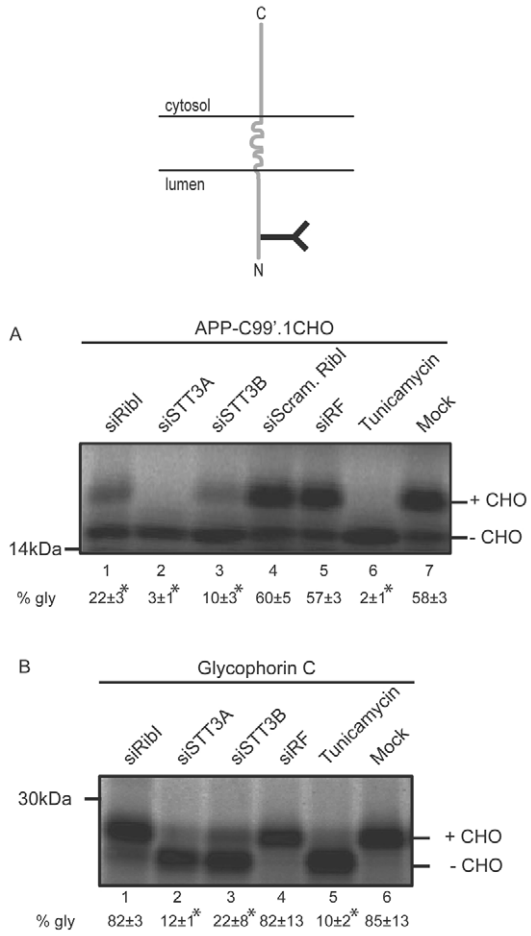


Fig. 3. Effect of OST subunit knockdown on the N-glycosylation of type I membrane proteins. Transmembrane topology and number of N-linked glycans (branched structures) for the two proteins studied. (A) APP-C99'.1CHO was synthesised as a radiolabelled polypeptide using rabbit reticulocyte lysate supplemented with semi-permeabilised HeLa cells prepared 48 hours after transfection with siRNAs specific for the mRNAs encoding ribophorin I (lane 1), STT3A (lane 2) or STT3B (lane 3), a scrambled form of the ribophorin I siRNA (siScram. RibI) (lane 4), a non-functional control siRNA (siRF) (lane 5) or following mock transfection (lane 7). As a positive control for loss of N-glycosylation, HeLa cells were incubated with 2 μg/ml tunicamycin for 12 hours before isolation on day 2 (lane 6). The resulting glycosylated (+ CHO) and non-glycosylated (– CHO) polypeptides are shown following SDS-PAGE. The relative proportion of glycosylated polypeptide was calculated for each sample and expressed as a percentage of the total protein recovered. The numbers below the lanes are the mean ± s.e.m. of three independent experiments. Levels of N-glycosylation that differ from the mock-treated control by a significance of at least 0.02 are indicated (*). (B) Human glycophorin C was synthesised as in A except that the scrambled ribophorin I siRNA control was omitted. The proportion of N-glycosylated chains was calculated as for A and symbols are as previously described.

molecular weight fractions of the gradient (supplementary material Fig. S1, cf. panels A and D), although the total cellular levels of Dad1 were unaltered (Fig. 1B, cf. lanes 4, 5, 6 and 7, Dad1 panel). The pellet fraction from the gradient of the STT3B knockdown contained a substantially higher proportion of Dad1 than the pellet from control cells (data not shown) and we deduce that although the knockdown of STT3B does not substantially destabilise the association of STT3A, ribophorin I, ribophorin II and OST48, it does cause some loss of Dad1 probably by the formation of detergent-insoluble aggregates.

Functional consequences of OST subunit knockdown

Having established that the levels of specific OST subunits could be significantly reduced by siRNA treatment with differing results upon the remaining complex, we investigated the functional consequences of these losses. On the basis of our previous study (Wilson et al., 2005), our working hypothesis was that any role of ribophorin I during N-glycosylation may be precursor specific. To test this hypothesis, we established a system to analyse the effect of OST subunit knockdown upon the modification of a variety of different glycoproteins. This consists of a novel assay that uses RNAi-treated HeLa cells in combination with an in vitro readout of N-glycosylation efficiency using siRNA-treated, digitonin-permeabilised, cells (cf. Wilson et al., 1995).

