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Incorporation of ceramides into yeast GPI anchored proteins can be monitored *in vitro*

*Régine Bosson, Isabelle Guillas[1], Christine Vionnet, Carole Roubaty and Andreas Conzelmann**

University of Fribourg, Department of Medicine, Ch. Du Musée 5, CH-1700 Fribourg, Switzerland

*Corresponding author: Dr. A. Conzelmann, Division of Biochemistry, Chemin du Musée 5, CH-1700 Fribourg, Switzerland

Tel. ++41 26 300 8630. Fax ++41 26 300 9735.

E-mail: andreas.conzelmann@unifr.ch

[1]Present address: Dr. Isabelle Guillas-Beaudouin, Université de Paris 6, UPMC Univ Paris 06, UMR 7180, PCMP, F-75005, Paris, France et CNRS, UMR 7180, PCMP, F-75005, Paris, France.

Running title: Microsomal incorporation of ceramide into GPI anchors

Abstract

After glycosylphosphatidylinositols (GPIs) are added to GPI proteins of *Saccharomyces cerevisiae*, a fatty acid of the diacylglycerol moiety is exchanged for a C26:0 fatty acid through the subsequent actions of Per1 and Gup1. In most GPI anchors the thus modified diacylglycerol-based anchor is subsequently transformed into a ceramide-containing anchor, a reaction, which requires Cwh43. Here we show that the last step of this GPI anchor lipid remodeling can be monitored in microsomes. The assay uses microsomes from cells having been grown in the presence of myriocin, a compound that blocks the biosynthesis of dihydrosphingosine (DHS) and thus inhibits the biosynthesis of ceramide based anchors. Such microsomes, when incubated with [^{3}H]DHS, generate radiolabeled, ceramide-containing anchor lipids of the same structure as made by intact cells. Microsomes from *cwh43*Δ or *mcd4*Δ mutants, unable to make ceramide-based anchors *in vivo*, do not incorporate [^{3}H]DHS into anchors *in vitro*. Moreover, *gup1*Δ microsomes incorporate [^{3}H]DHS into the same abnormal anchor lipids as *gup1*Δ cells synthesize *in vivo*. Thus, the *in vitro* assay of ceramide incorporation into GPI anchors faithfully reproduces the events that occur in mutant cells. Incorporation of [^{3}H]DHS into GPI proteins is observed with microsomes alone, but the reaction is stimulated by cytosol or bovine serum albumin, ATP plus coenzyme A or C26:0-Coenzyme A, particularly if microsomes are depleted of acyl-CoA. Thus, [^{3}H]DHS cannot be incorporated into proteins in the absence of acyl-CoA.

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Introduction

The lipid moieties of the GPI lipid at the stage when it is transferred by the transamidase to GPI proteins are different from those found on mature GPI-anchors of *Saccharomyces cerevisiae.* The free GPI lipids contain a phosphatidylinositol (PI) moiety, which comigrates in thin layer chromatography (TLC) with the free PI of yeast membranes and therefore probably contains the typical C16:0 and C18:1 fatty acids found in yeast PI (7, 12, 26, 29, 31). In contrast, the majority of mature protein-linked GPI anchors contain a ceramide moiety, and a minor fraction contains a diacylglycerol modified to have C26:0 fatty acids in *sn*-2 (9, 31). Ceramides of GPI anchors contain phytosphingosine (PHS) and C26 fatty acids as do the bulk of the free sphingolipids in yeast membranes, which are inositolphosphorylceramides (IPCs) and derivatives thereof (9, 32). Thus, all mature GPI proteins of yeast contain large lipid moieties with C26 fatty acids, either in the form of a ceramide or a special diacylglycerol and these lipids are introduced by remodeling enzymes (remodelases) that replace the primary lipid moiety of the anchor.

Recently, two gene products required for introducing C26:0 fatty acids into the primary GPI anchor have been identified as shown in Fig. 1. *PER1* encodes a phospholipase A_2 that removes the C18:1 fatty acid of the primary anchor (13). *GUP1* encodes an acyltransferase required for the addition of a C26:0 fatty acid to the liberated *sn*-2 position, thus generating a pG1 type anchor (4, 13)(Fig. 1). pG1-type anchors (Fig.1) may be the preferred substrate for the enzymes introducing ceramides, since the normal ceramide-containing anchor lipids are strongly reduced in *per1*Δ and *gup1*Δ mutants, but significant amounts of abnormal, more polar, base resistant, inositol-containing anchors are observed in *gup1*Δ cells (4, 13)(unpublished results).

Yeast cells lacking *CWH43* are unable to synthesize ceramide-containing GPI anchors, while the replacement of C18 by C26:0 fatty acids on the primary diacylglycerol anchor by Per1 and Gup1 is still intact (14, 34). *CWH43* comprises a 953 amino acid long open reading frame with 19 predicted transmembrane domains. Single amino acid substitutions in the hydrophilic, lumenally exposed C-terminal part (amino acids 666 to 953) completely abolish the introduction of ceramides into GPI anchors, whereas mutations in the N-terminal part tend to destabilize the protein (14, 21, 34). The *cwh43*Δ mutants grow well in rich media, do not secrete GPI proteins and some *cwh43*Δ strains are also able to grow in the presence of calcofluor white, quite unlike the *per1*Δ and *gup1*Δ mutants (14).

The yeast remodelase activity introducing ceramide (ceramide remodelase) can be monitored by metabolic labeling experiments using tritiated inositol ([^{3}H]inositol) or dihydrosphingosine ([^{3}H]DHS) (28, 31). When fed to intact cells, these tracers are rapidly taken up and incorporated into lipids and GPI proteins, but no other proteins. [^{3}H]DHS only labels those GPI proteins, which carry a ceramide in their anchors. All [^{3}H]inositol- or [^{3}H]DHS-derived label can be removed from the metabolically labeled proteins in the form of PIs or IPCs using nitrous acid, a reagent that releases the inositolphosphoryl-lipid moieties from GPI anchors by cleaving the link between glucosamine and inositol (10, 28, 31).

It presently is not clear what the substrates for the Cwh43-mediated remodelase reaction are. It appears that certain GPI proteins such as Gas1 are not receiving ceramide anchors (9) whereas many others do. It also is not clear if this is because Cwh43 itself discriminates between different protein substrates or if only certain proteins get access to Cwh43. Furthermore, it is unclear if Cwh43 replaces the phosphatidic acid or the diacylglycerol moiety of the GPI proteins and if it introduces

either a ceramide or only a long chain base, which latter would have to be acylated through a second biosynthetic reaction. Here we report on a microsomal assay that recapitulates the findings previously made in living cells and allows addressing these questions under defined conditions *in vitro*.

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Materials and Methods

Strains, media and materials. Strains with single deletions of non-essential genes (*CWH43, SUR2, GUP1*) were obtained from EUROSCARF (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/col_index.html) in BY4741, *MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0* or BY4742, *MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*. Other strains are shown in Table I. Strains were cultured at 24, 30 or 37°C in YPD medium or in minimal media supplemented with glucose (SD), galactose (SG) or raffinose (SR) and amino acids (aa), uracil (U) and adenine (A) (30). Selection for integration of KanMX4-containing deletion cassettes was performed on YPD plates containing 200 µg/ml G418 (CALBIOCHEM). Pepstatin was obtained from ALEXIS, octyl-Sepharose and concanavalin A-Sepharose from AMERSHAM Biosciences, Zymolyase from Seikagaku, C26:0-CoA from American Radiolabeled Chemicals; C26:0, myriocin, cerulenin and other chemicals were purchased from SIGMA. [^{3}H]DHS (25-60 Ci/mmol) was from ANAWA Trading SA, Switzerland, or RC TRITEC AG, Teufen, Switzerland, [^{3}H]DHS used for Figure 2A only was from NEN (2.5 Ci/mmol).

Preparation of microsomes. Cells exponentially growing in SDaaUA at 30°C were transferred to medium containing 40 µg/ml of myriocin and further incubated at 30°C

for 90 min. Aliquots of 8.4 x 10^8 cells were resuspended in spheroplasting buffer (10mM NaN_3, 1.4 M sorbitol, 50mM K_2HPO_4 pH 7.5, 40mM β-mercaptoethanol, 0.2mg/ml of zymolyase) and incubated at 30°C to make spheroplasts. Then the spheroplasts were resuspended in lysis buffer (10mM triethanolamine pH 7.2, 0.8 M Sorbitol, 1 mM EDTA and protease inhibitor cocktail) and were lysed by forcing them with a precooled syringe 10 times through a 0.4 mm x 19 mm wide needle. The microsomes were filtered through a 2 µm pore filter (Millipore, Millex®AP, n°SLAP02550) to avoid contamination with intact cells, centrifuged at 15'000 g for 40 min at 4°C and resuspended in lysis buffer. The supernantant was frozen to – 20°C and utilized as a source of cytosol in the remodeling assays.

Microsomal ceramide remodeling assay. Microsomes from X2180 were used unless indicated otherwise. The standard conditions for the *in vitro* remodeling assay were as follows: microsomes equivalent to 100µg of microsomal proteins were incubated for 1 hour at 30°C in 25 mM Tris-HCl, pH 7.5 containing 1 mM ATP, 1 mM GTP, 1 mM CoA, 30 mM creatine phosphate, 1 mg/ml of creatine kinase, cytosol (200 µg of proteins), 10 nmol of C26:0, 20 µg/ml myriocin, 200 µg/ml cycloheximide and 10 µCi (0.17 nmol) of [^{3}H]DHS in a final volume of 100µl. Myriocin and cycloheximide were added to inhibit endogenous DHS production and block incorporation of [^{3}H]DHS into newly made proteins during incubation. Where indicated, C26:0-CoA (10 nanomoles total) or $MgCl_2$ (2 mM) were also included. "Final conditions" for the assay were elaborated through the various attempts to optimize incorporation and to make the assay more defined. When done under "final conditions", assays contained 600µg bovine serum albumin (BSA) instead of cytosol, and in addition to the ingredients of the standard assay also contained $MgCl_2$ (2 mM), glutathione (5mM) and NADPH (1mM). To set up the assay, [^{3}H]DHS and C26:0 or C26:0-CoA were

dried in separate tubes, [^{3}H]DHS was resuspended in 25 µl of lysis buffer by vortexing, and then transferred to the tube containing the dried C26:0 or C26:0-CoA. After vortexing, other ingredients were added. Reactions were started by adding microsomes and stopped by the addition of 600 µl of $CHCl_3$:CH_3OH (1:1). Protein pellets were extensively delipidated by repeated extraction with organic solvents as described for GPI proteins of metabolically labeled intact cells (16). Labeled proteins were either analyzed by SDS-PAGE/fluorography or were further delipidated and purified by Concanavalin A-Sepharose affinity chromatography as described (16). Bound proteins were either released from Concanavalin A-Sepharose by boiling in SDS sample buffer and analyzed by SDS-PAGE/fluorography or were released from Concanavalin A-Sepharose using pronase and the radioactivity of the thus generated anchor peptides was detected by scintillation counting. In many assays, samples were divided into two parts to be analyzed separately by SDS-PAGE/fluorography and pronase treatment/scintillation counting, and the two methods always gave excellent agreement. For analytical purposes the anchor peptides were freed from hydrophobic peptides using octyl-Sepharose column chromatography and labeled anchor peptides were eluted with 25%, then 50% propanol (16). Anchor peptides are normally found only in the 50% eluate except for anchors from gup1Δ cells, which contain also abnormally polar anchor lipids so that the bulk of *gup1*Δ anchor peptides elute at 25% propanol (Fig. 5C)(4). Lipid moieties were released from peptides by nitrous acid treatment for analysis by TLC (16). Lipids were resolved by TLC on Silica 60 plates (20 x 20 cm) using solvent 1 = chloroform:methanol:NH_4OH 40:10:1 or solvents 2 and 3 = chloroform:methanol:0.25%KCl at ratios of 55:45:10 and 55:45:5, respectively. Radioactivity was detected by fluorography or radioimaging using the

BIO-RAD Molecular Imager FX. Radioactivity was quantified by one- or two-dimensional radioscanning in a Berthold radioscanner.

