Identification and Characterization of a Sphingolipid Δ4-Desaturase Family*

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Sphingolipids desaturated at the Δ4-position are important signaling molecules in many eukaryotic organisms, including mammals. In a bioinformatics approach, we now identified a new family of protein sequences from animals, plants, and fungi and characterized these sequences biochemically by expression in *Saccharomyces cerevisiae*. This resulted in the identification of the enzyme sphingolipid Δ4-desaturase (dihydroceramide desaturase) from *Homo sapiens*, *Mus musculus*, *Drosophila melanogaster*, and *Candida albicans*. This resulted in the identification of the enzyme sphingolipid Δ4-desaturase/C-4-hydroxylase from *M. musculus*. Among the sequences investigated are the *Homo sapiens* membrane lipid desaturase, the *M. musculus* degenerative spermatocyte, and the *Drosophila melanogaster* degenerative spermatocyte proteins. During spermatogenesis, but not oogenesis of *des* mutant flies, both cell cycle and spermatid differentiation are specifically blocked at the entry into the first meiotic division, leading to male sterility. This mutant phenotype can be restored to wild-type by complementation with a functional copy of the *des* gene (Endo, K., Akiyama, T., Kobayashi, S., and Okada, M. (1996) Mol. Gen. Genet. 253, 157–165). These results suggest that Δ4-desaturated sphingolipids provide an early signal necessary to trigger the entry into both meiotic and spermatid differentiation pathways during *Drosophila* spermatogenesis.

In eukaryotic cells, sphingolipids have recently become a focus of interest, because they are emerging as an important class of messenger molecules linked to many different cellular functions (1–6). Their chemical structure differs from the more commonly known glycerolipids in having a long-chain amino alcohol, the sphingoid base (Fig. 1), as a backbone. Sphingolipid biosynthesis starts in the endoplasmic reticulum from serine and a palmitic or stearic acid coenzyme A-thioester, which are condensed and subsequently reduced to form a ceramide base. The free sphingoid base is then N-acylated to form a ceramide. In a further step, a polar head group is added onto the primary hydroxy group of the ceramide to give a variety of complex sphingolipids such as cerebrosides, sphingomyelin, and phytylglycolipids. A significant proportion of these sphingolipids is finally found in the outer leaflet of the plasma membrane, where they have both structural and signaling functions (1).

In mammalian cells, sphingolipid-derived messengers, in particular ceramide and phosphorylated sphingoid bases, control activities such as cellular proliferation, differentiation, and motion (2), as well as cell cycle arrest and apoptosis (3). Ceramide can be generated either by *de novo* synthesis as described above or by hydrolysis of complex sphingolipids. Once released, ceramides may be hydrolyzed to free sphingoid base and fatty acid, which themselves give rise to messenger molecules such as phosphorylated sphingoid bases. In addition, free and phosphorylated sphingoid bases can also be generated by *de novo* synthesis.

Important postsynthetic modifications of sphingolipids are desaturation at the Δ4-position and hydroxylation at the C-4-position of the sphinganine, so that they contain (E)-sphing-4-enine or (E)-4-hydroxysphinganine (Fig. 1). In mammals, the (E)-Δ4-double bond contributes to the second messenger activity of ceramide (7). It is introduced by the enzyme sphingolipid Δ4-desaturase (8). This enzyme is frequently called dihydroceramide desaturase in mammals, because its activity is highest with dihydroceramide as substrate (9). The C-4-hydroxy group is introduced by the enzyme sphingolipid C-4-hydroxylase, which is encoded by the yeast *SUR2* gene and its plant homologues (10–12).

In the course of identifying enzymes involved in sphingolipid biosynthesis (12–14), we were interested in cloning the sphingolipid Δ4-desaturase. Its biochemical characteristics, for example, the requirement of NAD(P)H and O2 as cofactors (9), are typical for membrane-bound desaturases and hydroxylases. These enzymes belong to a large superfamily defined by three characteristic sequence motifs, the histidine boxes HX₃₋₅H, HX₃₋₅HH, and (H/Q)X₃₋₅HH (15). Members of this superfamily introduce double bonds or hydroxy groups into many different lipid substrates, including fatty acyl groups, sphingoid bases, and sterols (16). We therefore assumed the amino acid sequence of sphingolipid Δ4-desaturase to be similar to that of other known desaturases or hydroxylases which contain the histidine box sequence motifs.

In a first approach, we expected the sequence of this enzyme to be similar to the *Saccharomyces cerevisiae* sphingolipid C-4-hydroxylase *Sur2p* (10, 11), because desaturases and hydroxylases of identical regioselectivity and substrate specificity were believed to be very similar at the amino acid sequence level (16, 17). However, cloning and expression of sequences similar to
S. cerevisiae Sur2p resulted in the discovery of sphinganine C4-hydroxylases from plants (12), but not of the unknown Δ4-desaturase. This indicated that the sphingolipid Δ4-desaturase and Sur2p were not as similar as expected.

