LASS5 Is a \textit{Bona Fide} Dihydroceramide Synthase That Selectively Utilizes Palmitoyl-CoA as Acyl Donor\textsuperscript{*}

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Sujoy Lahiri and Anthony H. Futerman

From the Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel

We demonstrated recently (Riebeling, C., Allegood, J.C., Wang, E., Merrill, A. H. Jr., and Futerman, A. H. (2003) \textit{J. Biol. Chem.} 278, 43452–43459) that upon over-expression in human embryonic kidney cells, longevity assurance gene homolog 5 (LASS5, previously named \textit{TRH4}) elevates the synthesis of (dihydro)ceramides selectively enriched in palmitic acid. To determine whether LASS5 is a \textit{bona fide} dihydroceramide synthase or, alternatively, whether it modifies an endogenous dihydroceramide synthase, we over-expressed LASS5 with a hemagglutinin (HA) tag at the C terminus, solubilized it using digitonin, and purified it by immunoprecipitation. Solubilized LASS5-HA displays the same fatty acid selectivity as the membrane-bound enzyme. After elution from agarose beads, only one band could be detected by SDS-PAGE, and its identity was confirmed to be LASS5 by mass spectrometry. Dihydroceramide synthase activity of the eluted LASS5-HA protein was totally dependent on exogenously added phospholipids. Moreover, eluted LASS5-HA was highly selective toward palmitoyl-CoA as acyl donor and was inhibited by the (dihydro)ceramide synthase inhibitor, fumonisin B1. This study identifies LASS5 as a genuine dihydroceramide synthase and demonstrates that mammalian dihydroceramide synthases do not require additional subunits for their activity.

Ceramide, an important lipid second messenger (1–3), consists of a sphingoid long chain base to which a fatty acid is attached at carbon-2 via an amide bond. Ceramide is also a key metabolite in the pathway of sphingolipid (SL)\textsuperscript{2} biosynthesis (4), and within the past 2–3 years the molecular identities of most of the enzymes in this pathway have been identified (5). Among these, a family of mammalian genes that regulates ceramide synthesis has been discovered (6, 7). Surprisingly, over-expression of \textit{LASS1} leads to an increase in ceramides containing different fatty acids (8–10). Thus, over-expression of \textit{LASS1} leads to an increase in the synthesis of ceramides containing stearic acid (8), whereas over-expression of \textit{LASS5} leads to an increase of ceramide containing palmitic acid (9). However, it is not known whether these genes modify an endogenous ceramide synthase activity, and thereby confer fatty acid selectivity, or whether the LASS proteins themselves are \textit{bona fide} dihydroceramide synthases.

We have now purified, by immunoprecipitation, LASS5 with an HA tag at the C terminus and demonstrate here that it is a genuine dihydroceramide synthase that displays the same fatty acid selectivity as seen upon its over-expression in mammalian cells. This is the first biochemical isolation of a mammalian dihydroceramide synthase and paves the way for studying the role of LASS proteins in regulating ceramide production both for SL biosynthesis and for the regulation of the defined aspects of cell physiology in which it plays vital roles.

\section*{EXPERIMENTAL PROCEDURES}

Materials—1-[\textsuperscript{14}C]Palmitoyl-CoA (specific activity, 60 mCi/mmol) was from Amersham Biosciences, and 1-[\textsuperscript{14}C] stearoyl-CoA (specific activity, 55 mCi/mmol) was from American Radiolabeled Chemicals (St. Louis, MO). Fumonisin B1, defatted bovine serum albumin, phenylmethylsulfonyl fluoride, leupeptin, antipain, and aprotinin were from Sigma. Sphinganine, sphingosine, palmitoylsphingosine, and stearoyl-sphingosine were from Matreya (Pleasant Gap, PA). Digitonin was from Sigma or Calbiochem. A monoclonal anti-HA-agarose conjugate (clone HA-7) was from Sigma, a rabbit polyclonal anti-HA antibody (Y-11) and protein A-agarose were from Santa Cruz Biotechnology (Santa Cruz, CA), and peroxidase-conjugated AffiniPure goat anti-mouse IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA). Dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylserine (DOPS) were from Avanti Polar Lipids (Alabaster, AL). Silica gel 60 TLC plates were from Merck. All solvents were of analytical grade and were purchased from Biolab (Jerusalem, Israel).

Cell Culture and Transfection—Human embryonic kidney 293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 \mu g/ml streptomycin. Transfections using \textit{LASS5-HA} (9) were performed by the calcium phosphate method. Transfections were also performed with pcDNA-HA as a control.