Results

Microsomes incorporate [^{3}H]dihydrosphingosine into GPI proteins

Cell-free microsomes were prepared from X2180 wild type (wt) cells that had been treated for a few hours with myriocin, a specific inhibitor of the serine palmitoyltransferase, the key enzyme for DHS biosynthesis (18, 23) and were labeled with [^{3}H]DHS as described in the experimental procedures. As shown in Fig. 2A, many proteins were efficiently labeled with [^{3}H]DHS without any significant difference between 1 h or 4 h (lanes 1 - 4 vs. 5 - 8) of labeling time. The addition of cytosol increased the incorporation of radioactivity into the proteins, irrespective of whether it had been boiled (lanes 4 and 8) or not (lanes 1 and 5). However, when microsomes were boiled (lanes 3 and 7), labeling of the proteins was completely abolished. This strongly suggested that the remodeling, or at least the incorporation of DHS into proteins, is an enzymatic process and not a spontaneous reaction. Myriocin pretreatment of cells was critical (Fig. 2B, lanes 1 vs. 2), and this probably for two reasons: first, because the lack of sphingolipids, specifically of ceramides, delays the transport of GPI proteins from the ER to the Golgi and causes an accumulation of immature GPI proteins in the ER (18, 28, 33, 35); second, because myriocin pretreatment allows to starve cells of DHS, PHS and ceramides and therewith prevents the introduction of ceramides into GPI anchors by the ER based GPI anchor ceramide remodelase Cwh43. The appearance of distinct bands on SDS-PAGE argued that most of the labeled glycoproteins were still localized in the ER and had not reached the Golgi, where the glycan elongation transforms most GPI proteins as well as many other secretory proteins into diffuse and poorly migrating proteins of high molecular mass (6). Fig. 2B shows proteins labeled with [^{3}H]DHS *in vitro* side by side with GPI proteins of *sec18-1* cells metabolically labeled *in vivo* with [^{3}H]DHS

or [^{3}H]inositol at 37°C, at which temperature the vesicular transport of proteins from the ER to the Golgi is blocked in this mutant. This shows that the proteins labeled by [^{3}H]DHS in microsomes (Fig. 2B, lanes 1 - 5) were of similar mass as the ones labeled by [^{3}H]DHS or [^{3}H]inositol in intact cells (Fig. 2B, lanes 6 - 8). This is strong evidence that the microsomes incorporate [^{3}H]DHS into immature GPI proteins of the ER. Omission of C26:0, Coenzyme A (CoA) and ATP significantly reduced he incorporation of [^{3}H]DHS into proteins but did not abolish it, suggesting that a certain amount of these ingredients or of acyl-CoA was present the microsomes or the cytosol (Fig. 2B, lanes 1 vs. 3). Addition of C26:0-CoA improved the incorporation of [^{3}H]DHS into proteins only slightly (Fig. 2B, lanes 3 - 5), although it strongly enhanced the Lac1/Lag1/Lip1-dependent ceramide synthesis in these microsomes (Fig. 2C, lanes 1' vs. 4' and 5'). This suggests that ATP and CoA may stimulate the attachment of [^{3}H]DHS to GPI proteins not by allowing for acyl-CoA biosynthesis but via another mechanism.

Characterization of GPI anchors generated *in vivo*

The inositolphosphoryl-lipid moieties of GPI anchored proteins from wt cells labeled with [^{3}H]inositol are of three kinds, as described before (31); pG1, a remodeled form of PI containing C26:0 in *sn*2 of glycerol, IPC/B and IPC/C (Fig. 1, Fig. 3A, lane 4). IPC/B- and IPC/C-type GPI anchor lipids are formed, respectively, in the ER and the Golgi (28). The major IPC/B and IPC/C moieties of GPI anchors are believed to contain PHS-C26:0, and PHS-C26:0-OH ceramides, respectively, based on the chemical analysis of yeast GPI anchors, which were shown to contain PHS, C26:0 and smaller amounts of C26:0-OH (9, 28).

As a prelude to the analysis of the *in vitro* remodeled GPI lipids we wanted to confirm by genetic means that the *in vivo* generated, metabolically labeled anchor lipid previously named "IPC/B" indeed contains PHS and C26:0, not DHS and C26:0-OH. The genetic confirmation was sought using a *sur2*Δ mutant, deficient in the transformation of DHS into PHS and a *scs7*Δ mutant, which is unable to hydroxylate the a carbon of the fatty acid in ceramides (17, 22). Wild type and mutant cells were labeled *in vivo* with [^{3}H]inositol and their GPI lipids were isolated for analysis on TLC. The GPI lipids of the wt strain show the usual remodeled PI (pG1), IPC/B and three minor bands, one of which may represent IPC/C (Fig. 3A, lane 4). The anchor lipids of *scs7*Δ also contained IPC/B as their main anchor lipid (Fig. 3A, lane 2 vs. 4), confirming that the fatty acid of IPC/B is not hydroxylated. In contrast, IPC/B was no more present in sur2Δ cells (Fig. 3A, lane 3 vs. 4) confirming the presence of PHS in IPC/B. A more hydrophobic band named IPC/A was seen in this strain, which must represent DHS-C26:0. The result also indicates that DHS-containing ceramides can be utilized by the ER remodelase.

GPI anchors are remodeled to IPC/A and IPC/B *in vitro*

Anchor lipids from [^{3}H]DHS-labeled proteins generated in the *in vitro* assay were compared with anchor lipids generated in intact cells as shown in Fig. 3B.

Anchor lipids labeled *in vitro* in wt microsomes appeared as two bands, one comigrating with IPC/B the other being more hydrophobic and running at the position of IPC/A (Fig. 3B, lane 3). Both lipids were resistant to mild base treatment (Fig. 3B, lanes 3 and 4, Fig. 4B, lanes 3 and 6), as is expected for ceramide-containing anchor lipids. The two species were however destroyed by strong acid hydrolysis and yielded [^{3}H]DHS and traces of [^{3}H]PHS (Fig. 4A, lane 3 and 4B, lane 4). PHS was not

formed upon strong acid hydrolysis of sur2Δ derived anchor lipids (Fig. 4B, lane 7 vs. 4). When anchor peptides obtained from *in vitro* labeled GPI proteins were treated with PI-specific phospholipase C, two different anchor lipids were removed, which migrated in the region of ceramide standards on TLC (Fig. 3B, lane 7). This argues that the difference between IPC/A- and IPC/B-type anchor lipids resides in the ceramide moiety. To confirm that the two *in vitro* generated anchor lipids comigrating with IPC/A and IPC/B contain DHS-C26:0 and PHS-C26:0, respectively, we repeated the *in vitro* experiment in sur2Δ cells. As seen in Fig. 3C, deletion of *SUR2* eliminated the band comigrating with IPC/B, whereas IPC/A was still made. The predominance of IPC/A in anchor lipids generated by wt microsomes suggested that Sur2 hydroxylase may not be optimally working in the *in vitro* system. Sur2 belongs to a family of lipid desaturases and hydroxylases often requiring cytochrome b5 as electron carrier. By removing EDTA from the buffers used during cell lysis, we found that the proportion of IPC/B made *in vitro* was significantly increased (Fig. 3C, lanes 6, 7, vs. 2, 3). This is compatible with the view that EDTA partially inactivates Sur2 activity by removing an iron atom from an essential component of the hydroxylase.

Cwh43, Mcd4 and Gup1 are required for ceramide remodeling *in vitro*

Cwh43Δ cells lack the capacity to make ceramide-based GPI anchors so that all GPI anchors of cwh43Δ cells are of the pG1 type (Fig. 1)(14, 34). Cwh43Δ derived microsomes were entirely unable to incorporate [^{3}H]DHS into proteins (Fig. 5A, lane 3), although they made normal amounts of ceramides (Fig. 5B, lanes 1' vs. 3'). Incorporation of [^{3}H]DHS into proteins was restored, albeit only partially, by overexpressing Cwh43 in cwh43Δ from plasmid p*CWH43* (Fig. 5A, lanes 3, 4). The same plasmid completely restored the incorporation of [^{3}H]DHS into proteins in intact

cwh43Δ cells (not shown). Incomplete restoration *in vitro* may be caused by the fact that the overexpression of Cwh43 from the *GAL1* promoter may render the accumulation of not yet remodeled GPI proteins during preculture in myriocin less efficient.

The mdc4Δ strain lacks an ethanolamine-phosphate side chain on the α1,4 linked mannose of its GPI anchors and this has been found to be correlated with a complete absence of ceramide remodeling (36). Similarly, the microsomes of this cell line do not incorporate any [^{3}H]DHS into proteins (Fig. 5A, lanes 5, 6).

The *in vitro* remodelase test also faithfully reproduced the ceramide remodelase defect of gup1Δ cells (4). Microsomes of gup1Δ cells still incorporated [^{3}H]DHS into proteins, albeit with a lower efficiency than wt cells (Fig. 5A lanes 9, 10 vs. 7, 8), but most GPI proteins that were labeled in wt were also labeled in gup1Δ. It was previously reported that metabolic labeling with [^{3}H]inositol of gup1Δ cells yields GPI anchor peptides that elute from the preparative octyl-Sepharose column already at 25 % propanol and contain abnormally polar anchor lipids (4). This previous study showed that of the three polar anchor lipids of gup1Δ (Fig. 5C, lane 2), only the one of intermediate mobility is mild base sensitive, suggesting that it represents a *lyso*-PI (4). This lipid was not labeled with [^{3}H]DHS *in vitro*, but the two lipids that previously were characterized as mild base resistant were labeled, and were mild base resistant also when labeled *in vitro* (Fig. 5C, lanes 3, 4). Thus, these two anchor lipids seem to contain a long chain base and inositol but possibly lack a fatty acid.

The incorporation of [^{3}H]DHS into proteins in microsomes from per1Δ cells was also severely (> 5 fold) reduced, whereas the synthesis of ceramides was not affected (not shown).

Altogether, it appears that the microsomal ceramide remodeling assay faithfully reproduces the events that have been observed in intact cells.

Definition of optimal conditions for the microsomal ceramide remodelase activity.

Numerous experiments were carried out in an attempt to optimize incorporation of [^{3}H]DHS into proteins. Many ingredients were found to consistently either enhance or inhibit the incorporation of [^{3}H]DHS into proteins, but for some of them the effect was somewhat variable from one experiment to the next. For instance, omission of cytosol had very drastic effects in some, but less drastic effects in other experiments. Only those parameters, which gave consistent results in many experiments are described in the following.

Freezing microsomes prior to the assays resulted in an 80% loss of activity. The standard *in vitro* reaction was done in the presence of myriocin to prevent the biosynthesis of cold DHS from serine and NADPH present in the added cytosol, but omission of myriocin from the *in vitro* assay did not diminish the incorporation of [^{3}H]DHS (not shown). When cold DHS was added to the reaction, the incorporation of [^{3}H]DHS was strongly reduced as shown in Fig. 6A, B. Calculations show that the chemical amounts of DHS incorporated into GPI proteins increased, when the DHS concentration was raised from 0.17 nmol to 4.32 nmol (=1.3 µg/ml)(Fig. 6B).