N-glycosylation of type I membrane proteins following siRNA treatment

The first substrate to be analysed in this study was a C-terminal fragment of the amyloid precursor protein (APP) normally generated in vivo by β-secretase cleavage of the larger precursor. This APP derivative, APP-C99'.1CHO, is a type I membrane protein (see Fig. 3, diagram) with a cleavable, N-terminal, signal sequence, and was selected because we had previously discovered a stable interaction between the newly synthesised polypeptide and ribophorin I (Wilson et al., 2005). To confirm the sensitivity of our in vitro system, we synthesised APP-C99'.1CHO using rabbit reticulocyte lysate supplemented with semi-permeabilised (SP) HeLa cells that had either been treated with tunicamycin or mock treated. This comparison showed that although APP-C99'.1CHO is efficiently N-glycosylated in control cells (~60% modification, see Fig. 3A, lane 7), pre-treatment with tunicamycin results in a near-complete loss of this modification (Fig. 3A, lane 6). To precisely define the effect of the different treatments upon OST function, we required a quantitative measure of N-glycosylation that would be unaffected by any differences in the number of cells, or the even amount of ER membrane, present in the various SP cell preparations used to assay function. For this reason, we measured the amount of both the N-glycosylated and the unglycosylated forms of the precursor in each reaction, and then expressed the amount of N-glycosylated material as a percentage of the total membrane-associated products synthesised in that reaction (see Materials and Methods). In the case of tunicamycin treatment, such quantification revealed that N-glycosylation was reduced to 2% (Fig. 3A), and we concluded that our assay could faithfully report perturbations of normal OST function.

Having established the in vitro assay was robust, we investigated the effect of siRNA mediated depletion of specific OST subunits upon the N-glycosylation of APP-C99'.1CHO. It was immediately apparent that in this case, all of the specific siRNAs resulted in a dramatic reduction in N-glycosylation (Fig. 3A, cf. lanes 1-3 and 7). Specificity was confirmed by the normal levels of N-glycosylation detected with both a control siRNA and a scrambled version of the

ribophorin I specific duplex (Fig. 3A, cf. lanes 4, 5 and 7). N-glycosylation levels after the knockdown of ribophorin I were 22% and after STT3B knockdown were 10% (Fig. 3A). The most dramatic effect was observed with STT3A siRNA where N-glycosylation levels were only 3% (Fig. 3A), providing an inhibition approaching that seen with tunicamycin (Fig. 3A). Statistical analysis of multiple such experiments showed that the significance of the effects observed had a *P* value of 0.02 (Fig. 3A), and we conclude that for APP-C99'.1CHO, a reduction in the levels of any of the three OST subunits tested results in a significant loss of N-glycosylation.

In order to establish whether the three OST subunits under investigation have a global role in N-glycosylation, we analysed the effects of siRNA-mediated knock down on a second type I membrane protein. Glycophorin C is a type I membrane protein that lacks an N-terminal signal peptide but has a transmembrane domain that acts as a signal-anchor sequence. As previously observed, treatment of HeLa cells with siRNAs specific for STT3A or STT3B, or with the drug tunicamycin, all caused a substantial reduction in the levels of N-glycosylation when compared with control cells (Fig. 3B, cf. lanes 2-6). In this case, N-glycosylation after STT3A knockdown reduced to 12% and after STT3B knockdown to 22% (Fig. 3B). Given the reproducible impact of STT3 knockdown upon the N-glycosylation of the two type I membrane proteins, it was striking that the equivalent depletion of ribophorin I had no significant effect upon N-glycosylation (Fig. 3B, cf. lanes 1, 4 and 6). Taken together with our observation that the loss of ribophorin I does not substantially disrupt the OST complex (Fig. 2), these data provide the first direct experimental evidence in support of the idea that the role of ribophorin I during N-glycosylation is indeed substrate specific (Wilson et al., 2005).

N-glycosylation of type II membrane proteins

To further test our hypothesis, and determine the types of proteins acted upon by ribophorin I, we analysed the N-glycosylation of other distinct classes of precursors. The invariant chain of the MHC class II complex has a signal-anchor sequence and assumes a type II orientation in the ER membrane with two sites for N-glycosylation in its luminal C-terminal region (see Fig. 4, diagram). In contrast to the significant levels of N-glycosylation seen at both sites on the invariant chain with control semi-permeabilised cells (Fig. 4A, lanes 4 and 6), the depletion of ribophorin I, STT3A and STT3B all resulted in a substantial decrease (Fig. 4A. cf. lanes 1-4 and 6). Quantification and statistical analysis showed that glycosylation after knockdown of ribophorin I was 10%, after loss of STT3B was 2% and after loss of STT3A was 1% (Fig. 4A).