Solubilization of \textit{LASS5-HA}—293T cells, at ~90% confluency, were washed with phosphate-buffered saline and removed from culture dishes using trypsin (0.05%, w/v). After centrifugation (4 min, 150 \times g\textsubscript{av}) cell pellets were washed twice with phosphate-buffered saline by centrifugation (4 min, 150 \times g\textsubscript{av}) and then homogenized in 20 \mu l HEPES-KOH, pH 7.4, 25 \mu M KCl, 250 \mu M sucrose, and 2 \mu M MgCl\textsubscript{2} containing protease inhibitors (1 \mu M phenylmethylsulfonyl fluoride, 1 \mu g/ml leupeptin, 1 \mu g/ml antipain, and 100 kallikrein-inactivating units/ml aprotinin). Digitonin was added to the homogenate to give a final concentration of 1% (w/v), and after 1 h at 4 °C, the homogenate was centrifuged at 100,000 \times g\textsubscript{av} for 30 min. The resulting supernatant was used for subsequent purification steps.

Immunoprecipitation of \textit{LASS5-HA}—The digitonin-solubilized supernatant was concentrated using an iCON concentrator (Pierce) and then incubated with protein A-agarose for 1 h at 4 °C to reduce nonspecific binding. After removal of protein A-agarose by centrifugation, the supernatant was incubated with an anti-HA agarose conjugate overnight at 4 °C. The conjugate was then pelleted by centrifugation and washed with the same HEPES buffer used for homogenization followed by a wash with 1 \textit{M} NaCl. \textit{LASS5-HA} was eluted from the beads using 100 \mu l glycine, pH 2.5, and immediately neutralized using 1 \textit{M} Trizma
Purified LASS5 Is a Bona Fide Dihydroceramide Synthase

(Tris base), pH 11. The eluate was concentrated using an iCON concentrator, and in some cases, DOPC or DOPS liposomes prepared as described (11), were added to the concentrated eluate prior to analysis of dihydroceramide synthase activity. The composition of the eluate was analyzed by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide gel) and Western blotting using a rabbit polyclonal anti-HA antibody. Control immunoprecipitations were performed using cells that had been transfected with pcDNA-HA.

Mass Spectrometry—Protein bands were excised from the SDS gel and subsequently reduced, alkylated, and in-gel-digested for 18 h with bovine trypsin (sequencing grade, Roche Diagnostics) at a concentration of 12.5 ng/μl in 50 mM ammonium bicarbonate at 37 °C. An extracted peptide solution was dried for subsequent matrix-assisted laser desorption/time of flight ionization (MALDI-TOF) and electrospray ionization mass spectrometric analyses.

Aliquots of the extracted peptide mixture and electrophoresed proteins, dissolved in 0.1% trifluoroacetic acid or a mixture of formic acid/isopropanol/H2O (1/3/2; v/v/v), were used for MALDI-TOF mass spectrometry. Lipids were visualized using chloroform/methanol/2M ammonium hydroxide (40/10/1; v/v/v) as the developing solvent and using palmitoyl- sphinganine and 20:4 sphingosine as standards. Lipids were visualized using a rabbit polyclonal anti-HA antibody. Control immunoprecipitations were performed using cells that had been transfected using pcDNA-HA.

RESULTS

Initial experiments were performed to establish a means to solubilize membrane-bound LASS5-HA. Digitonin, which was previously used to solubilize Lag1p (13), a yeast LASS homolog (14, 15), was able to solubilize LASS5-HA and preserve dihydroceramide synthase activity. Most of the dihydroceramide synthase activity was recovered in the digitonin-solubilized supernatant, which correlated with levels of LASS5-HA detected by Western blotting (Fig. 1A). Digitonin-solubilized LASS5-HA displayed the same fatty acid specificity as membrane-bound LASS5, showing an ∼10-fold higher activity toward palmitoyl-CoA than toward stearoyl-CoA (Fig. 1B), and was inhibited by the (dihydro)ceramide synthase inhibitor fumonisin B1 (16) to a similar extent as observed in the homogenate (Fig. 1B).

Digitonin-solubilized LASS5-HA was subsequently immunoprecipitated using an anti-HA agarose conjugate and eluted using 100 mM glycine. A significant fraction of the immunoprecipitated LASS5-HA could be eluted from the beads (Fig. 2A), and analysis by SDS-polyacrylamide gel electrophoresis revealed only one band, with a molecular mass of ∼48 kDa (Fig. 2B), similar to the predicted molecular mass of LASS5-HA. No other bands could be detected reproducibly either by silver staining of the gel (Fig. 2B) or by Coomassie staining (not shown). The identity of the band as LASS5-HA was confirmed by Western blotting (Fig. 2C) and by MALDI-TOF mass spectrometry (TABLE ONE).

In addition, no other peptides co-migrated with LASS5 (TABLE ONE), which was confirmed by nano-liquid chromatography electrospray ionization tandem mass spectrometry (not shown).

Eluted LASS5-HA did not show any dihydroceramide synthase activity unless phospholipids were added to the eluate. Of the two phospho-
lipids tested, DOPC was more efficient than DOPS at preserving dihy-
roceramide synthase activity (Fig. 3A). Moreover, eluted LASS5-HA
was highly selective toward palmitoyl-CoA compared with stearoyl-
CoA (Fig. 3B), could be inhibited by fumonisin B1, and was able to
acylate both sphinganine and sphingosine (Fig. 3C).3

DISSCUSSION

In the current study we have demonstrated that LASS5 is a bona fide
(dihydro)ceramide synthase. This is the first time that a mammalian
dihydroceramide synthase has been purified, representing one of the
last recalcitrant enzymes in the SL biosynthetic pathway (5).

