Human Homologues of LAG1 Reconstitute Acyl-CoA-dependent Ceramide Synthesis in Yeast*

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Lag1p and Lac1p are two highly homologous membrane proteins of the endoplasmic reticulum. lag1Δ lac1Δ double mutants in Saccharomyces cerevisiae lack an acyl-CoA-dependent ceramide synthase and are either very sick or nonviable, depending on the genetic background. LAG1 and LAC1 are members of a large eukaryotic gene family that shares the Lag1 motif, and some members of this family additionally contain a DNA-binding HOX homeodomain. Here we show that several human LAG1 homologues can rescue the viability of lag1Δ lac1Δ yeast cells and restore acyl-CoA-dependent ceramide and sphingolipid biosynthesis. When tested in a microsomal assay, Lac1p and Lag1p had a strong preference for C24:0-CoA over C24:0-CoA, C20:0-CoA, and C16:0-CoA, whereas some human homologues preferred C24:0-CoA and CoA derivatives with shorter fatty acids. This suggests that LAG1 proteins are related to substrate recognition and to the catalytic activity of ceramide synthase enzymes. CLN8, another human LAG1 homologue implicated in ceroid lipofuscinosis, could not restore viability to lag1Δ lac1Δ yeast mutants.

A key role in the sphingolipid pathway is played by ceramide synthase, as it not only catalyzes an essential biosynthetic reaction, but also influences the levels of long chain bases and ceramides, which have signaling function. The simultaneous deletion of LAG1 and its close homologue LAC1 eliminates all detectable acyl-CoA-dependent ceramide biosynthesis in yeast microsomes (5, 6). Moreover, lag1Δ lac1Δ cells have a drastically reduced amount of normal ceramides and IPCs, but exhibit a marked accumulation of free DHS and a compensatory increase of C26:0 fatty acids, which seem to be used for making a new form of phosphatidylinositol (PI) that we call PI’ (5, 6). Whereas the single deletion of LAG1 or LAC1 had no abnormal growth phenotype, the concomitant deletion of LAG1 and LAC1 caused osmotic fragility, calcofluor white hypersensitivity, and a significant decrease of the growth rate in the genetic background of W303 cells, and the same double mutation was lethal in the background of YPK9 cells (5, 7, 8). Lethality of YPK9 lag1Δlac1Δ (herein named YPK9.2Δ) can be overcome by overexpression of LAG1 homologues from man (LAG1Hs, also named LASS1) or Caenorhabditis elegans (LAG1Ce-1, also named hyl-1), two genes showing 26 and 32% identity to yeast LAG1, respectively (8). A recent paper has shown that the murine UO1G1, which has 81% identity with LAG1Hs, and 27% identity with yeast LAG1, strongly induces a C18-CoA-specific ceramide synthase activity, when overexpressed in a mammalian cell line (9). Thus, the pivotal role of LAG1 proteins in the ceramide synthase reaction is established in both yeast and mammalian cells, but it presently is not understood whether LAG1 proteins are part of the catalytically active enzyme or are essential regulators of the ceramide synthase.

This question has led us to investigate the substrate specificity of human and yeast LAG1 proteins. We found that expression of some human homologues of hitherto unknown function in YPK9.2Δ not only restored viability but also allowed the acyl-CoA-dependent synthesis of ceramides that are not usually made but can be used for IPC biosynthesis.

EXPERIMENTAL PROCEDURES

Strains, Growth Conditions, and Materials—S. cerevisiae strains used in this study are listed in Table I. Cells were grown on rich medium (YPD) or minimal media SDaa or SGaa, containing 2% glucose or galactose and uracil (U) and adenine (A) if required (10, 11) at 30 °C. Chemicals, radiochemicals, and inhibitors were from sources described (5, 12). Plasmid pYES2/CT was from Invitrogen AG (Basel, Switzerland). Acyl-CoAs were synthesized as described (5). Oligonucleotides and DNA sequencing services were obtained at Microsynth (Balgach, Switzerland).

Conditional Expression of Clones 1 and 4, LASS2, and CLN8—Human cDNA clones were obtained from the Deutsches Ressourcenzentrum für Genomforschung (RZPD; www.rzpd.de/), namely clone 1 (NCBI entry BC010032; RZPD, IRAI96231927Q2), clone 4 (NCBI entry AK021251; RZPD, IRAI96231927Q2), and CLN8 (NCBI entry BC007725; RZPD, IMAG9858E241166Q2). LASS2 (NCBI entry...
XM (41889) was from Origene Technologies Inc. (www.origene.com/home.html). The conditional expression of human cDNAs in yeast was achieved by the insertion of the open reading frames behind the GAL110 promoter of the vector pYES6/CT carrying as selective marker a blasticidin resistance gene (Invitrogen AG, Basel, Switzerland). Primers to amplify the cDNA open reading frames between suitable restriction sites are shown in Table II. Reverse primers included a stop codon so that the constructs remained without any tag in pYES6/CT. The sequences of the inserts of all vectors constructed were verified by sequencing.