As before, we analysed a second glycoprotein from the same class (see Fig. 4): the asialoglycoprotein receptor (ASGPR). The readout of the effect of the siRNA-mediated reduction of OST subunits was very similar to that seen for the invariant chain. Hence, the knockdown of ribophorin I, STT3A and STT3B all resulted in a substantial reduction in N-glycosylation compared with control treatments (Fig. 4B, cf. lanes 1 to 6). Levels of N-glycosylation were 16% following the loss of ribophorin I, 17% for STT3B knockdown and 2% after the knockdown of STT3A (Fig. 4B). Thus, in contrast to the type I membrane proteins studied, all three OST subunits tested are required for wild-type levels of N-glycosylation of two representative type II membrane proteins.

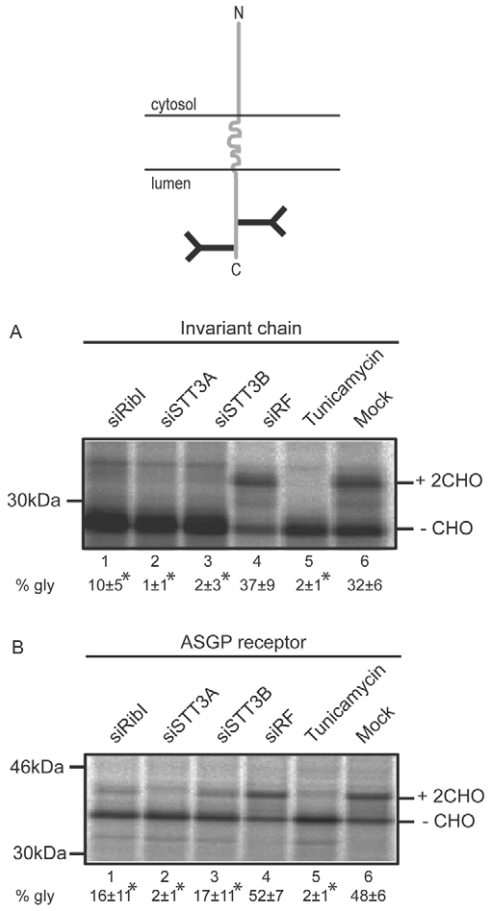


Fig. 4. Effect of OST subunit knockdown on the N-glycosylation of type II membrane proteins. Transmembrane topology and number of N-linked glycans (branched structures) for the two proteins studied. (A) Human invariant chain was synthesised as previously described to determine the extent of its N-glycosylation (see Fig. 3A). The resulting glycosylated (+ 2CHO) and non-glycosylated (– CHO) polypeptides are shown with the proportion of N-glycosylated products calculated as before (see Fig. 3A), and symbols are as previously described. (B) Human asialoglycoprotein receptor (ASGPR) was synthesised and products analysed as in A. The numbers below the lanes are the mean ± s.e.m. of three independent experiments. Levels of N-glycosylation that differ from the mock-treated control by a significance of at least 0.02 are indicated (*).

N-glycosylation of polytopic membrane proteins

Having analysed the effects of siRNA-mediated knockdown upon simple, single-spanning membrane proteins, we next investigated their role during the glycosylation of more complex polytopic proteins (see diagram above Fig. 5A). In the first case we studied opsin, a seven transmembrane G-protein coupled receptor (GPCR) with a non-cleavable signal anchor sequence and two sites for N-glycosylation. The behaviour of opsin was strikingly different to that of the two other classes of precursors studied. Specifically, only the knockdown of STT3A had a clear effect upon its efficient N-glycosylation (Fig. 5A, cf. lanes 1-6). Quantification confirmed this