We now employed a bioinformatics strategy to identify the protein sequence of the sphingolipid Δ4-desaturase. Using PSI-blast (18), an extensive collection of sequences characterized by the histidine box motifs was assembled and subsequently grouped into families according to sequence similarity. After identifying a new family with candidate sequences from animals, plants, and fungi, which will be called the DES family, several of these sequences were cloned and biochemically characterized by expression in S. cerevisiae. By this strategy, the first sphingolipid Δ4-desaturases, as well as a bifunctional sphingolipid Δ4-desaturase/C4-hydroxylase were identified.

Among the sphingolipid Δ4-desaturases investigated are the Drosophila melanogaster degenerative spermatocyte (des-1) (19), the Mus musculus degenerative spermatocyte (MDES) (20), and the Homo sapiens membrane lipid desaturase (MLD) (21) proteins. A considerable amount of information about these proteins is already present in the literature, generated without knowing their biochemical activity. Their identification as sphingolipid Δ4-desaturases now allows assignment of unexpected cellular functions to Δ4-desaturated sphingolipids.

We propose that Δ4-desaturated sphingolipids are involved in cell cycle control during Drosophila spermatogenesis.

**EXPERIMENTAL PROCEDURES**

**Bioinformatics**

*Initial PSI-Blast Searches*—The NCBIs non-redundant protein sequence data base (www.ncbi.nlm.nih.gov) was searched using PSI-blast (18) with the S. cerevisiae Sur2p (10, 11) and Arabidopsis thaliana.

1 Typographic rules for genes and their products differ among the species indicated in this study. Particularly in Drosophila, genes named after a recessive mutant phenotype, as well as the mutant itself, are typeset in lowercase Italicics. Any item in italics should therefore be considered a gene, messenger RNA, or complementary DNA, except when it is evident from the context that it refers to a mutant. Any item in Roman characters should be considered a protein.

2 The abbreviations used are: des-1, Drosophila melanogaster degenerative spermatocyte; ANS, 8-anilino-1-naphthalene sulfonic acid; MDES, M. musculus degenerative spermatocyte; MLD, H. sapiens membrane lipid desaturase; DNP, 2,4-dinitrophenyl; HPLC, high performance liquid chromatography; Ascan optical density at 600 nm; EGF, epidermal growth factor; contig, group of overlapping clones.

**Identification of a Sphingolipid Δ4-Desaturase Family**

Complete open reading frames were amplified from the cDNA clones indicated in the previous section (H. sapiens, M. musculus, D. melanogaster, and L. esculentum) or from genomic DNA (C. albicans strain CA14) by PCR with specific primers using proofreading Pfu Turbo DNA polymerase (Strategene). The primer sequences were: CGCGGATCCATGGGAATTTGAAGGGACTTTCG and GCTCTAGACTATTCGGACTTGTTTGCTT (DES) (21), CGCGGATCCATGGGATTTGAAGGGACTTTCG and GCTCTAGACTATTCGGACTTGTTTGCTT (M. musculus Des1), CGCGGATCCATGGGATTTGAAGGGACTTTCG and GCTCTAGACTATTCGGACTTGTTTGCTT (M. musculus Des2), CGCGGATCCATGGGATTTGAAGGGACTTTCG and GCTCTAGACTATTCGGACTTGTTTGCTT (C. albicans Des1), CGCGGATCCATGGGATTTGAAGGGACTTTCG and GCTCTAGACTATTCGGACTTGTTTGCTT (C. albicans Des2). The primers included adapter sequences containing the restriction sites BglII (forward primer) or KpnI and XbaI and were generated containing Des1p from C. albicans and open reading frame consisting of bases 17916–16535 of contig 9a58 encoding a Des1p homologue from N. crassa were identified. Sequence data for C. albicans were obtained from the Stanford Genome Technology Center website at sequence-www.stanford.edu/group/candida. Sequence data for C. crassa were obtained from the Neurospora Genome Project website at mips.gsf.de/ndrg/neurospora.

**Cloning**

SLD1 (13) protein sequences (GenBank™ protein accession NP_010583 and T47950) as queries. The search yielded two non-overlapping sets including a total of 397 sequences containing the three histidine box motifs. These sequences were grouped into families by manual inspection of multiple sequence alignments and phylogenetic trees generated with ClustalX (22). Putative biochemical functions were assigned to each family based on experimentally characterized member sequences. The new DES family was identified as described under “Results,” and initially consisted of 7 sequences from H. sapiens (DES1, GenBank™ protein accession NP_003667), M. musculus (DES1, NP_031879), D. melanogaster (DES1, CA63889), C. elegans (NP_493549 and NP_501256), A. thaliana (AAD17540), and Schizosaccharomyces pombe (T40335).