**Microsomal Ceramide Synthase Assay—**Microsomes were prepared and resuspended in lysis buffer as described (13). Unless stated otherwise, assays contained the equivalent of 35 μg of protein (3.3 μg of microsomes or 33–50 μg of detergent extract), 6–10 μM of [3H]DHS (2–3.3 nmol = 20–33 μM) and 10–13 nmol (100–130 μM) of acyl-CoA or free fatty acids in a final volume of 1 ml. Acyl-CoA or free fatty acids were added in 3 μl from a stock in 1% Zwittergent 3-16 so that assays contained 0.01–0.06 mM EGTA and 0.01–0.06 mM EDTA, at 10 mM, caused a 50% inhibition at 30 mM, and completely inhibited the activity found in membranes from YPK9 lag1Δ/YPK9.2 lac1Δ. The non-linearity at higher protein concentrations was expected, as most [3H]DHS was transformed into [3H]ceramide and, thus, [3H]DHS was exhausted. Maximal ceramide synthase activity could be observed over a broad pH range extending from pH 6.5 to 9.5 (Fig. 1D). The same broad pH profile was found in membranes from YPK9 lag1Δ and YPK9 lac1Δ single mutants, in which only either LacIp or LagIp is operating (data not shown). Thus, the two homologues cannot be distinguished in this respect. Although EGTA and EDTA, at 10 mM, had no effect on the assay, all divalent cations except for Mg2+, Ca2+, and Zn2+ at 5 mM reduced the activity to 85, 37, 31, 26, and 7% of the activity found without divalent cations. 2-Mercaptoethanol, a reducing agent, was without any effect at 7.5 mM, caused a 50% inhibition at 30 mM, and completely inhibited the synthase reaction at 60 mM. Our initial microsomal system contained ATP to maintain ionic gradients (5). However, ATP also allows for biosynthesis of acyl-CoA from a free fatty acid and CoA. By consequence, in assays where ATP is present, free CoA, generated by the ceramide synthase itself or through hydrolysis of acyl-CoA, could be used to activate other free fatty acids present in the microsomes and the acyl-CoA that finally is used to make [3H]ceramide may not be the one

**RESULTS**

**Characterization of the Microsomal Ceramide Synthase Assay—**The ceramide synthase assay described previously (5) contained microsomes, [3H]DHS, divalent cations, an ATP-regenerating system, and C26:0-CoA. As can be seen in Fig. 1A, when the amount of crude microsomes was varied in such an assay, a maximal amount of [3H]ceramide was generated, when the assay contained 33 μg of microsomal protein. At higher protein concentrations, the amount of product formed decreased. This can be explained by surface dilution, i.e. the fact that these substrates partition into the membranes and their effective concentration, expressed as mol % of membrane lipids, decreases when more membranes are added. Similar findings were also obtained by Morell and Radin (16). Fig. 1B shows that the reaction was linear with time for over 2 h, irrespective of the amount of [3H]DHS we added, and that the amount of [3H]ceramide formed was strictly proportional to the amount of radioactivity added. Thus, in our standard conditions (20 μM [3H]DHS, 2 h of labeling), [3H]DHS is limiting, as expected from the finding that the apparent $K_m$ value for DHS in detergent is very high (27 mol %) (5). Digitation was the only detergent that solubilized the activity in good yield. When using solubilized membrane proteins, we obtained 2–fold more activity/mg of protein than with crude microsomes. This may be explained by an enrichment of the enzyme when detergent-insoluble proteins are removed by centrifugation. When digitoni-n-solubilized microsomal proteins were used, the amount of product made increased with the amount of membrane proteins added into the assay up to 100 μg of protein/assay (Fig. 1C). The non-linearity at higher protein concentrations was expected, as most [3H]DHS was transformed into [3H]ceramide and, thus, [3H]DHS was exhausted. Maximal ceramide synthase activity could be observed over a broad pH range extending from pH 6.5 to 9.5 (Fig. 1D). The same broad pH profile was found in membranes from YPK9 lag1Δ and YPK9 lac1Δ single mutants, in which only either LacIp or LagIp is operating (data not shown).

### Table I

| Strains       | Genotype                     | Reference |
|--------------|------------------------------|-----------|
| W303–1A      | MATα can1–100 ade2–1 his3–11,15 leu2–3,112 trpl–1 ura3–1 | (29)      |
| YPK9         | MATα ade2–10::his3Δ200 leu2–311 lys2–81::trp1–363 ura3–52 | (29)      |
| YPK9 lac1Δ   | Same as YPK9, but lac1Δ::LEU2 | (8)       |
| YPK9 lag1Δ   | Same as YPK9, but lag1Δ::TRP1 | (8)       |
| YPK9 lac1lag1Δ pBM150::LAG1+ (here named YPK9.2A.LAG1) | Same as YPK9, but lac1Δ::LEU2 lag1Δ::TRP1 and containing pBM150::LAG1+ (here named YPK9.2A.LAG1) | (8)       |
| YPK9 lac1lag1Δ pBM150::LAG1Hs+ (here named YPK9.2A.LAG1Hs) | Same as YPK9, but lac1Δ::LEU2 lag1Δ::TRP1 and containing pBM150::LAG1Hs+ (here named YPK9.2A.LAG1Hs) | (8)       |
| YPK9.2A.C1   | As YPK9, but lag1Δ::LEU2 lag1Δ::TRP1 + pYES6/CT with GALIΔ::Clone 1 | This study |
| YPK9.2A.C4   | As YPK9.2A.LAG1 but with LASS2 under GAL1 promoter in pYES6/CT | This study |
| YPK9.2A.LASS2 | Same as YPK9.2A.LAG1 but with CLN8 under GAL1 promoter in pYES6/CT | This study |
| YPK9.2A.CLN8 | Same as YPK9.2A.LAG1 but with CLN8 under GAL1 promoter in pYES6/CT | This study |
| YPK9.2A.CEMY | MATα ura3 trp1 leu2 elo2::LEU2 | R. Schneiter |
| YPK9.2A.CEMY | MATα ura3 trp1 leu2 elo3::URA3 | R. Schneiter |
| YPK9.2A.CEMY | MATα acc1–2150 | R. Schneiter |