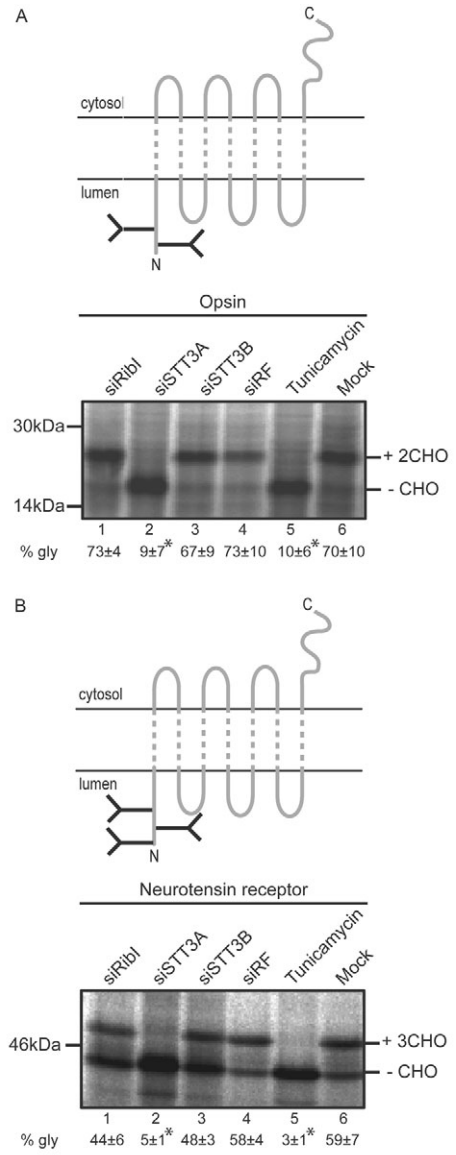


Fig. 5. Effect of OST subunit knockdown on the N-glycosylation of polytopic membrane proteins. (A) The extent of bovine opsin N-glycosylation was determined and quantified as before (Fig. 3A). (B) Rat neurotensin receptor N-glycosylation was determined as for A. The numbers below the lanes are the mean ± s.e.m. of three independent experiments. Levels of N-glycosylation that differ from the mock-treated control by a significance of at least 0.02 are indicated (*).

conclusion, and statistical analysis showed that only the knockdown of STT3A caused a significant reduction in the proportion of opsin chains that were N-glycosylated. Hence, the loss of STT3A resulted in an inhibition of N-glycosylation to 1%, akin to that seen with tunicamycin treatment (Fig. 5A).

To establish the specificity of alterations in the N-glycosylation of opsin, we also studied the neurotensin receptor, a second GPCR with the same topology and three N-glycosylation sites. As for opsin, we found that the efficient N-glycosylation of the neurotensin receptor requires full STT3A function but not that of ribophorin I or STT3B (Fig. 5B). Although the proportion of neurotensin receptor that was N-glycosylated in vitro was lower than for opsin (cf. Fig. 5A and Fig. 5B, lanes 4 and 6), quantification confirmed the significance of the STT3A knockdown (Fig. 5B). In short, our analysis of two GPCRs provides the first evidence that not only might ribophorin I function be unnecessary for efficient N-glycosylation, but also that STT3B is expendable for the modification of at least some precursors.

N-glycosylation of secretory proteins

To date, our analysis of OST subunit function had focused on the N-glycosylation of membrane proteins because these are known to associate with ribophorin I (Wilson et al., 2005). However, a large proportion of the proteins synthesised at the ER are soluble secretory proteins and many are N-glycosylated during their biosynthesis. The precursor of the yeast mating pheromone, pre-pro-alpha factor (ppαF) has a cleavable signal sequence and two sites for N-glycosylation, and has been widely used in mammalian systems. The precursor is converted into pro-alpha factor (pαF) by signal sequence cleavage during translocation across the ER membrane and released into the ER lumen in its glycosylated form. The pattern of pαF glycosylation resembles that seen with glycophorin C, and thus knockdown of both STT3A and STT3B result in a reduction in N-glycosylation whilst the loss of ribophorin I has no obvious effect (Fig. 6A, lanes 1-6). Quantification confirmed that the ribophorin I knockdown had no significant effect upon pαF glycosylation in contrast to the two STT3 isoforms – the loss of which each led to a ~tenfold reduction (Fig. 6A).

We studied a second secretory glycoprotein in the form of human γ interferon (γIFN), which also has a cleavable signal sequence and 2 sites for N-glycosylation. As with pαF, the knock down of ribophorin I had no effect upon N-glycosylation whereas the loss of either STT3A or STT3B had a clear effect (Fig. 6B, cf. lanes 1-6). As before, quantification confirmed the significance of the effects of the STT3A and STT3B siRNAs (Fig. 6B). Thus, as seen with a subset of the membrane proteins we have studied, the N-glycosylation of secretory proteins does not require normal levels of ribophorin I.