**Identification of Additional DES Homologues**—The TIGR Gene Indices (www.tigr.org/db) were searched with tblastn (18) for tentative consensus sequences with high similarity to the DES family at the protein level. Tentative consensus sequences corresponding to the DES family members identified in the PSI-blast searches (H. sapiens DES1, M. musculus DES1, and D. melanogaster DES-1) as well as tentative consensus sequences encoding new family members (DES2 homologue from H. sapiens, M. musculus DES2, and the DES homologue from Lycomersis esculentum) were found. Corresponding cDNA clones were sequenced and their nucleotide sequences were submitted to the GenBank™/EBI Data Bank. The sequence of I.M.A.G.E. Consortium cDNA clone cLET2D1 from the NSF Tomato Genome Project cDNA clone LP11871 (24) was obtained from Research Genetics. The cDNA clone cLET2D1 from the NSF Tomato Genome Project was supplied by the Clemson University Genomics Institute.

To find additional DES homologues from fungi, the Candida albicans and Neurospora crassa genomic sequences were searched with tblastn (18) for open reading frames showing high similarity to the DES family at the protein level. One open reading frame consisting of bases 7499–8619 of contig 6–2340 encoding Des1p from C. albicans and open reading frame consisting of bases 17916–16535 of contig 9a58 encoding a Des1p homologue from N. crassa were identified. Sequence data for C. albicans were obtained from the Stanford Genome Technology Center website at sequence-www.stanford.edu/group/candida. Sequence data for N. crassa were obtained from the Neurospora Genome Project website at mips.gsf.de/proj/neurospora.
carrying the open reading frames from \textit{C. albicans} and \textit{L. esculentum} were checked by sequencing. The insert sequence encoding the DES homologue from \textit{L. esculentum} was identical to the cDNA sequence. The insert sequence encoding \textit{C. albicans} Des1p differed in a single nucleotide (G or C, at position 985) from the sequence determined by the Stanford Genome Technology Center, but this had no effect on the predicted protein sequence. \textit{C. albicans} strain SC5314 was sequenced at the Stanford Genome Technology Center, whereas strain CAI4 was used in this study.

**Yeast Cultures**

Precultures of transformed \textit{sur}Δ2 cells were grown aerobically at 30°C in complete minimal uracil dropout medium (25) containing 2% (w/v) glucose. To induce expression under control of the \textit{GAL1} promoter, cells were harvested and transferred to complete minimal uracil dropout medium (25) containing 2% (w/v) raflinose and 2% (w/v) galactose as carbon sources. Cultures were grown aerobically at 25°C for 70 h (final \(A_{600} = 2.5-3\)), subjected to heat shock at 37°C for 90 min, and harvested by centrifugation. The heat shock treatment was performed to increase ceramide levels as observed in several studies (4, 5).

**Sphingoid Base Analysis**

HPLC Analysis—Analysis of the sphingoid base composition was performed as described in Ref. 13 with some optimizations. Yeast cells (570–520 mg fresh weight) were harvested by centrifugation, washed with \(\text{H}_2\text{O}\), and hydrolyzed directly with \(\sim 10\%\) (w/v) \(\text{Ba(OH)}_2\) in 3 ml of 1,4-dioxane/\(\text{H}_2\text{O}\) 1:1 (v/v) (20 h at 110 °C) (26). The free sphingoid bases were extracted by phase partitioning with CHCl\(_3\)/1,4-dioxane/\(\text{H}_2\text{O}\), 8:5:8 (v/v/v). The organic phase was washed with an equal volume of 0.1 M KOH + 0.5 M KCl. The sphingoid bases were converted to their DNP derivatives with 0.2 ml of 0.5% (w/v) methanolic 1-flouro-2,4-dinitrobenzene and 0.8 ml of 2 M boric acid/KOH, pH 10.5 (30 min at 60 °C) (27), extracted by phase partitioning with CHCl\(_3\)/methanol/\(\text{H}_2\text{O}\), 2:1:1 (v/v/v), and dissolved in 0.5 ml of methanol. Analysis by reverse-phase HPLC was performed on a Multospher RP18—MS 2.5 × 250 × 4-mm column (Cromatographie) with a flow rate of 0.8 ml/min and a concave gradient from 84 to 100% methanol/acetonitrile/2-propanol, 10:3:1 (v/v/v), against \(\text{H}_2\text{O}\) in 55 min. The elution was monitored with a UV detector (ThermoQuest) at 350 nm.