As can be seen in Fig. 6C, using 100µg of microsomal protein per assay we can observe a constant, close to linear increase of incorporated radioactivity during the first 30 – 60 min of incubation. Using less protein does not significantly reduce the rate of incorporation of [^{3}H]DHS (Fig. 6C). A likely explanation of this fact is that with fewer microsomes and hence less cold DHS and PHS, the specific activity of

[^{3}H]DHS in the assay becomes higher. The close to linear time course however and the total absence of incorporation with boiled microsomes (Fig. 2A, Fig. 6C) argue that in our standard conditions (60 min of incubation, 100µg of protein) we will observe less incorporation if one of the required enzyme activities or substrates becomes limiting.

On the technical side, adding [^{3}H]DHS to assays not directly, but incorporated into phosphatidylcholine-containing liposomes reduced its incorporation into proteins by a factor of about 2 (not shown). The addition of cytosol was strongly stimulatory (Fig 8A, 2 vs. 1); Fig. 8B, 7 vs. 1), but [^{3}H]DHS was incorporated into the same proteins in the presence or absence of cytosol (Fig. 2A). As boiled cytosol had the same enhancing effect (Fig. 2A), we initially assumed that the active principle might be an ion or small molecule. Fractionation of cytosol by gel filtration on Biogel-P2 (separating in the range of 100 - 1800 dalton) and testing individual fractions in the microsomal remodeling assay did however not reveal any stimulatory activity in the small molecular weight range (not shown). We also were unable to extract a stimulatory lipid from cytosol using organic solvent (Fig. 7, 7 and 8). Interestingly, cytosol was efficiently replaced by other proteins such as bovine serum albumin (BSA), defatted or boiled BSA, or rabbit serum (Fig. 7, 3 - 6). In our view the data suggest that proteins might stabilize the microsomes, e.g. by preventing their aggregation during the incubation, or that proteins protect the remodelases and/or GPI protein substrates from proteolytic degradation.

Preculture of cells with myriocin was found to be capital (Fig. 8B, 6 vs. 1), as already discussed before (Fig. 2B, lane 2).

Further experiments were done to evaluate the importance of CoA, ATP, GTP and C26:0-CoA. The simultaneous omission of CoA and ATP reduced the incorporation

of [^{3}H]DHS into proteins by about 35% (Fig. 8A, 5; Fig. 8B, 8), whereas omission of C26:0 was in most cases of no consequence (Fig. 8A, 4 vs. 1; 8B, 3 vs. 1). This suggests, that microsomal membranes contain sufficient C26:0-CoA or precursors thereof in order to attach a certain amount of [^{3}H]DHS to GPI proteins, but that exogenously added CoA and ATP can enhance the reaction. Addition of C26:0-CoA to the standard reaction was usually of no effect (not shown). However, C26:0-CoA usually enhanced the incorporation of [^{3}H]DHS when CoA and ATP were lacking (Fig. 8A, 6 vs. 5; Fig. 9B, 9 vs. 8), but not to levels higher than observed under the standard conditions. Curiously, C26:0-CoA consistently had little effect if added into reactions that contained ATP (Fig. 8A, 8 vs. 7; Fig. 8B, 10 vs. 8). Aureobasidin A, a specific inhibitor of IPC synthase (24) could be expected to increase the amount of ceramide available for the remodeling reaction by blocking the further metabolism of ceramide, but it did not stimulate the incorporation of [^{3}H]DHS into GPI proteins (Fig. 8A, 9 vs. 1; data not shown).

While many tests showed stimulation of the remodeling reaction by ATP, this stimulation was not dependent on the presence of Mg^{2+} suggesting that ATP, not -Mg^{2+}-ATP is required. As shown in Fig. 8B (done without Mg^{2+}), the simultaneous omission of C26:0, CoA, ATP and GTP again reduced the incorporation of [^{3}H]DHS into proteins quite significantly (Fig. 8B, reactions 1, 2, 8). Furthermore, the omission of either ATP and GTP or CoA caused a similar reduction (Fig. 8B, 1 - 5). Gel electrophoresis experiments also showed that the profile of labeled proteins was not significantly different when microsomes were deprived of the possibility to make acyl-CoA (Fig. 8C). Reactions became more dependent on exogenously added CoA and ATP, and became even dependent on C26:0, when microsomes were derived from cells that had been precultured in the presence not only of myriocin, but also of

cerulenin, a drug which blocks fatty acid biosynthesis by inhibiting the β-ketoacyl-ACP synthase (1, 20). As shown in Fig. 9, omission of C26:0 reduced the incorporation of [^{3}H]DHS into proteins significantly (Fig. 9, 4 vs. 3), omission of CoA or ATP led to a severe reduction (Fig. 9, 5, 6 vs. 3) and C26-CoA could restore some activity even though ATP was present (Fig. 9, 9 vs. 5). Thus, after preculture of cells with cerulenin the microsomes may be low in acyl-CoAs so that the remodeling reaction becomes more dependent on acyl-CoA synthesis. The very long chain fatty acid-specific acyl-CoA synthase Fat1 has recently been localized in the ER (25). We further investigated if other fatty acids than C26:0 would enhance the standard reaction (using microsomes from cells incubated with myriocin but not cerulenin). Compared to the reaction without added fatty acid the addition of C26:0 or C16:0 was of no consequence; only C24:0 slightly stimulated the incorporation of [^{3}H]DHS into proteins (not shown). Addition of physiological electron donors such as glutathione (GSH) or NADPH did not stimulate the reaction (not shown). Also, the TLC mobility of anchor lipids did not change when GSH, NADH or NADPH were added to standard reaction (not shown). After these studies we now utilize a slightly modified standard assay including Mg^{2+}, C24:0, NADPH, GSH and BSA instead of cytosol (final conditions, see methods section).

Discussion

Ceramides are found in the GPI anchors of certain plants, (e.g. pears), *Trypanosoma cruzi, Paramecium, Aspergillus fumigatus* and *Dictyostelium*, sometimes as the sole anchor lipid (3, 27). Recent studies show that, similar to yeast, the first steps of GPI biosynthesis in *A.fumigatus* and *T.cruzi* do not use ceramide as the lipid support,

suggesting that ceramide is added by remodeling at a later step not only in yeast but also in other species (2, 11).

In a recent report we described a microsomal assay for the Gup1-mediated addition of fatty acids in the *sn*2 position of GPI anchors, which revealed that the *GUP1* homologue of *Trypanosoma brucei* can remodel free GPI lipids as well as GPI anchors of proteins (19). Here we describe a further assay allowing to measure the replacement of diacylglycerol based GPI anchors by ceramide based anchors. These assays set the stage for further biochemical investigation and for reconstitution experiments of the various remodeling reactions.

The SDS-PAGE/fluorography profile of GPI proteins labeled *in vitro* is very similar to the one of GPI proteins labeled *in vivo* when the exit from the ER is blocked. This argues that the microsomal assay measures the GPI remodeling event occurring in the ER. While *in vivo* remodeling in the ER generates IPC/B-containing anchors, the *in vivo* remodeling in the Golgi generates IPC/C-containing anchors (28). The fact, that the *in vitro* labeled GPI anchors contain IPC/B but not IPC/C suggests that the *in vitro* test reproduces ER remodeling or else, that Scs7, the enzyme hydroxylating the fatty acid moiety of sphingolipids and required for the generation of IPC/C, is not operative in our assay. The current knowledge suggests that Scs7 is localized outside the ER in vesicles whereas Sur2, generating PHS from DHS and required for the generation of IPC/B-containing anchors is localized to the ER. (25).

The increase of DHS incorporation upon addition of cold DHS (Fig. 6B) might be thought to be due to a detergent effect of DHS, but the 4.3 nmol giving the highest incorporation correspond to a concentration of 0.0013% (w/v) in the assay. 4.3 nmol/assay correspond to 1.3 µg of DHS mixing in with a total of approximately 100 µg of membrane lipids. While 4.32 nmol of long chain bases/100 µg membrane lipid

is a 15 fold higher concentration than the physiological 42 pmol/A_{600} unit of cells (8), ceramide synthase deficient *lag1*Δ *lac1*Δ strains have > 20 fold increased long chain base levels and are living (15). Thus, adding 4.32 nanomoles to the assay is not expected to significantly alter the membrane structure. Moreover, adding 1.3 µg of *lyso*-phosphatidic acid, a natural detergent, had no influence on the incorporation (not shown). A more likely interpretation of results in Figs. 6 and 8 is that the DHS concentration in the standard assay is somewhat limiting the rate of DHS incorporation whereas C26:0 concentration is not. The concentration of non-remodeled GPI proteins that can serve as substrates is most likely also rate limiting, but it is impossible to test this by varying their concentration in our microsomal assay. C26:0-CoA or C26:0 required to synthesize C26-CoA by the ER-based acyl-CoA synthase Fat1 (25) had only major effects if the cells had previously been depleted of acyl-CoA by cerulenin treatment (Fig. 9), but the addition of C26:0-CoA to the standard assay was usually of no consequence. In contrast, addition of C26:0-CoA strongly enhanced the biosynthesis of [^{3}H]ceramide from [^{3}H]DHS in the standard assay Fig. 2C and (15). This argues that in this *in vitro* system, the acyl-CoA dependent ceramide synthesis pathway is not feeding directly into the ceramide pool that is utilized by the GPI ceramide remodelase activity. The same idea is supported by findings obtained in intact cells: a) the type of ceramides predominating in GPI anchors (PHS-C26:0) is different from the one predominating in IPCs (PHS-C26:0-OH)(28)(Fig. 3); b) GPI anchor lipids made by a *lag1*Δ *lac1*Δ *ydc1*Δ *ypc1*Δ strain kept alive by the murine *LAG1* homologue Lass5 contain the typical IPC/B moiety although the free sphingolipids of this strain almost exclusively contain C16:0 and C18:0 fatty acids in their ceramide moiety (5). These data suggest that Cwh43 is not just transferring ceramides made by the acyl-CoA- and Lag1- or Lac1-dependent

ceramide synthase, but may generate ceramides on GPI anchors through a different mechanism. However, the significant dependence on CoA and ATP of the incorporation of [^{3}H]DHS into proteins in microsomes from acyl-CoA depleted cells (Fig. 9, reactions 5 and 6) suggests that [^{3}H]DHS cannot be incorporated as such as proposed in the introduction, but that it has to be acylated before being added to proteins. Further studies are necessary to fully understand the operating mode of Cwh43.

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Key words not title: membrane orientation, lipid remodeling, endoplasmic reticulum

Abbreviations: AbA, aureobasidin A; BSA, bovine serum albumin; CoA, coenzyme A; DHS, dihydrosphingosine; IPC, inositolphosphorylceramide; MIPC, mannosyl-IPC; PHS, phytosphingosine; PI, phosphatidylinositol; wt, wild type.

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Figure legends

Figure 1. GPI anchor lipid remodeling in the ER of *Saccharomyces cerevisiae.* Yeast genes implicated in the various steps are indicated in italics. Anchors are designated according to the lipid moiety they release upon nitrous acid treatment. Bst1 generates pG2 type anchors, which are gradually transformed into pG1 and IPC/B type anchors over about 20 - 30 minutes (31). Upon arrival in the Golgi apparatus, a small fraction of GPI anchors with α-hydroxylated C26:0 fatty acid is generated (= IPC/C-type anchors, not shown).