*pBM150 is a CEN-ARS, URA3 vector possessing the yeast GAL110 promoter.*
we add into the assay. Here we find that ATP is not required in the yeast microsomal system and that as much [3H]ceramide is made in its absence as in its presence, although massive amounts of apyrase (20 units/ml) inhibited the enzyme by 50%. (This could be the result of a nonspecific effect, but ATP also may have an unidentified indirect effect.) Microsomes from lcb4 lcb5 cells lacking LC kinases displayed a normal incorporation of [3H]DHS into ceramides in the presence of C26-CoA, the synthase generated 77, 19, and 4% of the amount it made with C26-CoA. This is in agreement with the finding that W303–1A cells, making mostly C22 and C20, are viable and make a normal amount of IPC (18). We further wanted to compare the fatty acid specificity of Lag1p and Lac1p. For this, the fatty acid preferences of microsomes from W303–1A wild type (wt) microsomes was realized in presence of increasing amounts of the two preferred substrates C26-CoA and C24-CoA (Fig. 1). The quantification of these bands showed that, with C24-CoA, C20-CoA, and C18-CoA, the synthase generated 77, 19, and 4% of the amount it made with C26-CoA. This is in agreement with the finding that W303–1A wt cells (data not shown). This result is in contrast to the findings of Funato et al. (30), who found that lcb4 lcb5 microsomes could not incorporate [3H]DHS into ceramide. We have presently no explanation for this discrepancy.

Thus, the yeast ceramidase synthase is similar to the mammalian synthase in that it does not require a metal cofactor, ATP, or a thiol-protecting agent (16).

**Substrate Specificity of Lag1p and Lac1p—**IPCs found in S. cerevisiae almost exclusively contain PHS or DHS linked to a very long chain fatty acid, mostly C26:0 but also some C24:0, both of which can be hydroxylated (17, 18). To check whether this prevalence of very long chain fatty acids is the result of the specificity of the ceramide synthase, we tested the ability of the ceramide synthase to use acyl-CoAs of different length. It appeared that C26-CoA was the best substrate, but slightly fewer hydrophobic ceramides were also generated in the presence of C24-CoA, C20-CoA, and C18-CoA (Fig. 2A). The quantification of these bands showed that, with C24-CoA, C20-CoA, and C18-CoA, the synthase generated 77, 19, and 4% of the amount it made with C26-CoA. This is in agreement with the finding that W303–1A cells, making mostly C22 and C20, are viable and make a normal amount of IPC (18). We further wanted to compare the fatty acid specificity of Lag1p and Lac1p. For this, the fatty acid preferences of microsomes from lcb4Δ cells, making mostly C22 and C20, are viable and make a normal amount of IPC (18). We further wanted to compare the fatty acid specificity of Lag1p and Lac1p. For this, the fatty acid preferences of microsomes from lcb4Δ cells, making mostly C22 and C20, are viable and make a normal amount of IPC (18). We further wanted to compare the fatty acid specificity of Lag1p and Lac1p. For this, the fatty acid preferences of microsomes from lcb4Δ cells, making mostly C22 and C20, are viable and make a normal amount of IPC (18).

**TABLE II**

| Target gene | Name of primer | Oligonucleotide sequence | Size of product |
|-------------|----------------|--------------------------|-----------------|
| Clone 1 into pYES | 1F | CGGGGTACCCGATTCGAAATTTAAATG | 1140 |
| Clone 4 into pYES | 4F | CGCCAGATCGAAAAATGCTgtgctagtttgagg | 1182 |
| LASS2 into pYES | 2F | CCGGGTGACAAATAAAATGctgctactgtggataac | 756 |
| CLNS into pYES | 8F | CCGGGTGACAAATAAAATGgtgctactgtggataac | 858 |
| Yeast Lag1 | LAG1F | tggtagtaacgtaccatta | 155 |
| Yeast Lac1 | LAC1R | tggtagtaacgtaccatta | 721 |
| LASS2 into pRS | 102.F1 | GGGATCTAGTAgcgtactttacgtgtgtagggag | 884 |
| CLNS into pRS | 101.F1 | GGGATCTAGTAgcgtactttacgtgtgtagggag | 981 |