Our data strongly suggest that the roles of different subunits of the OST complex are substrate specific. However, we wished to obtain further evidence that the effects of the RNAi we observed are a direct consequence of OST subunit loss, and not an indirect effect reflecting a more general perturbation of ER function. We therefore determined the effect of the various treatments upon a second well-characterised ER processing event, which, like N-glycosylation, is closely associated with protein translocation into and across the ER membrane. To this end we analysed the efficiency of signal-sequence cleavage for the soluble secretory protein preprolactin (see supplementary material Fig. S2). Bovine preprolactin was specifically chosen for this analysis because it is not N-glycosylated. This means that the identification of polypeptides with and without an intact signal sequence is straightforward and not confounded by alterations in polypeptide mobility resulting from N-glycosylation as is the case for ppαF and γIFN (cf. Fig. 6A,B). Quantification showed that the ratio of signal sequence processed to signal sequence unprocessed forms was not significantly altered by any of the treatments used (supplementary material Fig. S2), and we conclude that the siRNA treatment causes a specific disruption of N-glycosylation and not a pleiotropic defect of ER function per se.

Discussion

Comparisons between eukaryotic and prokaryotic OSTs raise two issues of note: first, they predict that it is the STT3 subunit of the eukaryotic complex that acts as the actual transferase (Kelleher et al., 2003; Nilsson et al., 2003; Wacker et al., 2002; Yan and Lennarz, 2002b); second, they beg the question as to why eukaryotic OSTs require so many additional subunits to carry out a fundamentally similar process (Kelleher and Gilmore, 2006; Kowarik et al., 2006).

Ribophorin I function

Our own work has led us to question the role of the ribophorin I subunit of the mammalian OST complex during N-glycosylation (Wilson et al., 2005). In *S. cerevisiae*, conditional mutants of the ribophorin I equivalent, Ost1p, display pleiotropic under-glycosylation of several precursors consistent with a global role in N-glycosylation (Silberstein et al., 1995). Mammalian ribophorin I shows ~28% sequence identity to Ost1p and is assumed to perform a similar function (Silberstein et al., 1995). However, although previous studies show ribophorin I is always associated with the enzymatic activity of the mammalian OST complex when assayed in vitro (Kelleher and Gilmore, 1997; Kelleher et al., 2003; Kelleher et al., 1992), they provide no indication of its role.

In this study we have used siRNA to mediate a specific knockdown of ribophorin I in cultured HeLa cells, and then studied the effects upon the N-glycosylation of a spectrum of different substrates (Table 1). Glycerol gradient analysis shows that although the loss of the majority of ribophorin I might result in the release of some STT3A, the majority of the OST complex remains intact. We maintain that the effects of ribophorin I depletion observed are not due to a pleiotropic disruption of ER function because: (1) any perturbation of N-glycosylation is substrate specific and (2) signal sequence processing is unaffected. Our studies provide the first definitive evidence that ribophorin I is not essential for the process of N-glycosylation per se. Hence, of the eight substrates studied, five are normally glycosylated following ribophorin I depletion consistent with wild-type OST activity (Table 1). We conclude that these substrates do not require normal ribophorin I function to be efficiently glycosylated or that the actions of ribophorin I are redundant and other OST subunits can completely compensate for its loss during N-glycosylation.

In the case of three substrates, we find profound defects in N-glycosylation when ribophorin I function is compromised (Table 1). Given the integrity of the OST complex and the completely normal N-glycosylation of five other precursors, we conclude that the role of ribophorin I during N-glycosylation is substrate specific. We found that a subset of simple, single-spanning membrane proteins require ribophorin I to be efficiently glycosylated whereas secretory and more complex membrane proteins are unaffected. Interestingly, although a previous study of the *S. cerevisiae* ribophorin I orthologue, Ost1p, found conditional mutants were defective in the N-glycosylation of all three precursors studied, the defect in glycosylation of the secretory protein carboxypeptidase Y appeared qualitatively less severe than that of two membrane proteins (Silberstein et al., 1992). Thus, there is a strong case for suggesting that the actions of ribophorin I are specific to a subset of integral membrane proteins, as suggested by our previous crosslinking studies (Wilson et al., 2005).