Figure 2. [^{3}H]DHS incorporation into proteins in wt microsomes *in vitro*. A, native (n) or boiled (b) microsomes (micr.) were labeled with 100 µCi [^{3}H]DHS at 30°C for 1 h (lanes 1 – 4) or 4 h (lanes 5 – 8), without (-) or with cytosol (cyt.) that either had been boiled (b) or not (n). Proteins were extracted and glycoproteins were purified by Concanavalin A-Sepharose affinity chromatography before analysis by SDS-PAGE and fluorography. B, lanes 1 - 5, microsomes from wt cells prepared as in Fig. 2A were labeled with 10 µCi ^{3}H-DHS (D) under standard conditions (lane 1) or omitting different ingredients as indicated at the bottom. Reactions in lanes 4 and 5 contained 10 nanomoles of C26:0-CoA (26CA), lane 4 contained 0.5 U of apyrase in addition. B, lanes 6 - 8, *sec18* were grown at 24°C, aliquots of 21 x 10^6 cells were precultured with (+) or without (-) myriocin (40 µg/ml) for 90 min at 24°C, the preculture was continued at 37°C during a further 15 min, cells were labeled for 2 h with 25 µCi [^{3}H]DHS (D) or 25 µCi [^{3}H]inositol (I) at 37°C under same conditions as used during the preculture. Cells were broken using glass beads, proteins extensively delipidated with 10:10:3 and boiled in sample buffer before being analyzed by SDS-PAGE/fluorography. C, aliquots (10%) of lipid extracts from microsomal labeling

reactions shown in Fig. 2B, lanes 1, 3, 4 and 5 were deacylated with NaOH or control incubated, desalted and analyzed by TLC in solvent 1 followed by radioimaging.

Figure 3. Characterization of GPI anchor lipids made by microsomes *in vitro*. A, exponentially growing wt, scs7Δ and sur2Δ cells were metabolically labeled with [^{3}H]inositol. After extensive delipidation, the proteins were concentrated by Concanavalin A-Sepharose affinity chromatography and digested by pronase. GPI anchor peptides were purified over octyl-Sepharose column, anchor lipids were released by nitrous acid (HNO_2) treatment (16) and released anchor lipids were analyzed by TLC (solvent 3) and fluorography (16). Lane 1 of all panels contains an aliquot of the lipid extract of [^{3}H]inositol-labeled wt cells (free lipids, FL). B, lanes 1 - 4: X2180 cells were labeled with [^{3}H]inositol at 30°C (lanes 1, 2) and microsomes from the same cells were labeled for 1 h with 10 µCi [^{3}H]DHS under standard conditions (lanes 3, 4). Anchor lipids were prepared as in panel A. Part of the samples was deacylated with mild NaOH treatment as indicated. All samples were desalted before analysis on TLC (solvent 3)/fluorography. B, lanes 5 - 8: 100'000 cpm aliquots of [^{3}H]DHS-labeled anchor peptides generated in an *in vitro* assay were treated (+) or control incubated (-) for 3h at 37°C with PI-specific phospholipase C (PI-PLC). Released ceramide moieties were analyzed by TLC (solvent 1). Lane 5, anchor peptides kept on ice; lane 6, incubation for 3h at 37°C without PI-PLC. [^{3}H]DHS used for the labeling of microsomes is in lane 8. C, microsomes were prepared from wt or sur2Δ cells either using the standard procedure or omitting EDTA from the lysis buffer used to break the cells (-EDTA). Microsomes were labeled with [^{3}H]DHS, anchor lipids were released, treated with NaOH for O-deacylation and analyzed as in panel A.

Figure 4. Strong acid hydrolysis destroys *in vitro* labeled anchor lipids. A, microsomes from X2180 wt cells were labeled with [^{3}H]DHS and anchor lipids were treated by strong acid hydrolysis (1M HCl in MeOH:H_2O; 9:1, 16 h at 80°C)(32) along with a sample of [^{3}H]DHS used for labeling. FL = lipid extract from [^{3}H]inositol-labeled wt cells. Part of [^{3}H]DHS also is transformed into apolar material during hydrolysis (lanes 4, 5). B, the same wt as well as anchor lipids from *sur2*Δ cells were treated with either mild base (mb) or strong acid (sa) hydrolysis. All lipids were analyzed by TLC in solvent 3 and fluorography. Acid hydrolysis leads to the appearance of DHS derived from IPC/A while most PHS (contained in IPC/B) is probably destroyed.

Figure 5. Ceramide remodeling *in vitro* using microsomes from mutants deficient in *in vivo* ceramide remodeling. A, microsomes from wt (X2180), cwh43Δ (Δ), cwh43Δ complemented with a plasmid born Cwh43 (Δ^+), mcd4Δ or gup1Δ cells were used for an *in vitro* remodeling assay under standard conditions, except that cytosol was omitted in reaction 2. Proteins were extracted and analyzed by SDS-PAGE/fluorography. B, lipid extracts from the microsomal reactions of panel A, lanes 1, 3, and from a further reaction using boiled (b) wt microsomes instead of native (n) ones were deacylated by mild NaOH treatment (+) or control incubated (-), and were analyzed by TLC in solvent 1 and radioimaging. C, gup1Δ cells were labeled *in vivo* with [^{3}H]inositol (lane 2), and gup1Δ derived microsomes were labeled *in vitro* using [^{3}H]DHS. Lipid moieties were released from polar anchor peptides eluting from the octyl-Sepharose column at 25% (lanes 2 - 4) or from the routinely used ones eluting at

50% propanol (lane 1). Lipids were deacylated or not and analyzed by TLC in solvent 2 and fluorography.

Figure 6. The concentration of DHS limits the ceramide remodeling reaction X2180 wt cells were preincubated with myriocin and microsomes were prepared. A, microsomal remodeling assays were preformed under standard conditions (lane 1) with 10µCi (0.17 nanomol) of [^{3}H]DHS. Lane 2 contained boiled microsomes; various amounts of cold DHS were added in lanes 3 - 5. 80% of the reactions were used for SDS-PAGE/fluorography. Chemical amounts of exogenously added DHS (including [^{3}H]DHS) are indicated at the bottom. B, anchor peptides were prepared from the remaining 20% and their radioactivity determined by scintillation counting. The chemical amounts of DHS incorporated into proteins was calculated by multiplying the specific activity of added DHS ([^{3}H]DHS + cold DHS) with the total incorporated radioactivity for those values, which were significantly above background. This amount is a minimal estimate because the microsomes potentially contained endogenous DHS or PHS as well. C, microsomal remodeling assays were preformed under final conditions (see materials and methods) using 25µCi (1 nanomole) of [^{3}H]DHS and 30 or 100 µg of microsomal protein per assay. Incubations lasted 4, 10, 24, 60 or 120 min. Anchor peptides were prepared and their radioactivity determined by scintillation counting. All conditions were tested in duplicate and standard deviations are indicated.

Figure 7. Dependence of microsomal remodeling assay on cytosol. A, X2180 wt cells were precultured with myriocin and microsomes were prepared. Microsomal remodeling assays were preformed under standard conditions (lane 1) or replacing

cytosol by various kinds of BSA or rabbit serum (all added at 600 µg/assay, lanes 3 – 6). Cytosol was extracted with chloroform:methanol:water, 10:10:3, the extract was dried in a rotary evaporator and desalted by butanol/water partitioning. Apolar lipids of the butanol phase or polar lipids of the butanol/water interphase were used instead of cytosol in lanes 7 and 8. Anchor peptides were prepared from 20% or reactions and their radioactivity determined by scintillation counting. B, the remaining 80% of reactions were analyzed by SDS-PAGE/fluorography.

Figure 8. Dependence of microsomal remodeling assay on exogenous ATP, Coenzyme A, and C26:0. A, X2180 wt cells were preincubated with myriocin and microsomes were prepared. Microsomal remodeling assays were preformed under standard conditions with Mg^{2+} (lane 1) or omitting/adding ingredients as indicated at the bottom. The amount of radioactivity in anchor peptides was determined by scintillation counting and plotted as a percentage of incorporation under standard conditions (lane 1). Error bars indicate standard deviations, where the assay conditions were tested in duplicate or triplicate assays. Reaction 1 contained a total of 36'000 cpm of anchor peptides. B, as A, but assays were carried out in the absence of Mg^{2+}, reaction 5 contained apyrase, and microsomes for reaction 6 were from cells not precultured with myriocin. The columns indicate the mean of 3 to 5 independent assays for reactions 1 – 7 and duplicate assays for 8 – 10. Reaction 1 contained a mean of of 54'000 cpm of anchor peptides. C, standard assays using microsomes from X2180 cells were performed omitting some ingredients as indicated at the bottom. Labeled proteins were analyzed by SDS-PAGE and fluorography.

Figure 9. Cerulenin enhances the need for ATP and C26:0 in microsomal assay

X2180 wt cells were precultured for 180 min with cerulenin (10µg/ml) and for the last 90 min with myriocin (40µg/ml) in addition (reactions 3 - 9) or only with myriocin for 90 min (reactions 1, 2). Microsomal remodeling assays were run in duplicate under standard conditions (lanes, 1, 3) or omitting/adding ingredients as indicated at the bottom. The amount of radioactivity in anchor peptides was determined by scintillation counting and plotted as a percentage of incorporation under standard conditions (lane 1). Column 6 contained 0.5 U of apyrase in addition, and 2 or 10 nanomoles of C26:0-CoA were added in conjunction with 0.1 mg of purified yeast acyl-CoA binding protein (ACBP).

Tables

Table I. *Saccharomyces cerevisiae* strains

Strain name	**Genotype**
X2180 (S288c)	*MAT***a** *suc2, mal, gal2, cup1*
164 - 1C = sec18	*MAT*α *sec18^{ts} his4 leu2*
2039 = wt	*MAT***a** *ura3-52 trp1 leu2 his4*
2039-sur2 = sur2Δ	*MAT***a** *sur2::TRP1 ura3-52 leu2 his4*
2039-scs7Δ = scs7Δ	*MAT***a** *scs7::URA3 trp1 leu2 his4*
FBY4128 = mcd4Δ	*MAT***a** *his3Δ1 leu2Δ0 lys2Δ0 MET15 ura3Δ0 YKL165c::kanMX4* harboring p425met-TbGPI10 (36)
FBY4179	*cwh43*Δ + p*CWH43* (14).