**Fig. 1. Characterization of the microsomal ceramide synthase assay.**

Crude microsomes (A, B, and D) or detergent-solubilized microsomal proteins (C) from YPK9 (A–C) or W303–1A wt cells (D) were assayed in the presence of C26:0-CoA (130 μM) at 37 °C. A, assays contained between 0 and 5 μg of microsomal proteins and 0.6 μCi of [3H]DHS (20 μCi). The relative concentrations of C26-CoA and [3H]DHS were expressed as mol % of total lipid, a variable that was calculated by summing up the molarities of membrane lipids, detergent, and substrates in each tube. B, 33 μg of protein were incubated with variable amounts of [3H]DHS (1 to 9 μCi, 3.3–30 μCi, 0.23 to 2.1 mol %) during 10, 45, or 140 min. C, assays contained 6 μCi of [3H]DHS and a variable amount of microsomal proteins solubilized in 0.3% digitonin. Final digitonin concentration was 0.3% in all assays. D, 33 μg of protein were incubated with 6 μCi of [3H]DHS in buffers (100 mM) of different pH. Lipids were extracted, de-salted, and developed in solvent system 1. The band corresponding to [3H]DHS-C26 was quantified, and results are given in percentage of [3H]DHS-C26 as compared with the radioactivity in the entire lane.
cells were labeled with [3H]DHS in the presence of various acyl-CoAs and quantified by radioscanning. Results were plotted as percentage of Fig. 1 legend).

.. figure:: FIG.2.png

**Fig. 2. Ceramide synthase specificity.** A, microsomes of YPK9 wt cells were labeled with [3H]DHS in the presence of various acyl-CoAs of different length (0.1 mM). 0.1 mM corresponding free fatty acids were used as control. The amount of ceramide made was quantified by Berthold radioscanning and is indicated as % of total radioactivity recorded in the entire lane at the bottom of some lanes. [3H]DHS used for the assay is shown in lane 9. B, microsomes of YPK9 (lanes 1–3), YPK9 lag1Δ (lanes 4–6), and YPK9 lac1Δ (lanes 7–9) were incubated with [3H]DHS and C26-CoA, C24-CoA, C20-CoA, or the corresponding free fatty acids. Lipids were extracted and analyzed by TLC (solvent 1). C, [3H]ceramides generated in presence of various acyl-CoAs in panel B were quantified by radioscanning and are expressed as fraction (%) of total radioactivity in the lane.

.. figure:: FIG.3.png

**Fig. 3. Rate of formation of ceramide as a function of acyl-CoA concentration.** 100 µg of microsomal proteins (crude microsomes) of YPK9 wt cells were incubated with 6 µCi of [3H]DHS in the presence of C26-CoA or C24-CoA at concentrations ranging from 0.014 to 1.4 mM (0.6–39 mol %). Zwittergent concentration was the same in all assays (0.89 mM). Lipids were extracted, analyzed by TLC in solvent system 1, and quantified by radioscanning. Results were plotted as percentage of [3H]DHS-C26 formed as compared with total radioactivity in the corresponding lane versus mol % of acyl-CoA of total lipids (for definition see Fig. 1 legend).

what is used in standard assays (5 mol %). Additionally, the lower activity of the synthase with C24-CoA could not be overcome by increasing the concentration of C24-CoA, as with higher concentrations of acyl-CoA the activity reached a maximum and even dropped, possibly because of a detergent effect of acyl-CoA. A similar inhibition at higher concentrations of C24-CoA were noted by Morell and Radin (16). In view of this possible inhibition, $K_m$ values can only be estimated roughly but the linear increase of activity up to 13 mol % nevertheless argues that ceramide synthase has a $K_m$ value for C26-CoA, which is certainly higher than the concentration of 0.1–0.13 mM (4–6 mol %) that is used in our standard assay. According to our former studies (5), the yeast ceramide synthase displays an apparent $K_m$ value for DHS of 27 mol % (when measured with the digitonin-solubilized enzyme). As mentioned above, the relatively high apparent $K_m$ values for DHS and acyl-CoA explain why we lost synthase activity in Fig. 1A, when high amounts of microsomes were added.

LAG1Hs Induces Ceramide Synthase Activity in YPK9—As expression of LAG1Hs (LASS1) rescues the lethality and restores the replicative life span of YPK9.2Δ cells (8), we undertook to examine the lipid profile and ceramide synthase activity of the rescued YPK9.2Δ.LAG1Hs strain. As shown in Fig. 4, LAG1Hs could restore an IPC profile to YPK9.2Δ as quantitatively and qualitatively similar as that for yeast LAG1 (lanes 2 and 5 versus lanes 3 and 6). Moreover, the cells containing LAG1Hs did not make PI', lipids a and b, or IPC/B, lipids that are typically found in cells lacking ceramide synthase (5). Indeed, YPK9.2Δ.LAG1Hs cells grow at the same rate as YPK9.2Δ.LAG1 (not shown). Thus, it would appear that LAG1Hs can replace the activity of LAG1 in ceramide synthesis, although we cannot ensure that this would also be true, if LAG1Hs were not overexpressed. When analyzing the microsomal ceramide synthase of YPK9.2Δ.LAG1Hs cells, we found that they contained an acyl-CoA-dependent activity that preferentially uses C26-CoA and C24-CoA (Fig. 5). The preference for C26-CoA over C24-CoA was even more pronounced with LAG1Hs than with yeast LAG1. As human cells do not usually contain ceramides with C26 fatty acids, we wondered whether the membrane environment of the yeast cell may have an influence on the acyl specificity of the synthase, e.g. by recruiting acyl-CoAs of a certain chain length. We argued that, if this were the case, detergent solubilization may liberate the enzyme from its lipid environment and may thus change the acyl chain specificity. Therefore, the synthase of YPK9.2Δ.LAG1Hs was tested after solubilization in digitonin. As shown in Fig. 6, Lag1Hsp again showed a very pronounced preference for C26-CoA and this suggested that Lag1Hsp has an intrinsic specificity for very long chain fatty acids. However, YPK9 microsomes showed a relatively higher activity with C18-CoA after