For NMR analysis, \textit{S. cerevisiae} sur2Δ cells (12 g fresh weight) expressing \textit{C. albicans} Des1p were hydrolyzed in a total reaction volume of 120 ml (24 h under reflux) as described above. The free saturated and desaturated sphingoid bases were separated by TLC on Silica Gel 60 plates in CHCl\(_3\)/methanol, 9:1 (v/v), followed by phase partitioning with CHCl\(_3\)/methanol/0.1 M KOH, 2:1:1 (v/v/v), and dissolved in 0.5 ml of methanol. Analysis by reverse-phase HPLC was performed on a Multospher RP18—MS 2.5 × 250 × 4-mm column (Cromatographie) with a flow rate of 0.8 ml/min and a concave gradient from 84 to 100% methanol/acetonitrile/2-propanol, 10:3:1 (v/v/v), against \(\text{H}_2\text{O}\) in 55 min. The elution was monitored with a UV detector (ThermoQuest) at 350 nm.

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**RESULTS**

**Identification of a Putative Sphingolipid Δ4-Desaturase Family with Members from Animals, Plants, and Fungi**—We employed a bioinformatics strategy to identify candidate sequences for sphingolipid Δ4-desaturases. Using PSI-blast (18) with the \textit{s. cerevisiae} sphingolipid C4-hydroxylase Sur2p (10, 11) and the \textit{A. thaliana} sphingolipid Δ8-desaturase SLD1 (13) protein sequences as queries, we assembled an extensive collection of sequences showing the histidine box sequence motifs characteristic for membrane-bound desaturases and hydroxylases. The final collection of 397 sequences from various organisms was grouped into families as described under “Experimental Procedures,” and each family was assigned a putative biochemical function based on experimentally characterized member sequences (Fig. 2). A candidate family for sphingolipid Δ4-desaturases was identified based on the following criteria. 1) The family should consist exclusively of sequences with so far unknown biochemical function. 2) It should contain sequences from animals, plants, and fungi, because Δ4-desaturated sphingolipids have been found in all these organisms. 3) The family should not contain sequences from \textit{S. cerevisiae}, because this is one of the few eukaryotic organisms lacking Δ4-desaturated sphingolipids.

One family with sequences from \textit{H. sapiens}, \textit{M. musculus}, \textit{Rattus norvegicus}, \textit{D. melanogaster}, \textit{Caenorhabditis elegans} (animals), \textit{A. thaliana}, \textit{Lycopersicon esculentum} (plants), \textit{S. pombe}, \textit{C. albicans}, \textit{N. crassa} (fungi), and \textit{Toxoplasma gondii} conformed to all three criteria (Fig. 2, red branch, and Fig. 3).

Three of these sequences, DES-1 from \textit{D. melanogaster}, MDES from \textit{M. musculus}, and MLD from \textit{H. sapiens}, have previously been studied without knowing their biochemical function (19–21). To unify nomenclature, we will call this new family the DES family and refer to mouse MDES and human MLD both as DES1, to the second mouse homologue as DES2, and to the DES homologue from \textit{C. albicans} as Des1p.4 There is also a DES2 homologue in \textit{H. sapiens}, but because we could not obtain its full-length sequence, we did not characterize it further.

The DES Homologues from Animals and Fungi Show Sphingolipid Δ4-Desaturase Activity—To investigate if the sequences in the DES family in fact encode sphingolipid Δ4-desaturases, the complete open reading frames coding for \textit{H. sapiens} DES1, \textit{M. musculus} DES1 and DES2, \textit{D. melanogaster} DES-1, the DES homologue from \textit{L. esculentum}, and \textit{C. albicans} Des1p were cloned into the yeast expression vector pYES2 under the control of the inducible \textit{GAL1} promoter. The resulting plasmids were used to transform the \textit{S. cerevisiae} sur2Δ mutant (10). In this mutant, the sphingolipid C4-hydroxylase gene \textit{SUR2} (10, 11) is disrupted, so that sphinganine is the only sphingoid base found in the sphingolipids. It was expected that these sphinganine-containing sphingolipids would be suitable as substrates for Δ4-desaturation. Expression of \textit{H. sapiens} DES1, \textit{M. musculus} DES1 and DES2, \textit{D. melanogaster} DES-1, and \textit{C. albicans} Des1p indeed resulted in the formation of sphingolipids containing the Δ4-desaturated sphingoid base (E)-sphing-4-ene (\textit{H. sapiens} DES1, 2.1 mol %; \textit{M. musculus} DES1, 0.2 mol %; \textit{M. musculus} DES2, 0.7 mol %; \textit{D. melanogaster} DES-1, 5.4 mol %; \textit{C. albicans} Des1p, 6.3 mol %) (Fig. 4). In surΔ2 cells expressing the DES homologue from \textit{L. esculentum} and in cells transformed with the empty vector pYES2 (control), no (E)-sphing-4-ene could be detected. In summary, \textit{H. sapiens} DES1, \textit{M. musculus} DES1 and DES2, \textit{D. melanogaster} DES-1, and \textit{C. albicans} Des1p show sphingolipid Δ4-desaturase activity, whereas no such activity could be detected with the DES homologue from \textit{L. esculentum} under these experimental conditions.