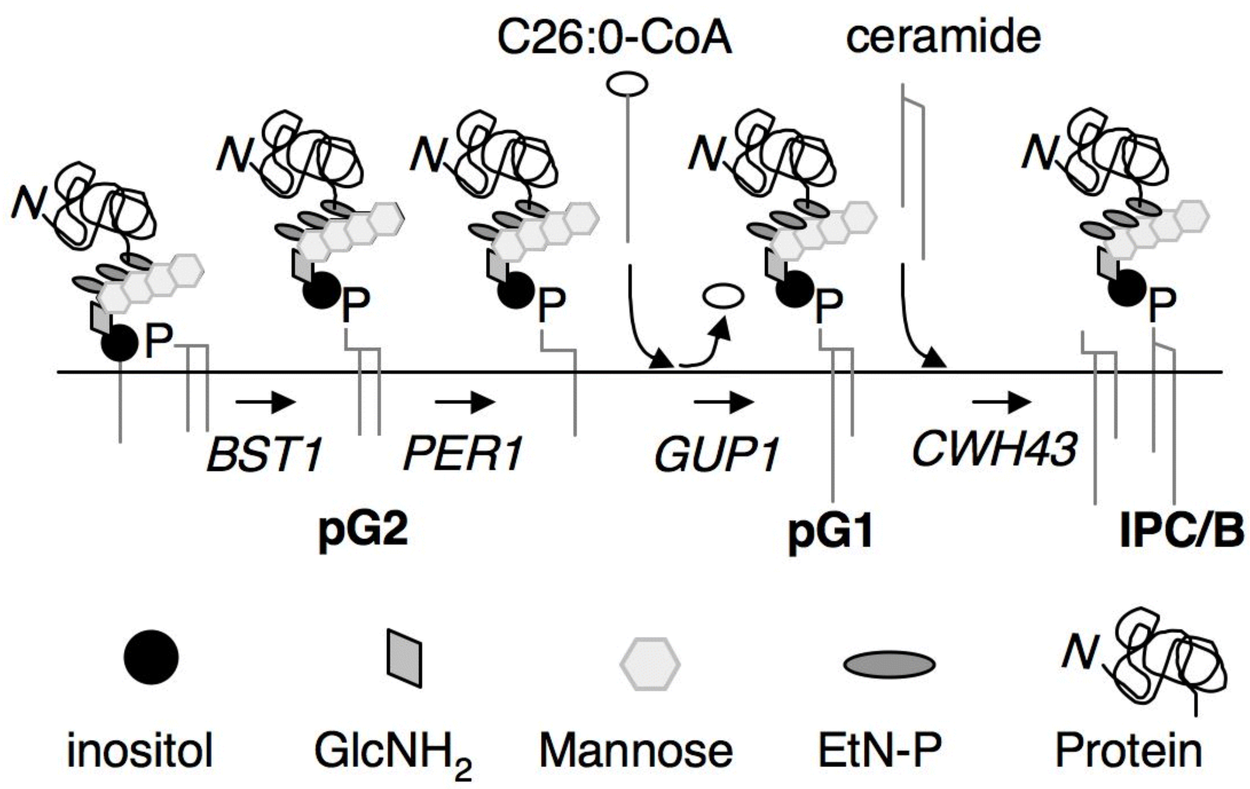

C26:0-CoA
ceramide
N
P
BST1
PER1
GUP1
CWH43
pG2
pG1
IPC/B
inositol
GlcNH2
Mannose
EtN-P
Protein

Fig. 1

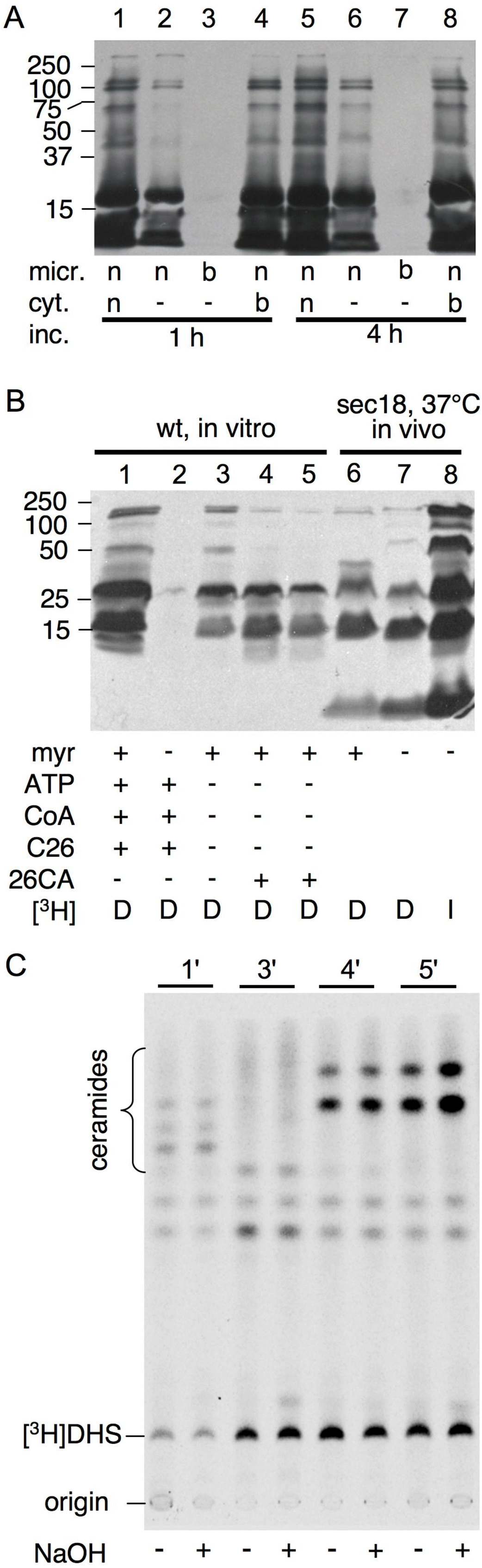
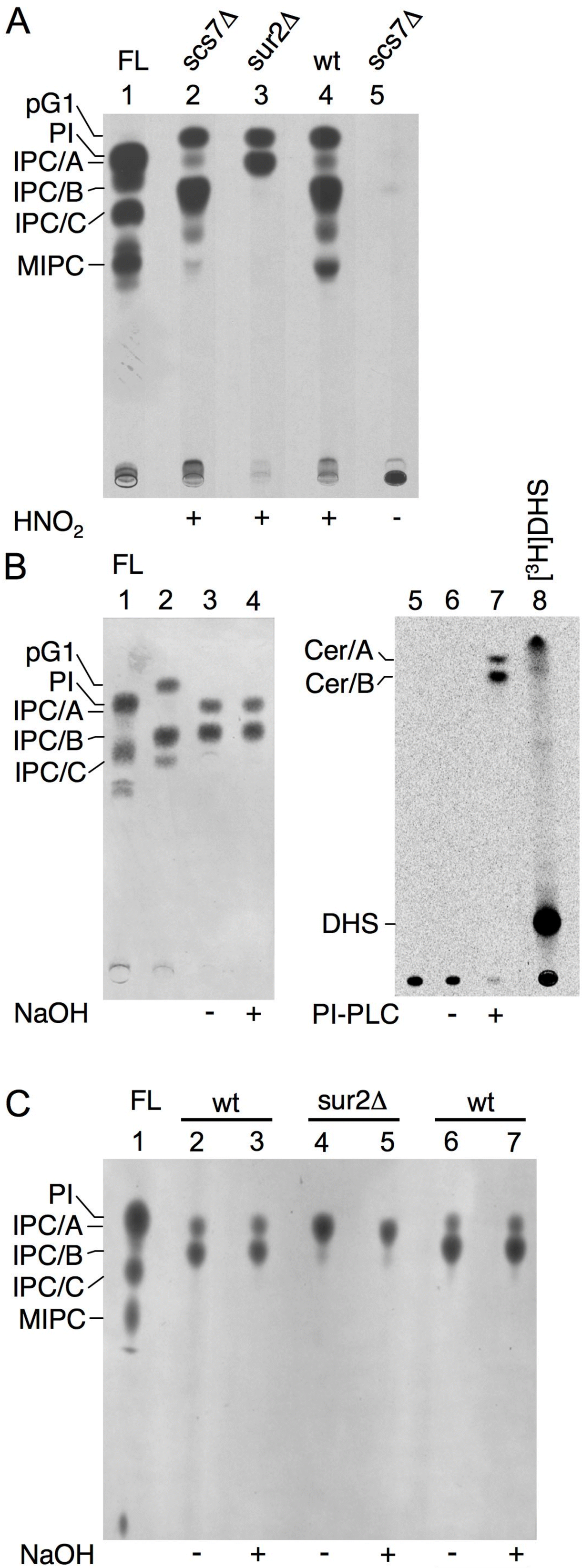

A
1 2 3 4 5 6 7 8
250
100
75
50
37
15
micr. n n b n n n b n
cyt. n - - b n - - b
inc. 1 h 4 h
B
wt, in vitro
sec18, 37°C in vivo
1 2 3 4 5 6 7 8
250
100
50
25
15
myr + - + + + + - -
ATP + + - - -
CoA + + - - -
C26 + + - - -
26CA - - - + +
[3H] D D D D D D D I
C
1' 3' 4' 5'
ceramides
[3H]DHS
origin
NaOH - + - + - + - +

Fig. 2


A
FL
scs7Δ
sur2Δ
wt
scs7Δ
1
2
3
4
5
pG1
PI
IPC/A
IPC/B
IPC/C
MIPC
HNO2
+
+
+
-
B
FL
[3H]DHS
1
2
3
4
5
6
7
8
pG1
PI
IPC/A
IPC/B
IPC/C
Cer/A
Cer/B
DHS
NaOH
-
+
PI-PLC
-
+
C
FL
wt
sur2Δ
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1
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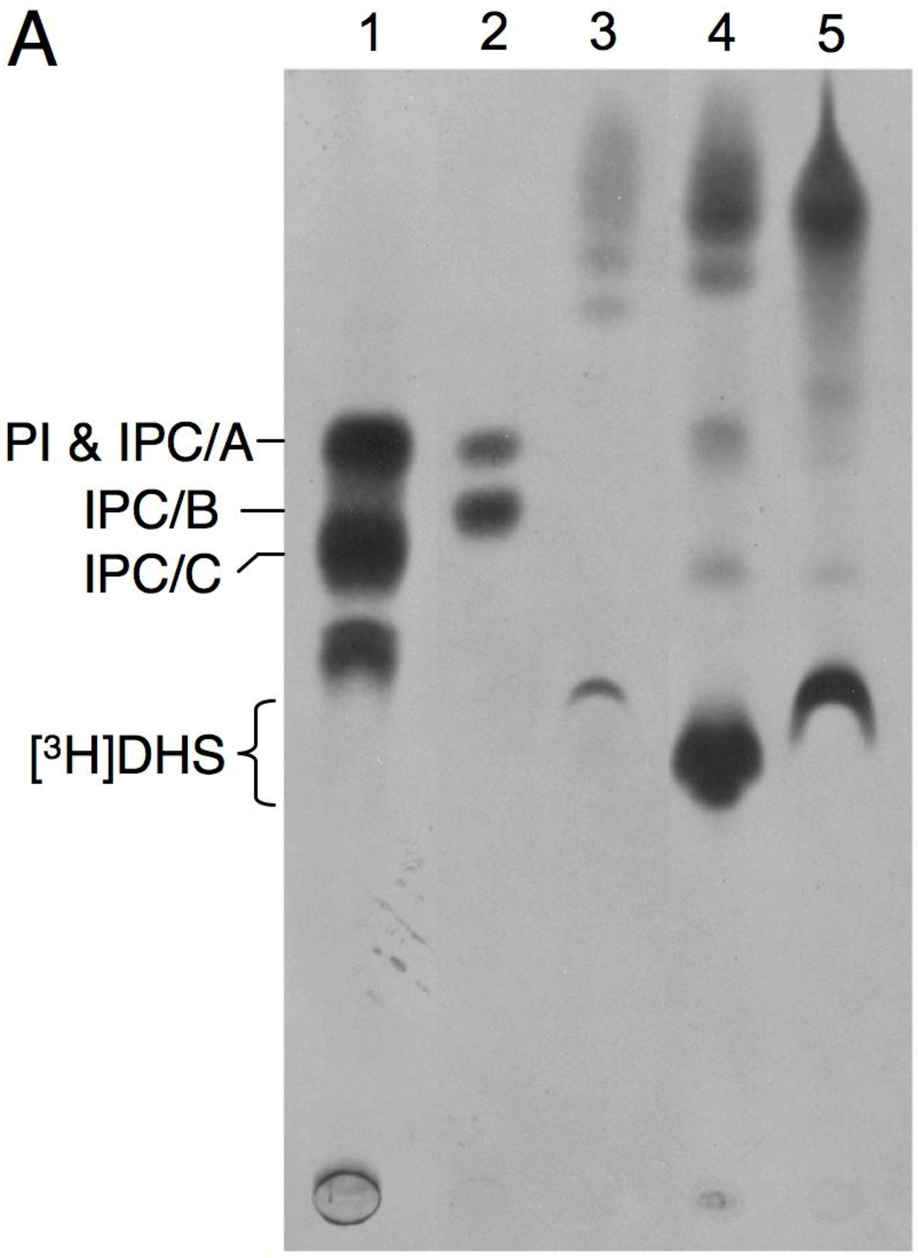
3
4
5
6
7
PI
IPC/A
IPC/B
IPC/C
MIPC
NaOH
-
+
-
+
-
+
-EDTA