![Image](FIG.3.png)

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![Image](FIG.2.png)

**Fig. 2. Ceramide synthase specificity.** A, microsomes of YPK9 wt cells were labeled with [3H]DHS in the presence of various acyl-CoAs of different length (0.1 mM). 0.1 mM corresponding free fatty acids were used as control. The amount of ceramide made was quantified by Berthold radioscanning and is indicated as % of total radioactivity recorded in the entire lane at the bottom of some lanes. [3H]DHS used for the assay is shown in lane 9. B, microsomes of YPK9 (lanes 1–3), YPK9 lag1Δ (lanes 4–6), and YPK9 lac1Δ (lanes 7–9) were incubated with [3H]DHS and C26-CoA, C24-CoA, C20-CoA, or the corresponding free fatty acids. Lipids were extracted and analyzed by TLC (solvent 1). C, [3H]ceramides generated in presence of various acyl-CoAs in panel B were quantified by radioscanning and are expressed as fraction (%) of total radioactivity in the lane.

![Image](FIG.4.png)

**Fig. 4. Overexpression of LAG1Hs restores a normal sphingolipid profile to YPK9.2Δ.** 10 OD₆₀₀ of YPK9 wt, YPK9.2Δ.LAG1, or YPK9.2Δ.LAG1Hs cells from cultures growing exponentially in galactose (SGaaA medium) were labeled with 60 µCi of [3H]inositol at 30 °C for 2 h. Lipids were extracted, deacylated or not with MMA and developed in solvent system 3. G-P-Ins is glycerylphosphorylinositol liberated by the MMA treatment.

**Fig. 4.** Overexpression of LAG1Hs restores a normal sphingolipid profile to YPK9.2Δ. 10 OD₆₀₀ of YPK9 wt, YPK9.2Δ.LAG1, or YPK9.2Δ.LAG1Hs cells from cultures growing exponentially in galactose (SGaaA medium) were labeled with 60 µCi of [3H]inositol at 30 °C for 2 h. Lipids were extracted, deacylated or not with MMA and developed in solvent system 3. G-P-Ins is glycerylphosphorylinositol liberated by the MMA treatment.
Holcine 37087

Function of Human LAG1 Homologues

Acyl-CoA specificity of yeast Lag1p/Lac1p was changed in mutant and wt microsomes are assayed in parallel. Although ceramide synthase of wt and elo2Δ cells always showed a distinct preference for C26-CoA as compared with CoA derivatives of shorter fatty acids, the synthase of elo3Δ cells had a distinct preference for C24-CoA over C26-CoA and seemed to utilize also some shorter fatty acid derivatives such as C18-CoA. Curiously, mtr7-1, which harbors a thermosensitive allele of ACC1, a gene encoding the acetyl-CoA carboxylase, prefers C24-CoA when grown at permissive temperature, but rather prefers C26-CoA when grown at restrictive temperature. As we have no real measure of the lipid environment of Lag1p and Lac1p in these different strains, it is not possible to say whether the observed differences correlate with the lipid environment of the synthase. Nevertheless, it is obvious that the deprivation of very long chain fatty acids in these strains did not drastically change the specificity of the endogenous yeast ceramide synthase with regard to acyl-CoA substrates.

HOX Domain-containing Human LAG1 Homologues Induce Ceramide Synthase Activity—Since 1998, when LAG1Hs was first identified, a large number of proteins with homology to LAG1 have been found in many species (8, 19). Their homology usually extends over approximately 200 amino acids and contains at least five predicted membrane-spanning domains. The homology domain has recently been named TLC (TRAM, LAG1, and CLN8 homology domains, SMART accession no. SM0724). In the human genome, the TLC homology domain is found in 18 open reading frames. As pointed out recently (19, 20), many of these homologues in addition contain a HOX homeodomain (SMART accession no. SM0389), a domain that is typical of DNA-binding factors that are involved in the transcriptional regulation of key developmental processes. Neither the yeast LAG1 nor LAG1Hs possesses a HOX domain, although HOX domains are present in other yeast proteins.

Among the human homologues of Lag1p, we collected cDNAs of two clones, here dubbed clone 1 and 4 (with NCBI www3.ncbi.nlm.nih.gov/entrez/query.fcgi accession nos. BC010032 and AK022151), both of which contain a first transmembrane domain followed by a HOX homeodomain and a TLC domain (Fig. 7A). These clones show ~30% identity over 210 and 240 amino acids, respectively, when compared with yeast LAG1, whereas LAG1Hs had a similar degree of homology over only 123 amino acids (8). In addition, two further human genes lacking a HOX domain, CLN8 and LASS2 (HUGO gene nomenclature committee (www.gene.ucl.ac.uk/nomenclature/) accession nos. NM_018941 and NM_013384) were chosen. LASS2 is in fact a shorter version of clone 1, lacking the first 150 amino acids of clone 1 and thus lacking the HOX domain contained in amino acids 69–132 of clone 1. CLN8 is ubiquitously expressed and encodes a 33-kDa ER protein (21). A missense mutation (R24G) in CLN8 is responsible for an autosomal recessive form of neuronal ceroid lipofuscinoses, a class of diseases causing progressive neurodegenerative disorders characterized by the accumulation of autofluorescent lipopigment in various tissues (22). As can be seen in Fig. 7A, all clones utilized contain a TLC domain comprising five hydrophobic regions.