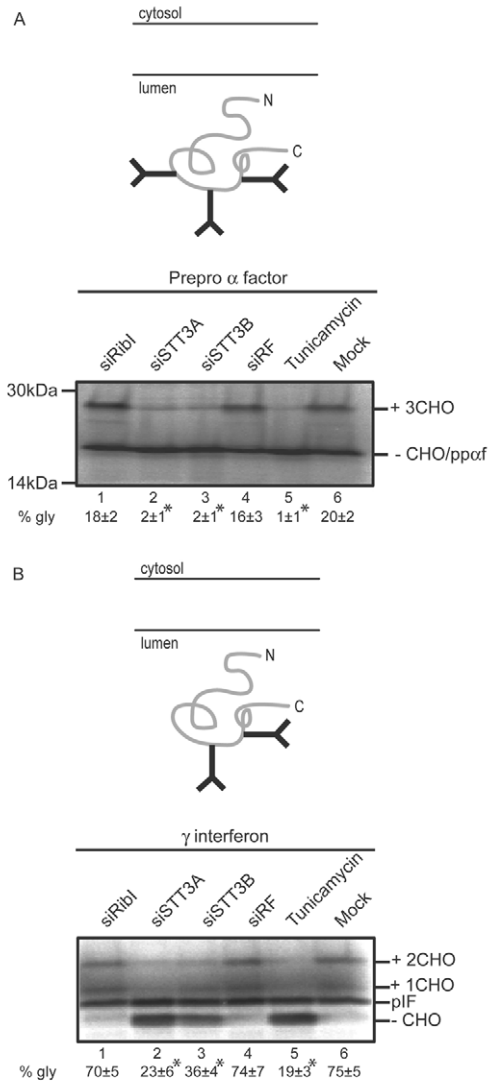
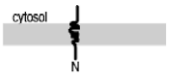
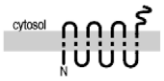
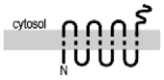


Fig. 6. Effect of OST subunit knockdown on the N-glycosylation of secretory proteins. (A) Yeast prepro α factor was synthesised as before to determine the extent of its N-glycosylation (cf. Fig. 3A). The resulting fully glycosylated (+ 3CHO) and non-glycosylated (– CHO) polypeptides are shown. In this case any untranslocated prepro α factor where the signal sequence has not been cleaved co-migrates with the translocated signal sequence cleaved form that has not been glycosylated (see products labelled CHO/ppαf). This means that the measured proportion of correctly translocated, N-glycosylated, chains is an underestimate of the true value (cf. Fig. 6B). The proportion of N-glycosylated pro α factor was calculated as before (Fig. 3A) with symbols as previously defined. (B) Human γ-interferon was synthesised as in A, four forms of the protein can be resolved, the doubly glycosylated form (+ 2CHO), a singly glycosylated form (+ 1CHO), the non-glycosylated form (– CHO) and an untranslocated form where the pre-sequence has not been cleaved (pIF). Data analysis of γ-interferon was performed as in A. Numbers below the lanes are the mean ± s.e.m. of three independent experiments. Levels of N-glycosylation that differ from the mock-treated control by a significance of at least 0.02 are indicated (*).

Where ribophorin I depletion causes a reduction in N-glycosylation, some degree of residual modification always remains and the loss is never as acute as that seen upon

Table 1. Effect of siRNA depletion for all substrates studied*

Substrate	Topology	siRibl	siSTT3A	siSTT3B
APP-C99′.1CHO		Defect	Defect	Defect
Glycophorin C		Normal	Defect	Defect
Invariant chain		Defect	Defect	Defect
ASGP receptor		Defect	Defect	Defect
Opsin		Normal	Defect	Normal
Neurotensin receptor		Normal	Defect	Normal
Prepro α factor		Normal	Defect	Defect
γ interferon		Normal	Defect	Defect

*A statistically significant effect of siRNA depletion upon N-glycosylation is indicated by ‘Defect’ and no effect by ‘Normal’.

tunicamycin treatment or STT3A knockdown. We cannot rule out the possibility that this reflects some residual ribophorin I activity remaining after siRNA intervention. Alternatively, the role of ribophorin I may be to specifically enhance the N-glycosylation of sub-optimal substrates, and thus in the absence of ribophorin I, low level modification could still occur. Given the completely normal glycosylation of many substrates following substantial ribophorin I depletion we favour the latter possibility.