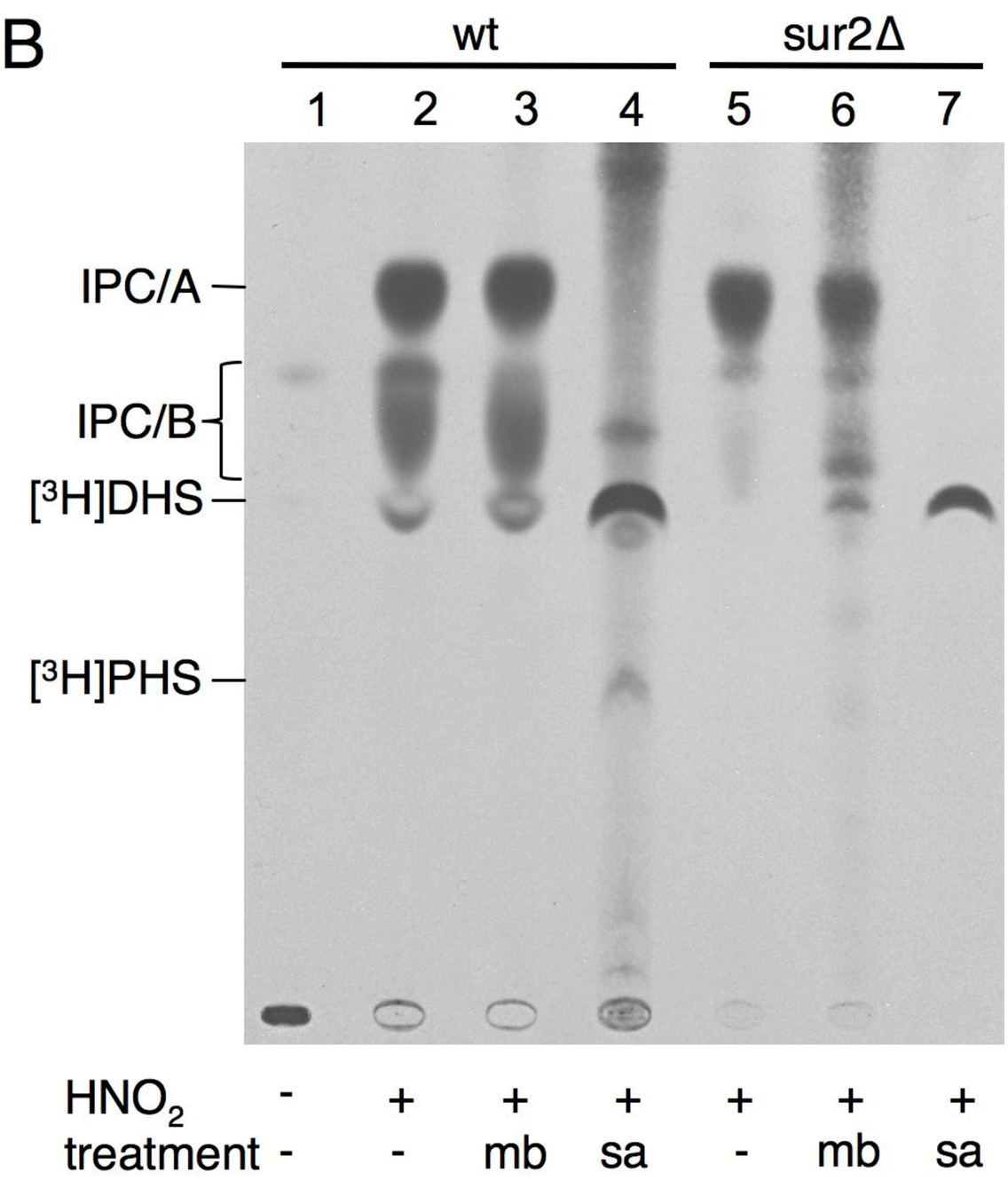
Fig. 3

A



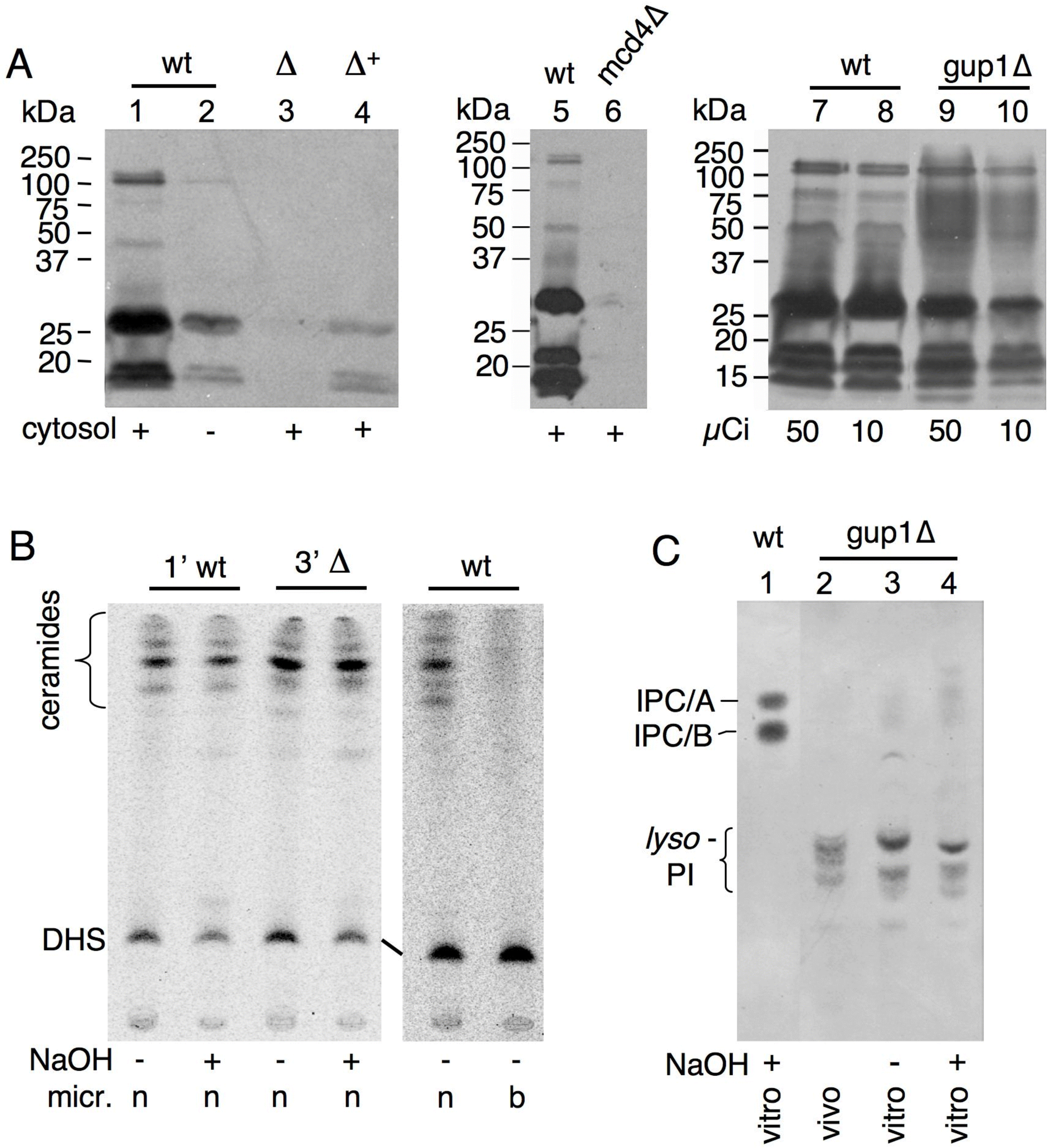
strong acid	-	+	-	+
	FL	anchor lipids		[3H]DHS