Clones 1 and 4, LASS2, and CLN8 were inserted into the yeast multicopy shuttle vector pYES6/CT behind the yeast GAL1,10 promoter, and the resulting vectors were transformed into strain YPK9.2ΔLAG1 (YPK9 lag1ΔLAC1 harboring pBM150:LAG1, an URA3-based vector expressing yeast LAG1 from the GAL1,10 promoter). YPK9.2ΔLAG1 grows rapidly on galactose but is unable to grow on glucose and cannot grow on fluoroorotic acid (FOA), as it depends on the presence of yeast LAG1 (5). (FOA kills cells harboring an intact URA3 gene and eliminates all cells that cannot lose the pBM150 plasmid that carries URA3.) After transfection, the cells containing pYES6/CT plasmids containing the human LAG1 homologues were selected on SGAaA plates containing blasticidin, as the pYES6/CT contains a blasticidin resistance gene and (compare Fig. 6 to Figs. 5 and 8), indicating that detergent solubilization may allow for specificity changes in certain cases.

The same issue was addressed by trying to see whether the acyl-CoA specificity of yeast Lag1p/Lac1p was changed in mutants, which can be expected to have lower amounts of very long chain fatty acids. Data are summarized in Table III. As we cannot guarantee that the acyl-CoA substrates are completely stable from one experiment to the next, we only can draw conclusions from results obtained in the same experiment where mutant and wt microsomes are assayed in parallel. Although ceramide synthase of wt and elo2Δ cells always showed a distinct preference for C26-CoA as compared with

![Diagram](image-url)
marker. Growing colonies were then streaked out on SGaaUA containing FOA. As can be seen on Fig. 8A, YPK9.2/H9004.LAG1 and YPK9.2/H9004.CLN8 did not grow significantly, whereas YPK9.2/H9004.C1 and YPK9.2/H9004.C4 grew as vigorously as YPK9. On glucose, YPK9.2/H9004.C1 and YPK9.2/H9004.C4 grew much slower than YPK9 (Fig. 8A). The absence of LAG1 sequences in YPK9.2/C1 and YPK9.2/C4 was confirmed by whole cell PCR, indicating that these strains had lost the plasmid that

TABLE III

| Experiment | Strain       | C26-CoA | C24-CoA | C22-CoA | C20-CoA | C18-CoA | C16-CoA | CoA Boiled microsomes |
|------------|--------------|---------|---------|---------|---------|---------|---------|-----------------------|
| 1<sup>a</sup> | wt           | 50      | 26      | NT<sup>c</sup> | 9       | 4       | 3       | NT<sup>c</sup>         |
|            | elo2Δ        | 60      | 23      | NT<sup>c</sup> | 14      | 8       | 2       | NT<sup>c</sup>         |
|            | elo2Δ        | 76      | 50      | NT<sup>c</sup> | 35      | 12      | 10      | NT<sup>c</sup>         |
|            | elo3Δ        | 7       | 16      | NT<sup>c</sup> | 10      | 8       | 2       | NT<sup>c</sup>         |
| 2<sup>a</sup> | wt           | 63      | 22      | NT<sup>c</sup> | 6       | 3       | —       | NT<sup>c</sup>         |
|            | elo2Δ        | 49      | 9       | NT<sup>c</sup> | 4       | 1       | —       | NT<sup>c</sup>         |
|            | mtr7-1, 20 min at 37 °C | 11  | 33      | NT<sup>c</sup> | 5       | 2       | 6       | NT<sup>c</sup>         |
|            | mtr7-1, 24 °C | 12      | 30      | 13      | 11      | 4       | 3       | 3         |
|            | mtr7-1, 3 h at 37 °C | 25      | 12      | 10      | 8       | 3       | 5       | 3         |
|            | mtr7-1, 3 h at 37 °C + terbinafine | 12      | 11      | 5       | 6       | 4       | 5       | 0         |

<sup>a</sup> No activity was seen with free fatty acids instead of acyl-CoAs (not shown).
<sup>b</sup> All cells were kept alive by growing them in 1 M sorbitol for osmotic stabilization.
<sup>c</sup> NT, not tested.
were prepared from YPK9, YPK9.2/H9004 radioactivity recorded in the entire lane at the top of each lane was quantified by radioscanning and is indicated as percentage of total microsomes was included as a control. The amount of ceramide made with delipidated BSA and assayed in presence of acyl donors or free CoA prepared from cells grown as in panel A, but they were preincubated and analyzed by ascending TLC with solvent 1.