The identity of the sphingoid bases indicated in Fig. 4 was confirmed by HPLC/MS. In the negative ion mode, peptidomolecular ions with \((m/z = M_\text{r} – 1)\) corresponding to the DNP

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4 Yeast nomenclature requires gene and protein names consisting of tree letters followed by a number. The number in \textit{C. albicans} Des1p does not imply that it is a mouse or human DES1 homologue. \textit{C. albicans} Des1p is equally related to both DES1 and DES2, as evident from Fig. 3.
Identification of a Sphingolipid Δ4-Desaturase Family

Evolution of Sphingolipid Desaturases and Hydroxylases—Until now, the yeast sphingolipid C-4-hydroxylase Sur2p (10, 11) and its plant homologues (12) were the only enzymes known to be capable of synthesizing C-4-hydroxylated sphingolipids. In particular, no mammalian Sur2p homologue could be found in the public sequence databases, although some mammalian tissues, in particular kidney and the intestine, contain considerable amounts of D-4-hydroxysphinganine (1), which was demonstrated biochemically (30). It was unclear how C-4-hydroxylated sphingolipids were biosynthesized in mammals. In this study, we have identified the M. musculus DES2 protein, which is a bifunctional sphingolipid Δ4-desaturase/C-4-desaturase family protein. DES2 in transgenic yeast resulted in a 4-double bond gave rise to a pseudomolecular ion with m/z = 482, being consistent with its designation as 4-hydroxy-sphinganine. The ESI/MS/MS fragmentation spectrum of this ion is distinguished from that of the DNP-derivatized D-4-hydroxysphinganine reference substance, whereas it is clearly different from the secondary ion spectra of sphinganine and (E)-sphing-4-enine (data not shown). These data confirm that expression of M. musculus DES2 in transgenic yeast resulted in the formation of 4-hydroxysphinganine in addition to (E)-sphing-4-enine. We did not determine the stereochemistry of the 4-hydroxysphinganine formed in this reaction, but we assume the C-4-hydroxy group to be of the D-configuration, as this is the configuration found in all naturally occurring trihydroxylated sphingoid bases investigated to date (12). Because the reaction mechanisms of desaturation and hydroxylation are very similar (16), we conclude that M. musculus DES2 is a bifunctional sphingolipid Δ4-desaturase/C-4-hydroxylase. Expression of H. sapiens DES1, D. melanogaster DES, and C. albicans Des1p also resulted in the formation of small amounts (0.03–0.09 mol %) of 4-hydroxysphinganine in addition to (E)-sphing-4-enine, with a (E)-sphing-4-enine/4-hydroxysphinganine ratio of 60–70:1. This indicates that a bifunctional Δ4-desaturase/C-4-hydroxylase activity might be a general feature of sphingolipid Δ4-desaturases.

FIG. 2. Dendrogram showing similarities between selected membrane-bound desaturases and hydroxylases. Red, sphingolipid Δ4-desaturases (DES family, sequences 1–3 in bold); blue, fatty acid desaturases and sphingolipid Δ8-desaturase (sequence 9); green, sphingolipid C-4-hydroxylases; brown, sterol desaturases and hydroxylases. N, N-terminal cytochrome b5, fusion protein; C, C-terminal cytochrome b5, fusion protein; ER, endoplasmic reticulum. The pink and light blue background indicates groups of desaturase and hydroxylase sequences distinguished by a different spacing between the first and second histidine box. The small pictures illustrate the distance between these histidine boxes, which is longer (25–34 amino acids) in the sequences with pink background, and shorter (7–18 amino acids) in the sequences with light blue background. The red box represents the insertion/deletion responsible for the different spacing, the pink box represents the insertion/deletion number (not drawn to scale). The dendrogram has been constructed from pair wise similarities of full-length amino acid sequences using T-Coffee (46). GenBank protein accession numbers are: 1) AA656377; 2) AF466379; 3) AAD17340; 4)CAA18198; 5) P20388; 6) P48623; 7) P46822; 8) P46313; 9) T47950; 10) AAD31282; 11) AAF70457; 12) AAD01410; 13) Ref. 32; 14) P21395; 15) AAB62299; 16) CAB51047; 17) AAA34826; 18) NP_033154; 19) BAA25181; 20) NP_013999; 21) T01359; 22) CAB56960; 23) O88822; 24) NP 015157; 25) AAD38120; 26) NP_003947; 27) NP_015157; 28) AAP43928; 29) NP_010583; 30) AAC24374.