B



HNO_2	-	+	+	+	+	+	+
treatment	-	-	mb	sa	-	mb	sa

Fig. 4


A
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mcd4Δ
kDa
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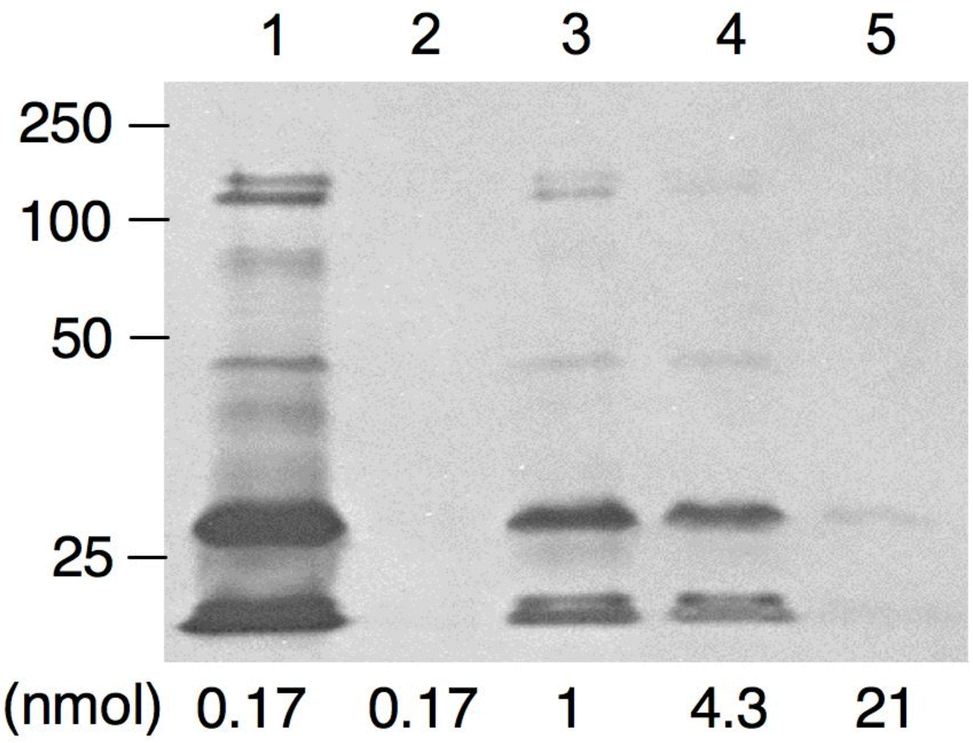
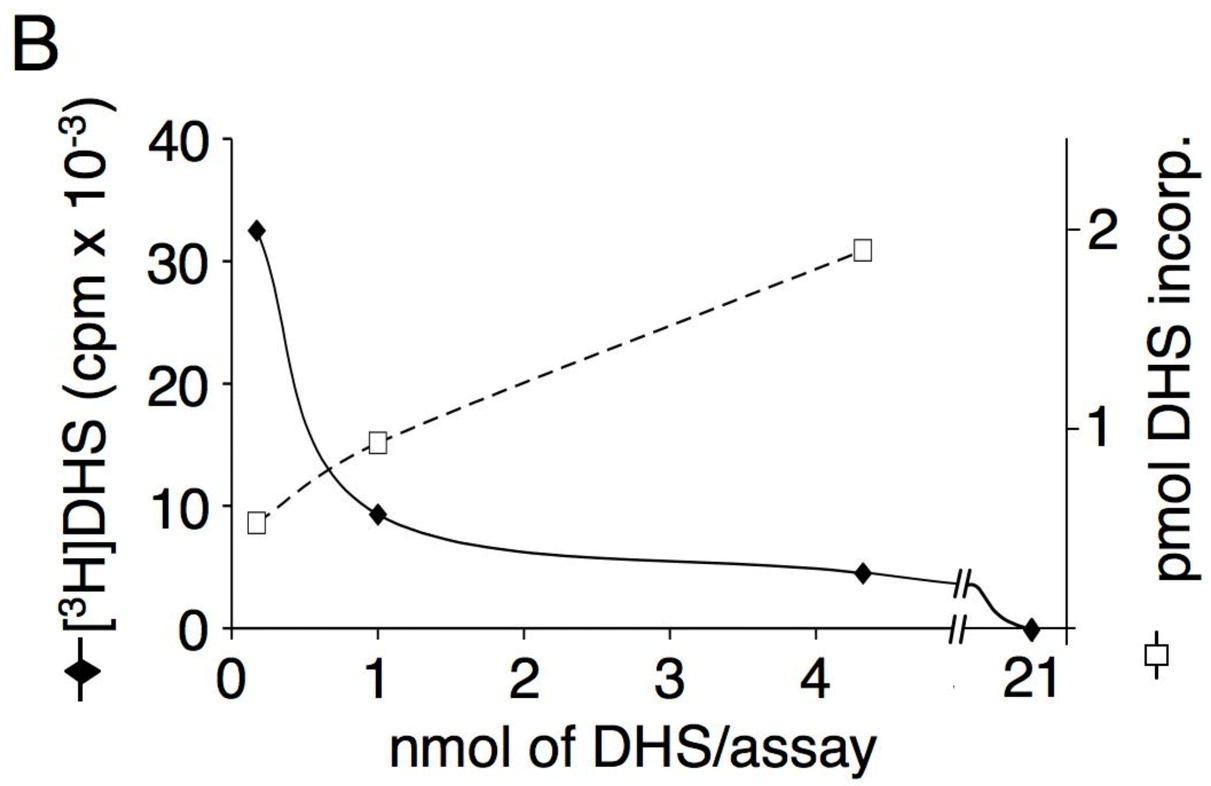
6
250
100
75
50
37
25
20
+ +
wt
gup1Δ
kDa
7
8
9
10
250
100
75
50
37
25
20
15
μCi 50 10 50 10
B
1' wt
3' Δ
wt
ceramides
DHS
NaOH - + - + - -
micr. n n n n n b
C
wt
gup1Δ
1
2
3
4
IPC/A
IPC/B
lyso - PI
NaOH + - +
vitro
vivo
vitro
vitro

Fig. 5

A


1
2
3
4
5
250
100
50
25
DHS (nmol) 0.17 0.17 1 4.3 21

B


[3H]DHS (cpm x 10-3)
40
30
20
10
0
0
1
2
3
4
21
pmol DHS incorp.
2
1
nmol of DHS/assay

C

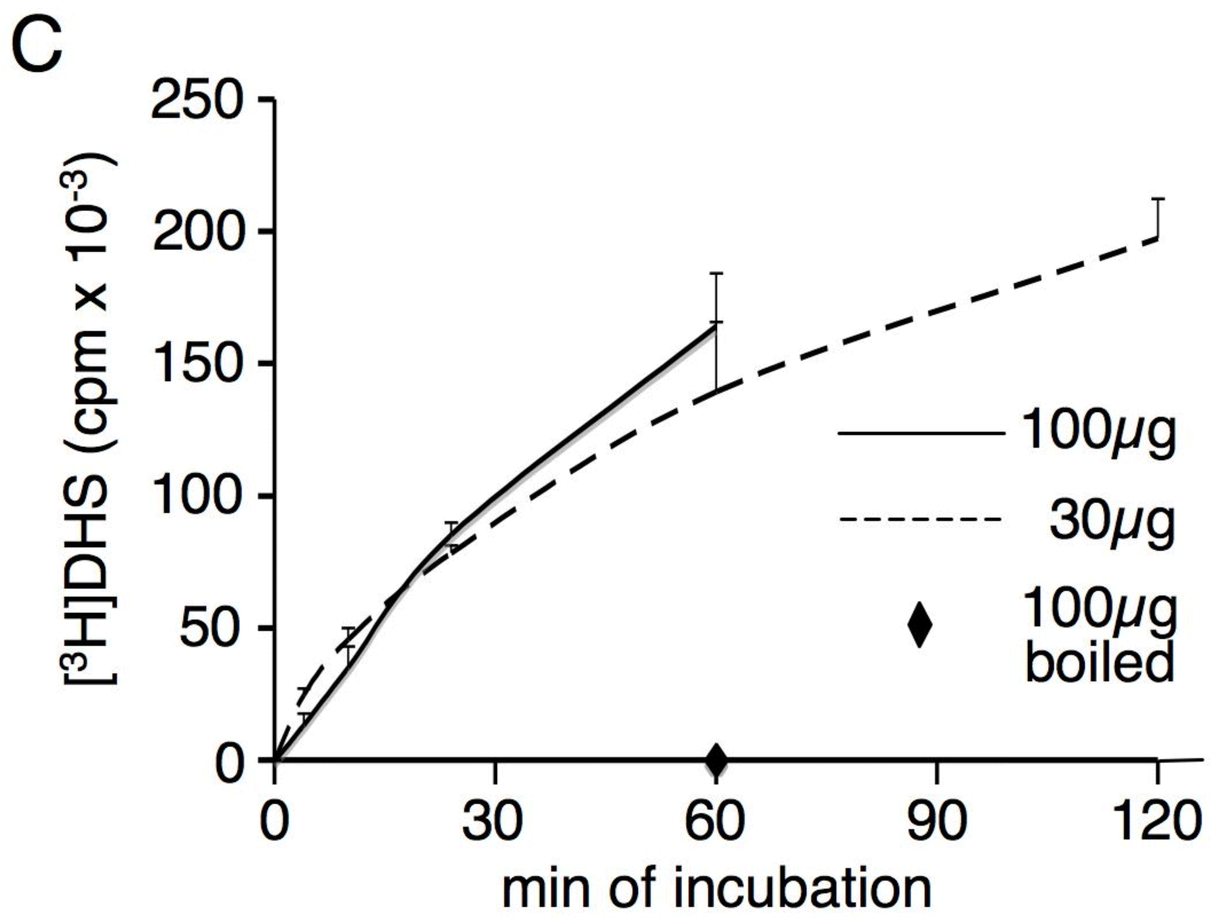

[3H]DHS (cpm x 10-3)
250
200
150
100
50
0
100µg
30µg
100µg boiled
0
30
60
90
120
min of incubation

Fig. 6

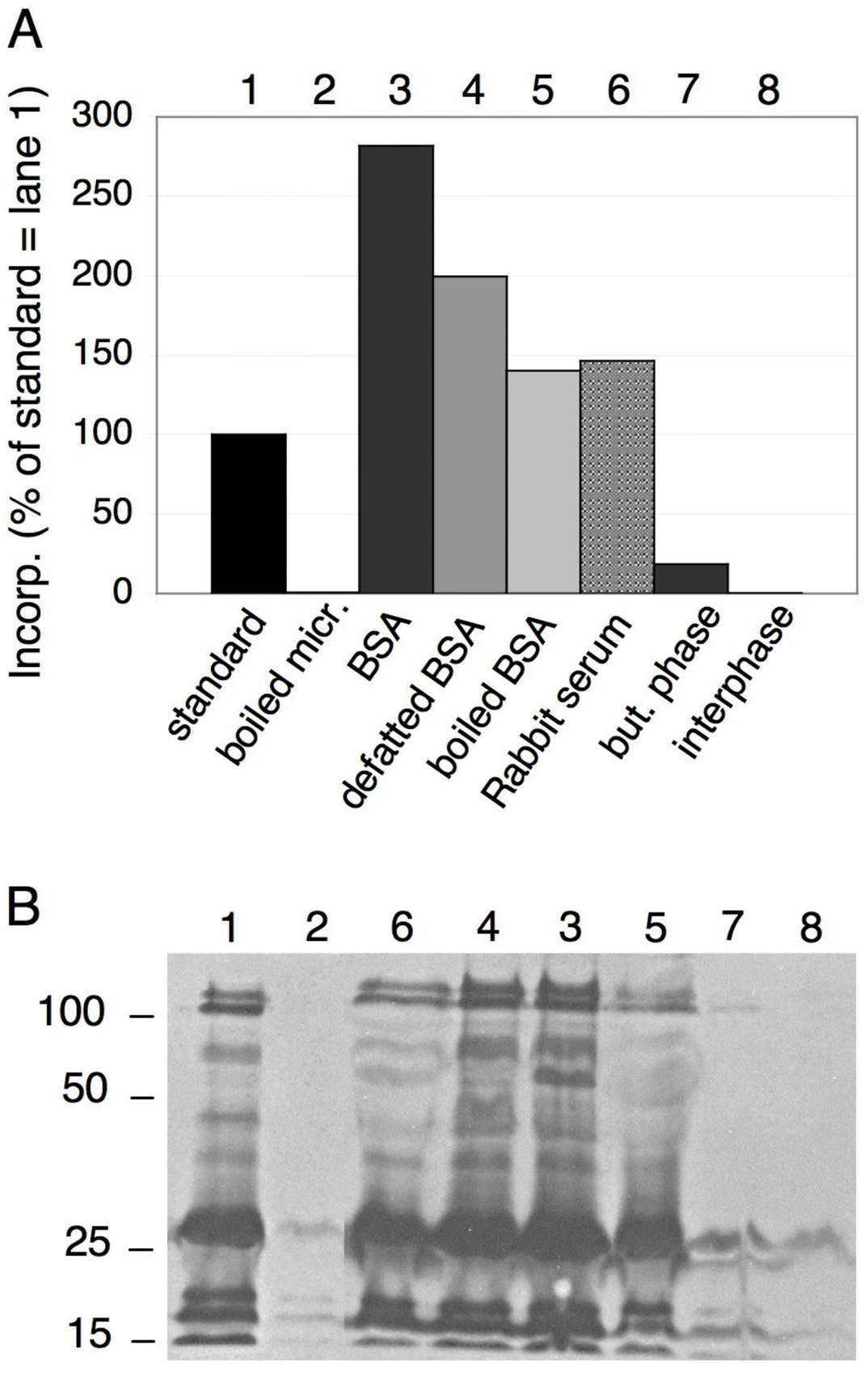
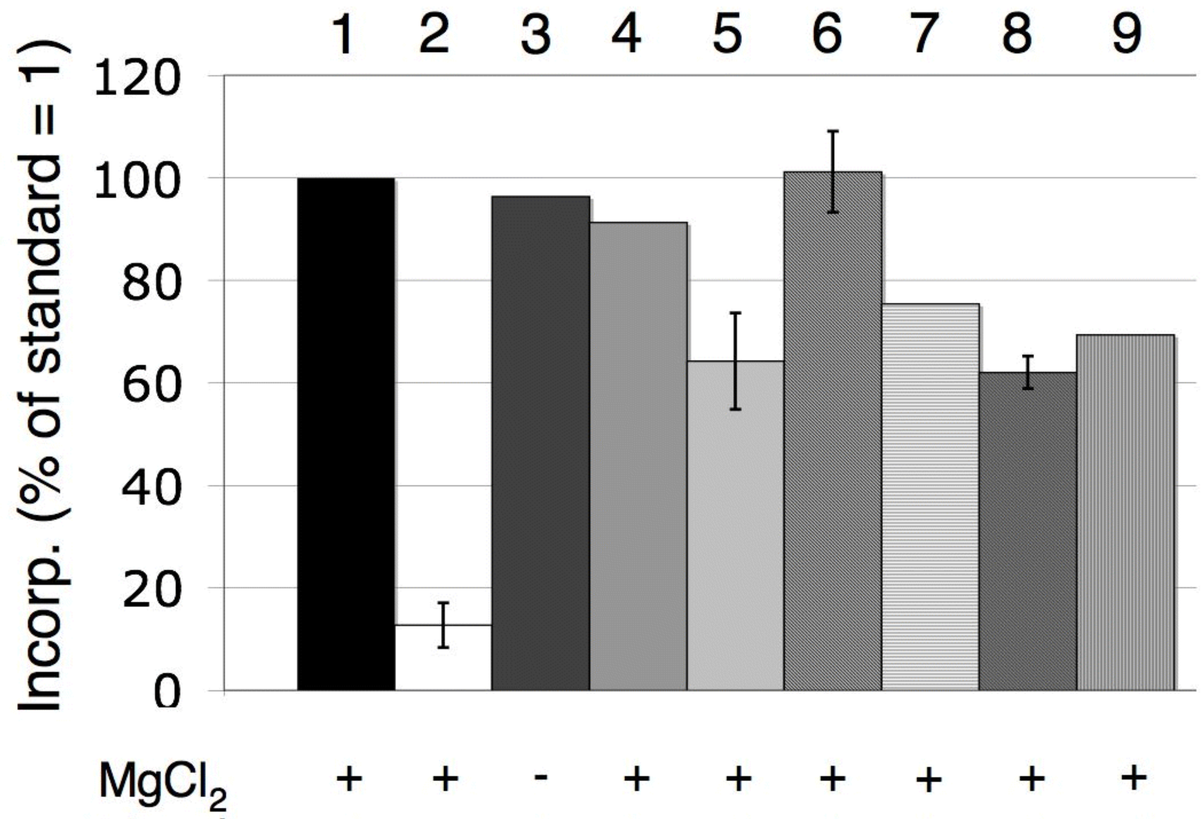