contains yeast LAG1 (Fig. 8C). When cells from SGaaUA + FOA plates were streaked out further on the same or other tester media (Fig. 8D), YPK9.2A.C1 and YPK9.2Δ.C4 growing exponentially on SGaaUA. Ceramides synthase assays using crude microsomes and [3H]DHS were done without ATP and in the presence of the various acyl-CoAs or free fatty acids indicated at the top of the lanes. The radiolabeled lipids were extracted, desalted, and analyzed by ascending TLC with solvent 1. B, microsomes were prepared from cells grown as in panel A, but they were preincubated with delipidated BSA and assayed in presence of acyl donors or free CoA using a previously described method (14). A sample containing boiled microsomes was included as a control. The amount of ceramide made was quantified by radioimaging and is indicated as percentage of total radioactivity recorded in the entire lane at the bottom of each lane.

function of Human LAG1 Homologues

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Microsomes from cells rescued by human LAG1 homologues were prepared and utilized for a microsomal ceramide synthase assay. As can be seen in Fig. 9, all clones had significant activity with CoA-C26, CoA-C24, and C20-CoA, as well as some CoA derivatives of shorter or unsaturated fatty acids, whereas there was little activity in presence of non-activated free fatty acids. We further corroborated the preference of clones 1 and 4 for C24-CoA in several experiments using microsomes that had been preincubated with delipidated bovine serum albumin (BSA). Preincubation with BSA enhanced the activity, particularly in microsomes of YPK9.2Δ.C4 (Fig. 9B). Several possibilities can be invoked to explain this stimulation of activity. Our calculations indicate that the previously described accumulation of cold DHS in lag1Δ lac1Δ cells (5) would at most achieve 2 nmol of DHS (=1.3 mol%) in our assay, an amount that is too low to cause an inhibition of the microsomal synthase assay. However, BSA may remove some other inhibitory lipid, e.g. the abnormal ceramides that accumulate in these cells (Ref. 5 and Fig. 11). BSA carried over into the assay may pull the reaction for ceramide synthases, especially if they are product-inhibited, or may facilitate the insertion of lipid substrates into microsomes and thus change substrate availability for the synthase.

The ceramides made by microsomes from YPK9.2Δ.C1 and YPK9.2Δ.C4 were scraped from the thin layer chromatography plates and found to be resistant to mild base but to yield [3H]DHS upon strong acid treatment, in keeping with the notion that they are ceramides (data not shown). The ceramide synthase activity in microsomes of YPK9.2Δ.C1 and YPK9.2Δ.C4 was inhibited to more than 50% at <1 and <0.25 μM fumonisin B1, respectively, a finding that agrees with data obtained previously for certain mammalian ceramide synthases (23). Microsomes of YPK9.2Δ.C1 and YPK9.2Δ.C4 also made ceramides with unsaturated acyl-CoAs or C16-CoA, acyl-CoAs that are not utilized by the yeast Lag1p, further suggesting that clones 1 and 4 encode ceramide synthases of different specificity than yeast Lag1p, further suggesting that clones 1 and 4 encode ceramide synthases of different specificity than yeast Lag1p. Some of the lipids made by microsomes of YPK9.2Δ.C1 and YPK9.2Δ.C4 are rather unusual as they are significantly more hydrophobic than DHS-C24 (Fig. 9B, pointed out by circles). Synthesis of these lipids is not dependent on added acyl-CoAs or fatty acids and may reflect the utilization of endogenous acyl-CoAs that cannot be utilized by yeast Lag1p or Lac1p. It is possible that this activity is conferred by the transfected human genes, but other possibilities also exist. For instance, it is conceivable that these ceramides are made by yeast enzymes that are induced in these cells as a consequence of the removal of the physiological ceramide synthase, especially as these cells do not seem to be fully complemented by the presence of the human homologues and retain an abnormal lipid profile (see below). Whatever the structure of these lipids and whatever the identity of the enzymes that produce them, it would appear that the presence of C24-CoA and C26-CoA inhibits their appearance.

To see whether the ceramides made in the various strains could be used for the synthesis of inositol phosphoceramides (IPCs), the cells were labeled with [2-3H]myo-inositol and [3H]serine. As can be seen from Fig. 10, YPK9.2Δ.LAG1, when depleted of Lag1p, makes much less of the normal IPCs (IPC/C and MIPC) than wt but instead makes abnormal base-resistant IPCs (IPC/B, IPC/C, and IPC/D) of wt cells and which are representative of stress in cells lacking the normal supply of ceramides (5). YPK9.2Δ.C1 and YPK9.2Δ.C4 continue to make lipids a, b, and IPC/D, which are resistant to mild base (data not shown), suggesting that they represent abnormal IPCs. Among them, the ones marked with a square and an x (Fig. 10, lanes 3 and 4) migrate as expected for IPCs having fatty acids that are shorter than C26.

Previous studies had shown that W303 lac1Δlag1Δ accumulated very high amounts of some kind of ceramide, despite its lack of the acyl-CoA-dependent ceramide synthase (5). Here we used metabolic labeling with [3H]serine to detect ceramides. As shown in Fig. 11, YPK9.2Δ.C1 and YPK9.2Δ.C4 accumulated the same amounts of abnormal ceramides as...
Lag1p-depleted YPK9.2ΔLAG1. This suggests that YPK9.2ΔC1 and YPK9.2ΔC4 maintain abnormal modes of ceramide synthesis despite the presence of the human LAG1 homologues.