We can only speculate as to the exact function of ribophorin I, but propose that it facilitates the N-glycosylation of ‘difficult’ substrates by acting as a chaperone or escort that presents these polypeptides to, and/or stochastically retains

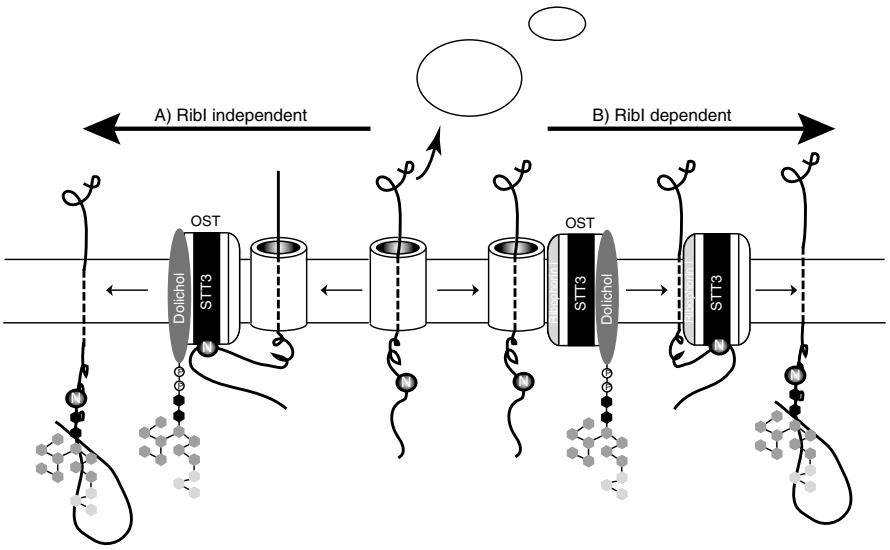


Fig. 7. Model for the role of ribophorin I during N-glycosylation at the OST. During translocation of the nascent polypeptide through the Sec61 translocon, the catalytic subunit of the oligosaccharyltransferase, STT3 scans the nascent chain for potential N-glycosylation sites. (A) If the nascent polypeptide is efficiently N-glycosylated or retention at the OST complex is mediated by a different OST subunit, then an interaction with ribophorin I is not required for efficient N-glycosylation. (B) Where N-glycosylation is inefficient, binding of the substrate to ribophorin I increases the time available for the STT3 subunit to recognise and modify the newly synthesised protein even after it has exited the ER translocon (Wilson et al., 2005).

them in proximity of, the catalytic STT3 subunits (Fig. 7). In this context it is worth noting that ribophorin I is not conserved in all eukaryotic OST complexes, being absent in organisms such as Trypanosomes (Kelleher and Gilmore, 2006). This observation further supports our hypothesis that the role of ribophorin I is to facilitate the N-glycosylation of particular proteins, hence where such proteins are not synthesised, its function would be redundant.

Our model refines the recent proposal that ribophorin I acts to funnel substrates to the catalytic core of the OST complex (Chavan and Lennarz, 2006), by incorporating our discovery that ribophorin I only acts upon selected precursors. It is also consistent with known links between protein folding and the efficiency of N-glycosylation (Kowarik et al., 2006; Petrescu et al., 2004). Where proteins have multiple N-linked glycans, the effect of ribophorin I depletion is all or nothing, with each potential site equally affected (this study). This is entirely consistent with ribophorin I acting to delay precursors at the OST complex providing an improved opportunity for the N-glycosylation of all potential sites.

Mammalian STT3 isoforms are not equivalent

The evidence that the STT3 subunit of eukaryotic OST complexes acts as the catalytic core is now compelling (Nilsson et al., 2003; Wacker et al., 2002; Yan and Lennarz, 2002b). Many higher eukaryotes have two distinct isoforms, STT3A and STT3B, and although these isoforms display distinct kinetic properties in vitro, any distinction in their roles in vivo was previously untested (Kelleher and Gilmore, 2006; Kelleher et al., 2003). At a fundamental level our data using siRNA-mediated knockdown of the two mammalian isoforms of STT3 strongly support the idea that both can act catalytically (Kelleher et al., 2003). However, our analysis identifies an unexpected level of complexity in terms of their roles, and shows that the two isoforms are not functionally equivalent in vivo. Specifically, we find that although knockdown of STT3B substantially reduces the glycosylation of most substrates, the N-glycosylation of two GPCRs is completely unaffected (Table 1). Thus, N-glycosylation of a limited set of precursors can occur in the absence of normal STT3B levels, presumably reflecting the ability of STT3A to function in the absence of STT3B.