In contrast to yeast, in which SLs contain only one kind of fatty acid,
namely C26, mammalian (dihydro)ceramides contain a wide fatty acid
spectrum (2, 4). It was formerly assumed that this was due to a lack of
specificity of dihydroceramide synthase with respect to the use of fatty-
acyl-CoAs. However, the discovery of a family of mammalian LASS
genomes that each synthesizes dihydroceramides containing different fatty
acids (at least those characterized to date (8–10)) demonstrates that this
is not the case. In contrast, yeast have only two highly homologous
ceramide synthases, LAG1 and LAC1, which together are responsi-
ble for the synthesis of C26-ceramides.

Evidence is accumulating that ceramides containing specific fatty
acids are involved in defined cell functions in mammalian cells (2,
17). This being the case, it might be expected that individual LASS
genes would be expressed in either a tissue-specific (9, 18) or tem-
poral manner so as to supply specific ceramides for the distinct
events in which they are involved. Our demonstration that LASS5 is
a bona fide dihydroceramide synthase that selectively utilizes palmi-
toyl-CoA supports this likelihood and strengthens the hypothesis
that specific LASS proteins play distinct roles either in ceramide

3 We attempted to determine the extent of recovery and -fold purification of LASS5 from
the diglucosin-solubilized supernatant. However, because of the low amounts of puri-
ified LASS5 protein and the inherent difficulties in estimating the amount of protein
after silver staining of the SDS-gel, we can only provide estimates of these values. In
three separate experiments, the -fold increase in specific activity of the immunopreci-
cipitated protein compared with the diglucosin-solubilized supernatant varied
between 5,000 and 12,000, and the recovery of LASS5 activity (toward 1-[14C]palmit-
toyl-CoA as substrate) varied between 26 and 49%. These values are probably under-
estimates because we have not systematically determined the conditions for optimal
activity of purified LASS5.

signaling or in SL metabolism. The evidence for the latter has already
been obtained by the observation that C18-ceramide formed by
LASS1 is selectively channeled into neutral glyco-SLs but not gan-
gliosides. The availability of purified LASS5 will permit systematic
characterization of the reaction mechanism and modes of regulation
of a mammalian dihydroceramide synthase (19).

The yeast ceramide synthase, Lag1p/Lac1p, was recently purified, and
was shown to co-immunoprecipitate with an additional subunit, Lip1p
(13), which was absolutely required for yeast ceramide synthesis, at least
using C26-fatty acids. No mammalian homologs of Lip1p are found by data
base searches, and our demonstration that no other protein is required for
the dihydroceramide synthase activity of purified LASS5 indicates that LASS5 by
itself is sufficient for catalytic activity in mammalian cells. However, the
dihydroceramide synthase activity of LASS5 is totally dependent on exog-
enaously added phospholipids, whereas no such requirement was observed
for yeast Lag1p/Lac1p (17). Together, these data suggest a unique role for
Lip1p in yeast that is not required in mammalian cells, and suggest that the
regulation of mammalian (dihydro)ceramide synthase may differ signifi-
cantly from that in yeast.

### Table One

|MALDI-TOF MS identification of LASS5|
|---|
|Experimentally determined values and the corresponding theoretical masses of tryptic peptides are listed; data base analysis confirmed the protein as LASS5 (AAH46797). Mass accuracy (ppm) and amino acid sequences are shown for each peptide.|
|Measured mass | Theoretical mass | Mass accuracy | Position in the sequence | Peptide sequence |
|---|---|---|---|---|
|694.342 | 694.347 | ~500 | 62–66 | MLFER|
|710.342 | 710.342 | 0 | 62–66 | MosxFER|
|902.462 | 902.461 | +1 | 114–120 | QLDWSVR|
|908.432 | 908.432 | 0 | 122–127 | IQCWF|
|914.472 | 914.472 | 0 | 398–405 | ATGFLHFR|
|1030.462 | 1030.556 | ~94 | 114–121 | QLDWSVRK|
|1014.402 | 1014.405 | ~3 | 141–147 | FCESMoxWR|
|1036.522 | 1036.527 | ~5 | 121–127 | KIQCF|
|1074.592 | 1074.600 | ~8 | 67–75 | FIAKPCALR|
|1140.582 | 1140.613 | ~31 | 131–140 | NQDKKTLT|
|1296.672 | 1296.714 | ~42 | 130–140 | RNNQDKPTLTK|
|1410.652 | 1410.739 | ~87 | 95–106 | VFVSTKYDEK|
|1566.742 | 1566.840 | ~98 | 95–107 | VFVSTKYDEK|
|1683.762 | 1683.831 | ~69 | 80–94 | DSPNVKVPNDTLEK|
|1773.722 | 1773.793 | ~71 | 173–185 | CWYNYPQPLSR|
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