A
1 2 3 4 5 6 7 8
300
250
200
150
100
50
0
Incorp. (% of standard = lane 1)
standard
boiled micr.
BSA
defatted BSA
boiled BSA
Rabbit serum
but. phase
interphase
B
1 2 6 4 3 5 7 8
100
50
25
15

Fig. 7

A



	1	2	3	4	5	6	7	8	9
$MgCl_2$	+	+	-	+	+	+	+	+	+
cytosol	+	-	+	+	+	+	+	+	+
CoA	+	+	+	+	-	-	-	-	+
ATP	+	+	+	+	-	-	+	+	+
GTP	+	+	+	+	+	+	+	+	+
C26	+	+	+	-	-	-	-	-	+
C26-CoA	-	-	-	-	-	+	-	+	-
AbA	-	-	-	-	-	-	-	-	+

B

	1	2	3	4	5	6	7	8	9	10
myr prec.	+	+	+	+	+	-	+	+	+	+
cytosol	+	+	+	+	+	+	-	+	+	+
CoA	+	-	+	-	+	+	+	-	-	-
ATP	+	-	+	+	-	+	+	-	-	+
GTP	+	-	+	+	-	+	+	+	+	+
C26	+	-	-	+	+	+	+	-	-	-
C26-CoA	-	-	-	-	-	-	-	-	+	+

Fig. 8A, B

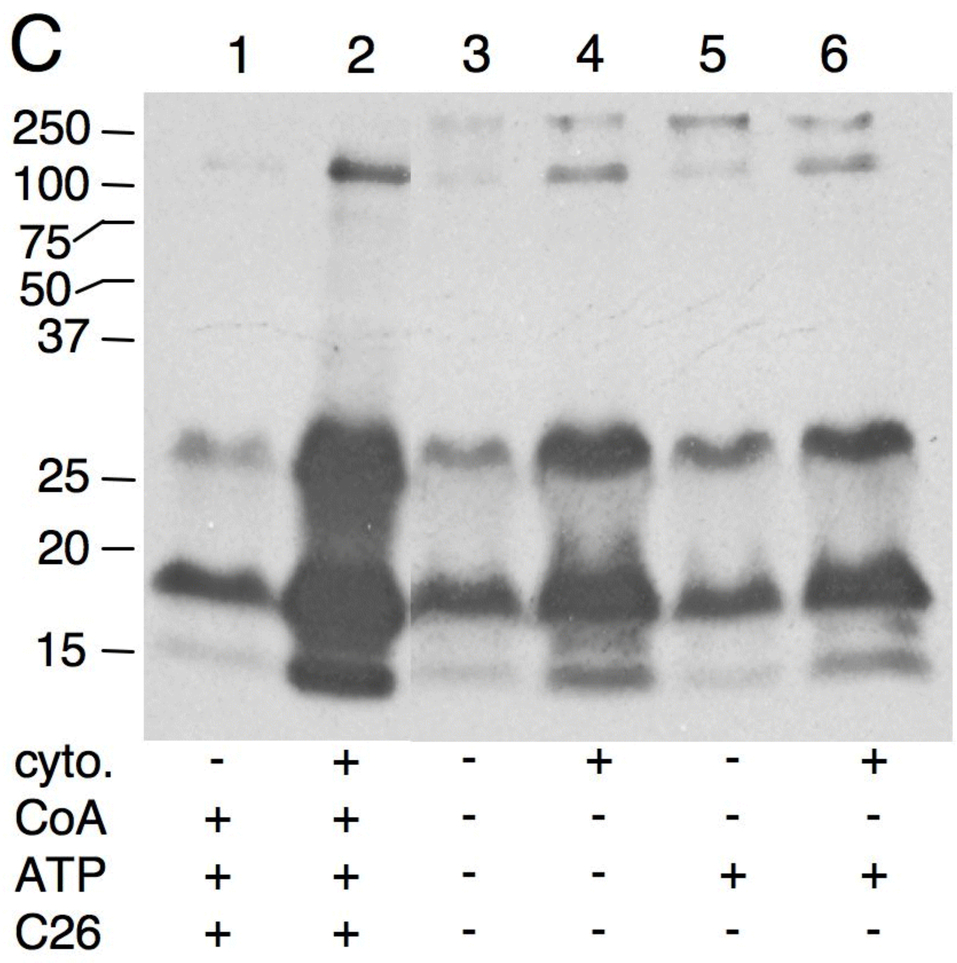


Fig. 8C

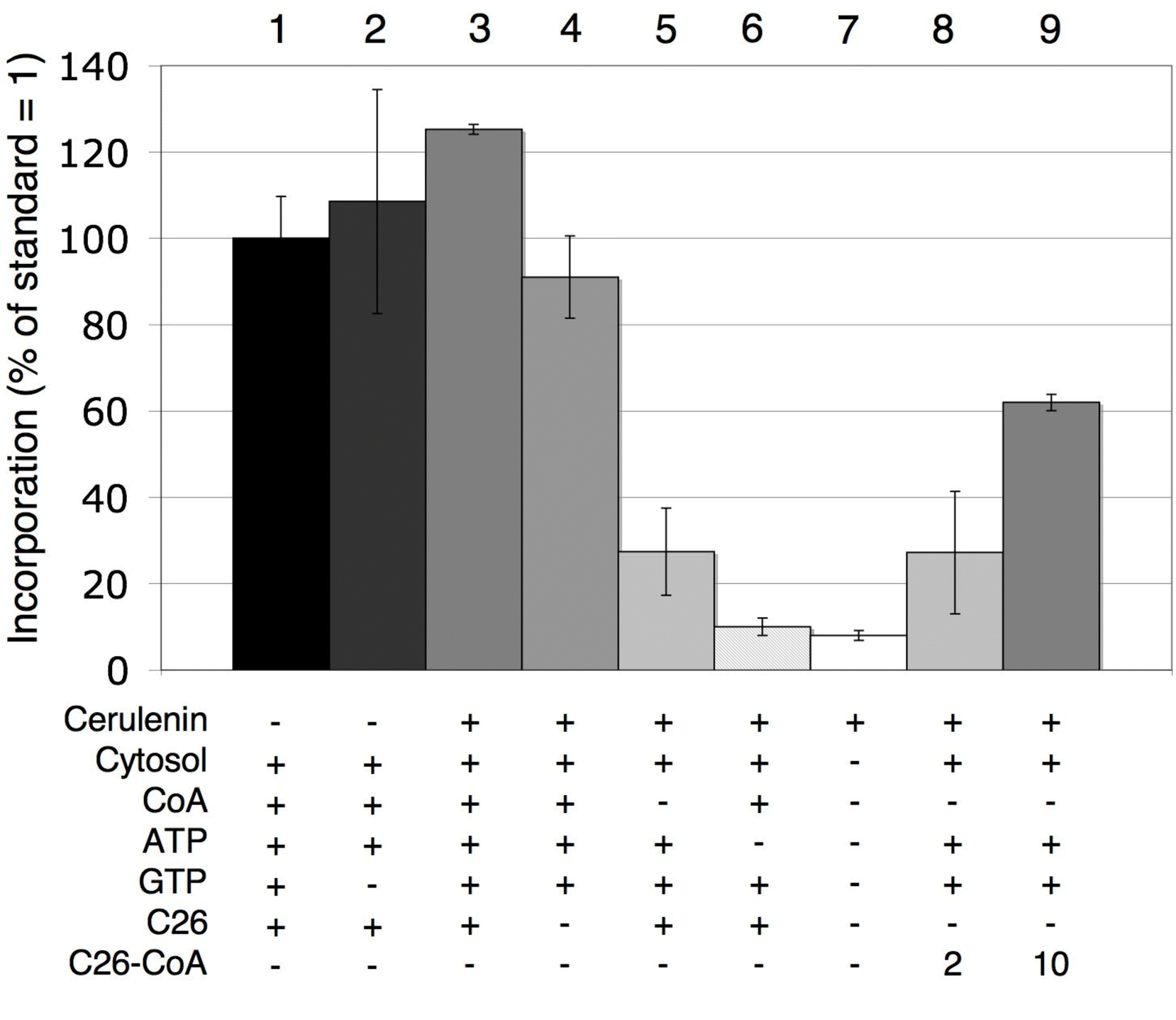

1 2 3 4 5 6 7 8 9
Incorporation (% of standard = 1)
140
120
100
80
60
40
20
0
Cerulenin - - + + + + + + +
Cytosol + + + + + + - + +
CoA + + + + - + - - -
ATP + + + + + - - + +
GTP + - + + + + - + +
C26 + + + - + + - - -
C26-CoA - - - - - - - 2 10

Fig. 9