To measure ceramide synthase activity of CLN8 and LASS2 in vitro in microsomes of Lag1p-depleted YPK9.2ΔLAG1, we placed CLN8 and LASS2 behind the strong TPI1 promoter on a multicopy vector. YPK9.2ΔLAG1 cells overexpressing CLN8 and LASS2 from these vectors, started to grow on glucose, but the cells could absolutely not grow on FOA, demonstrating that the CLN8 promoter (Fig. 8), CLN8 and LASS2 overexpression did not restore viability to YPK9.2A. The microsomes made from these cells, grown on galactose or glucose, made the ceramides that are observed in microsomes from YPK9.2ΔLAG1 grown on galactose, but no other novel ceramides. We suspect that the simultaneous presence of the centromeric pBM150 vector and the TPI1,UAS-CLN8 vector may lead to recombination events, thus multiplying the copy number of yeast CLN8 genes so that the cells express sufficient amounts of Lag1p for normal ceramide biosynthesis even on glucose. However, it still remains a possibility that the presence of CLN8 or LASS2 would allow YPK9.2ΔLAG1 cells to survive with much lower amounts of Lag1p than are normally required. This possibility is presently being investigated further. Altogether, the results strongly argue that CLN8 and LASS2 cannot induce a significant ceramide synthase activity on their own.

**DISCUSSION**

Our studies on the specificity of ceramide synthase clearly indicate a distinct preference of the yeast ceramide synthase for C26-CoA, and this is true for both Lag1p- and Lac1p-dependent activities. Human clones, in contrast, seem to have different preferences, with LAG1Hs preferring C26-CoA, clone 1 preferring C24-CoA, and clone 4 preferring C24-CoA and C20-CoA. Most human ceramides and sphingolipids contain C18 or C24 fatty acids (24), but ceramides made by keratinocytes contain mainly C30 and C32 (25, 26). In microsomal ceramide synthase assays using rat microsomes, it was found that C24-CoA and C18-CoA did not compete with each other and that the ratio of C18-CoA over C24-CoA-dependent ceramide synthase activity was very different in different tissues and cell types (27). This led to the postulate that mammals contain several ceramide synthases of different specificity (27). The different specificities of LAG1Hs, clone 1, and clone 4 seem to lend some support to this notion. In this respect, the preference of LAG1Hs for C26-CoA may be surprising as overexpression of UOG1, a close mouse homologue of LAG1Hs was shown to stimulate incorporation of stearoyl-CoA but not palmitoyl-CoA into ceramides in vitro and in vivo (9). It is quite possible, however, that the specific increase of C18-ceramide induced by UOG1 does not reflect the specificity of the ceramide synthases of the cell but is observed because only limited amounts of longer chain acyl-CoAs are available in these cells. It also will be interesting to test the effect of UOG1 expression on microsomal assays in greater detail because it is conceivable that in such assays C26-CoA would be an even better substrate than C18-CoA.

If LAG1 were a mere regulator of a catalytic subunit of yeast ceramide synthase, its replacement by human LAG1 homologues would not be expected to induce a ceramide synthase activity of different acyl-specificity. To us, the most likely interpretation of the different specificity profiles induced by different LAG1 homologues is to assume that Lag1p itself is the catalytic subunit of the acyl-CoA-dependent ceramide synthases. However, other possibilities cannot lightly be discarded. The microsomal in vitro assay is rather complex, and specificity changes may be brought about by parameters that do not directly depend on the transfected LAG1, but rather the general state of cells and in particular the membrane environment of the ceramide synthase. This environment may be changed because the partial lack of ceramides induces adaptive changes in lipid homeostasis of the membrane, e.g. the increase in glycerophospholipids with C26 fatty acids (5).

There are four human LAG1 homologues having a transmembrane domain followed by a HOX domain, and it has been suggested that they may act as ceramide-regulated transcription factors (20). Their sequence indeed predicts the HOX domain to be on the cytosolic side of the membrane and an interaction of clones 1 or 4 with DNA thus can be envisaged (Fig. 7, A and B). It is clear, however, that the role of some of these proteins is not only to act as sensors but also to be capable of inducing ceramide synthase activity.

Of the human homologues tested here, CLN8 and LAG1Hs
contain a classical KKXX or KKKX ER retrieval signal, whereas clone 1 and LASS2 contain a somewhat degenerate signal (RKND) at their C terminus and clone 4 has no such retrieval signal. Nevertheless, clones 1 and 4 efficiently restore sphingolipid biosynthesis to the cells, and this was not dependent on strong expression of these genes, as YPK9.2.

Thus, clone 1 and 4 proteins may not exit the ER efficiently, or, if they do, the ceramides they make may reach the relevant compartments by lipid traffic.

The negative results obtained with CLN8 cannot be considered as definitive evidence against a potential ceramide synthase activity of the product of this gene. It maybe that the Cln8p simply is not properly folded, or that it needs acyl-CoAs for activity, or that some regulatory modification, which induces the activity, does not take place in yeast. It also may be that CLN8 indeed allows for the synthesis of small amounts of ceramides in yeast, which however are not of the right kind to restore cell viability and are not made in substantial amounts in vitro. This possibility is presently under further investigation.

S. cerevisiae seems to be a convenient organism and YPK9.2ΔLAG1 an especially useful strain to assay ceramide synthase activity and specificity of candidate ceramide synthase genes. This particular strain is of particular value because, when maintained on galactose, it does not accumulate suppressors that induce alternative ways of ceramide biosynthesis that may obscure the effects of transfected genes.

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