derivatives of the sphingoid bases were detected at the expected retention times, with m/z = 466 for sphinganine, m/z = 464 for (E)-sphing-4-enine, and m/z = 482 for 4-hydroxysphinganine. In addition, expression of C. albicans Des1p in S. cerevisiae sur2Δ allowed the isolation of (E)-sphing-4-enine in quantities sufficient for NMR analysis. The 1H NMR spectra of peracetylated (E)-sphing-4-enine isolated from transgenic yeast were indistinguishable from spectra obtained with peracetylated (E)-sphing-4-enine reference substance (data not shown) and were consistent with data from the literature (29). Diagnostic inter alia resonances of the Δ4-double bond gave rise to signals at 5.372 ppm (H-4) and 5.772 ppm (H-5) expressing a coupling constant of J1,5 = 15.2 Hz characteristic for an (E)-configuration. These data show that the members of the DES family indeed catalyze the formation of (E)-sphing-4-enine. M. musculus DES2 Is a Bifunctional Sphingolipid Δ4-Desaturase/C-4-Hydroxylase—Expression of M. musculus DES2 in S. cerevisiae sur2Δ unexpectedly resulted in the formation of both (E)-sphing-4-enine (0.7 mol %) and 4-hydroxysphinganine (0.3 mol %) (Fig. 2e). The (E)-sphing-4-enine/4-hydroxysphinganine ratio was 2.5:1. In HPLC/MS analysis, the putative 4-hydroxysphinganine gave rise to a pseudomolecular ion with m/z = 482, being consistent with its designation as 4-hydroxysphinganine. The ESI/MS/MS fragmentation spectrum of this ion is indistinguishable from that of the DNP-derivatized D-4-hydroxysphinganine reference substance, whereas it is clearly different from the secondary ion spectra of sphinganine and (E)-sphing-4-enine (data not shown). These data confirm that expression of M. musculus DES2 in transgenic yeast resulted in the formation of 4-hydroxysphinganine in addition to (E)-sphing-4-enine. We did not determine the stereochemistry of the 4-hydroxysphinganine formed in this reaction, but we assume the C-4-hydroxy group to be of the D-configuration, as this is the configuration found in all naturally occurring trihydroxylated sphingoid bases investigated to date (12). Because the reaction mechanisms of desaturation and hydroxylation are very similar (16), we conclude that M. musculus DES2 is a bifunctional sphingolipid Δ4-desaturase/C-4-hydroxylase. Expression of H. sapiens DES1, D. melanogaster DES, and C. albicans Des1p also resulted in the formation of small amounts (0.03–0.09 mol %) of 4-hydroxysphinganine in addition to (E)-sphing-4-enine, with a (E)-sphing-4-enine/4-hydroxysphinganine ratio of 60–70:1. This indicates that a bifunctional Δ4-desaturase/C-4-hydroxylase activity might be a general feature of sphingolipid Δ4-desaturases.

DISCUSSION

Evolution of Sphingolipid Desaturases and Hydroxylases—Until now, the yeast sphingolipid C-4-hydroxylase Sur2p (10, 11) and its plant homologues (12) were the only enzymes known to be capable of synthesizing C-4-hydroxylated sphingolipids. In particular, no mammalian Sur2p homologue could be found in the public sequence databases, although some mammalian tissues, in particular kidney and the intestine, contain considerable amounts of 4-hydroxysphinganine (1), and a mammalian sphinganine C-4-hydroxylase activity has been demonstrated biochemically (30). It was unclear how C-4-hydroxylated sphingolipids were biosynthesized in mammals. In this study, we have identified the M. musculus DES2 protein, which is a bifunctional sphingolipid Δ4-desaturase/C-4-
hydroxylase. This indicates that sphinganine C-4-hydroxylase is mediated by Sur2p in yeast and by Sur2p homologues in plants, but by DES2 in mammals.

The superfamily of membrane-bound desaturases and hydroxylases can be divided into two major groups, which are distinguished by a different spacing between the first and the second histidine box (Fig. 2). This difference likely represents an insertion/deletion event very early in the evolution of this superfamily. From this, it can be concluded that yeast Sur2p and its plant homologues (10)–(12) with a short spacing (Fig. 2, green branch) and mammalian DES2 with a long spacing between the first and second histidine box (DES family, in Fig. 2, red branch) have evolved their sphingolipid C-4-hydroxylase activity independently of each other.