We can draw less definitive conclusions regarding STT3A function, because, in contrast to STT3B depletion, depletion of STT3A causes a loss of both the STT3A and STT3B isoforms. Recent studies suggest that the native OST complex is dimeric, being composed of two multisubunit complexes each with around eight different subunits (Chavan et al., 2006; Spirig et al., 2005). Thus, the loss of STT3B in response to the depletion of STT3A might reflect the presence of both STT3 isoforms in a single native OST complex that is destabilised by the loss of STT3A, but not by the loss of STT3B. We have clearly shown that ribophorin I acts to enhance the N-glycosylation of specific substrates at the mammalian OST. Our future studies will seek to establish whether other eukaryote-specific OST subunits play a similar role for other substrates (cf. Fig. 7).

Materials and Methods

Reagents and antibodies

Cell culture reagents were from GIBCO BRL or Cambrex, RNA polymerase and rabbit reticulocyte lysate from Promega and EasyTag L-[^{35}S]methionine from NEN Dupont. All other chemicals were from Sigma or BDH/Merck. Rabbit polyclonal antisera recognising ribophorin I, ribophorin II, DadI, and OST48 were made to order by Invitrogen (Paisley, UK), those specific for STT3A and STT3B were a gift from Reid Gilmore (University of Massachusetts Medical School). Monoclonal anti-α-tubulin (TAT1) was kindly provided by Keith Gull (University of Oxord). 21-nucleotide duplexes corresponding to human ribophorin I (Rib1A, aagcgcacagtggacctaagc; Rib1B, aatgaggacgtgaagcgcaca), STT3A (STT3A1, gcagtaggatcatatttgatt; STT3A2, gacaataacacatggaatatt), STT3B (STT3B1, tatcaacgatgaaagagtatt; STT3B2, catgaggactctagatgtatt), Scrambled ribophorin I and (aggaatgcgcacccggactaa) and the Risc-free siCONTROL were from Dharmacon Research.

Glycerol gradient analysis of OST subunits

HeLa cells grown to 60% confluence in 150-mm cell culture dishes were treated with siRNAs to ribophorin I, STT3A or STT3B for 48 hours and then prepared for glycerol gradient analysis in the presence of digitonin as previously described (Kelleher and Gilmore, 1997; Kelleher et al., 1992). The resulting fractions were run on 14% SDS-polyacrylamide Tris-glycine gels and analysed by immunoblotting with antisera specific for ribophorin I, ribophorin II, STT3A, STT3B, OST48, Dad1.

RNA interference and in vitro translation

HeLa cells (60% confluent) grown in 10-cm^2 dishes seeded 24 hours before treatment were transfected with 60 μl of 20 μM siRNA duplex using Oligofectamine (Invitrogen, Paisley, UK) as described (Elbashir et al., 2001). RNAi-treated cells were incubated for 48 hours and tunicamycin (2 μg/ml) was added 12 hours before preparation of semi-permeabilised cells (Wilson et al., 1995). A rabbit reticulocyte lysate system (Promega) was used to translate mRNAs encoding a number of substrates for 60 minutes at 30°C in the presence of 0.75 mCi/ml [^{35}S]methionine and RNAi-treated semi-permeabilised HeLa cells. Aurintricarboxylic acid (ATCA) was then added to a final concentration of 100 μM to inhibit further initiation, and the samples were incubated at 30°C for 10 minutes. Membrane-associated products were isolated by centrifugation for 1 minute at 16,000 ***g*** and washed by resuspension in KHM (20 mM HEPES pH 7.2, 110 mM potassium acetate, 2 mM magnesium acetate). All samples were incubated with 25 μl sample buffer for 10 minutes at 70°C (except Opsin was for 30 minutes at 37°C) and analysed on 14% SDS-polyacrylamide Tris-glycine gels. The resulting gels were dried and then visualised using a Fuji BAS 3000 PhosphoImager system (Fuji Photo Film, Tokyo, Japan). Protein knockdown was analysed by western blotting either with rabbit polyclonal antisera specific for ribophorin I, ribophorin II, STT3A, STT3B, OST48, Dad1 or mouse monoclonal antisera for α-tubulin as indicated.

Statistical analysis

All N-glycosylation assays were carried out in at least three independent experiments and a statistical analysis (two-sample *t*-test) was carried out using SPSS 10.1 software.

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