Sphingolipid Δ4-desaturase activity has evolved independently of sphingolipid Δ8-desaturase activity. The plant sphingolipid Δ8-desaturase SLD1 (13) is very similar to the plant and animal fatty acid Δ5- and Δ6-desaturases, all of which are cytochrome b5 fusion proteins (Fig. 2, sequences 9–12) (17, 31). In contrast, the sphingolipid Δ4-desaturases share only limited similarity with any other proteins characterized by the histidine box motifs, and they do not contain a cytochrome b5 domain (DES family, Fig. 2, red branch).

It has been believed that regioselectivity placed a higher constraint on the evolution of membrane-bound desaturases and hydroxylases than substrate specificity or desaturase versus hydroxylase activity (16, 17), i.e. that enzymes with identical or similar regioselectivity are also similar in their amino acid sequence. The fatty acid Δ4-desaturase, which has recently been cloned from Thraustochytrium sp. (32), is closely related to the fatty acid Δ5/Δ6-desaturases, and like them, is a cytochrome b5 fusion protein (Fig. 2, sequence 13). Although it cannot be completely excluded on phylogenetic reasons that their most recent common ancestor had a Δ4-regioselectivity, the fatty acid Δ4-desaturase and the sphingolipid Δ4-desaturases (DES family, Fig. 2, red branch) most likely have evolved their Δ4-regioselectivities independently of each other. Δ4/C-4-Regioselectivity has thus evolved independently three times: in the fatty acid Δ4-desaturase (32), in the sphingolipid Δ4-desaturases (DES family), and in the sphingolipid C-4-hydroxylase Sur2p (10–12). Convergent evolution must therefore be taken into account when viewing the whole superfamily of membrane-bound desaturases and hydroxylases.

**Bifunctional Desaturase/Hydroxylase Activity**—Certain families of membrane-bound desaturases and hydroxylases seem to be predisposed to evolving a bifunctional desaturase/hydroxylase activity. These are, in particular, the sphingolipid Δ4-desaturase (DES) and the fatty acid Δ12-desaturase families. In the DES family, *M. musculus* DES2 is a bifunctional desaturase/hydroxylase. In the fatty acid Δ12-desaturase family, all variations from a hydroxylase in *Ricinus communis* (33)
via a bifunctional desaturase/hydroxylase in Lesquerella fendleri (34) to a desaturase in A. thaliana (35) exist. In both families, even the “pure” desaturases seem to have a minor hydroxylase activity (Ref. 36 and this study). The ease with which a fatty acid Δ12-desaturase can be converted into a C-12-hydroxylase and vice versa by exchanging just a few amino acids shows how similar the active sites of desaturases and hydroxylases must be (36, 37).

Two amino acid residues have been identified in the A. thaliana fatty acid Δ12-desaturase that, when exchanged with different amino acids, are sufficient to convert the desaturase into a bifunctional desaturase/hydroxylase (36). However, inspection of amino acid alignments indicates that neither of the equivalent residues in the DES family is likely to be important in determining the desaturation/hydroxylation ratio. Instead, when comparing the amino acid sequences of M. musculus DES1 (a desaturase) and DES2 (a bifunctional desaturase/hydroxylase), three single amino acid differences in the vicinity of the histidine box motifs (Ser or Ala at position 121, Ile or Thr at position 122, and Asn or Met at position 260) are striking. This indicates that rather than particular amino acid side chains, the overall architecture of the active site might determine the desaturation/hydroxylation ratio. A high resolution structural analysis of a membrane-bound desaturase would be needed to address this issue in more detail.

Cellular Functions of Sphingolipids—Based on detailed cytological and molecular studies carried out with members of the DES family from three different organisms (19–21), it is now possible to get a first insight into the diverse cellular functions of sphingolipid Δ4-desaturases. The human member of the DES family, DES1 (MLD), has been isolated in a yeast two-hybrid screen (21). The DES1 protein interacts physically with the epidermal growth factor (EGF) receptor. Overexpression of DES1 in 293 EBNA cells reduces expression of the EGF receptor by a post-transcriptional mechanism. EGF receptor signaling involves (E)-sphing-4-enine-1-phosphate as messenger, which itself is perceived by the EDG receptor family (2). It has recently been shown that the EGF receptor is translocated to the nucleus upon ligand binding, where the EGF receptor-ligand complex functions as transcription factor (38). Internalization of the EGF receptor is mediated by a Cbl-CIN85-endophilin complex and probably proceeds via clathrin-coated pits (39). The second well studied case is D. melanogaster, in which the des gene is inactivated in a mutant defective in spermatogenesis (19). In this mutant, the cell cycle of primary spermatocytes is blocked at the G2/M transition of meiosis I just before chromosome condensation is initiated. The mutant phenotype can be reverted to wild-type by complementation with a functional copy of the des gene (19). Finally, the expression pattern of the Des1 (Mdes) transcript from M. musculus has been studied in mouse testis and was found to be very similar to that of des-1 from D. melanogaster (20).

The phenotype of the D. melanogaster des mutant is very similar to that of four other Drosophila mutants, always early (aly), cannonball (can), meiosis I arrest (mia), and spermatocyte arrest (sa), except that in these mutants the chromosomes become partially condensed before the cell cycle is similarly arrested at the G2/M transition (40). These four mutants, as well as the des mutant fail to initiate spermatid differentiation, whereas mutants in the core cell cycle machinery such as cdc2 and twine show a cell cycle arrest at the G2/M transition, but still undergo spermatid differentiation (40). It is therefore argued that meiosis and spermatid differentiation are controlled separately, and that ALY, CAN, MIA, and SA are linked to a checkpoint triggering the simultaneous initiation of both processes (40). Because the des phenotype is very similar to that of the aly, can, mia, and sa mutants, but the cell cycle arrests before even a partial chromosome condensation can be observed, DES-1 is likely linked to the G2/M checkpoint more directly than ALY, CAN, MIA, or SA. In this context, it might well be that Δ4-desaturated sphingolipids provide a key signal to trigger passage past this G2/M checkpoint.

It is important to note that in contrast to males, female des mutant flies are fertile, which means that meiosis is blocked only in spermatid, but not in egg cell formation. In contrast, the des phenotype is combined with semilethality (20–50% lethality) during embryonic stages in both males and females. The additional defects causing this semilethality have not been investigated in detail, but because sphingolipids are required for many different functions (structural functions as well as signaling), pleiotropic effects may be anticipated. In mice, a knock-out of the DNA mismatch repair gene Pms2 similarly leads to sterility in males, but not in females (41). In Pms2-/-mice, however, formation of the synaptonemal complex is disturbed, which becomes apparent at a later time point than the cell cycle arrest in the des mutant. In addition to male sterility, Pms2-/-mice are predisposed to certain types of cancer because of genomic instability (41, 42).

In humans, male infertility is often connected with a phenotype very similar to that of the D. melanogaster aly, can, mia, sa, and des mutants, with a cell cycle arrest at the G2/M transition and partially condensed chromosomes (40). If also in humans, Δ4-desaturated sphingolipids can be shown to play a role in progression past the G2/M checkpoint, this will help in elucidating the molecular basis of one type of human male sterility.

C. elegans has two DES homologues (sequences 6 and 7 in Fig. 3, not functionally characterized in this study). Interestingly, in a combined analysis of 552 microarray experiments performed under diverse conditions (43), one of the DES homologues (GenBank™ accession NP_493549) was found to be coexpressed with typical lipid metabolism genes, whereas the other (NP_501256) was assigned to a functionally diverse group of genes highly expressed in the germline. This indicates that the function of D. melanogaster DES-1 in gamete formation may indeed be conserved among entirely different phyla.

Investigating DES functions in other organisms and other genetic backgrounds will provide important insights. The G2/M transition has been studied in detail with microarray experiments during meiosis (sporulation) of S. cerevisiae (44). Expression of the so-called mid-sporulation genes starting at the G2/M transition is regulated by the transcription factor Ndt80p, which controls both meiotic division and subsequent cellular differentiation (spore formation) (45). It will be interesting to see if there are similarities between the control of the G2/M transition in S. cerevisiae and D. melanogaster, because in yeast, no differentiation between male and female is possible and Δ4-desaturated sphingolipids do not occur.

Higher plants, unlike mammals, D. melanogaster, and C. elegans, are diplohaplont organisms, in which a short haploid stage separates meiosis and mitotic gamete formation. In the plant kingdom, only certain algae, like diatoms and the brown alga Fucus, are diplonts which, except their gametes, have only diploid life stages. It is therefore of particular interest to see whether plant DES homologues have similar functions as D. melanogaster DES-1, and if their activity is required in the sporophyte (meiosis) or gametophyte (gamete formation).

As described in the beginning, (E)-sphing-4-enine derivatives do not only interfere with cell cycle control, but also with many biochemical activities not linked to cell division. A recently discovered example from plants is the control of stomatal opening, which involves (E)-sphing-4-enine-1-phos-
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S. cerevisiae sphingolipid signaling. By showing that Δ4-desaturated sphingolipids are involved in cell cycle control during Drosophila spermatogenesis, the discovery of the DES family has unexpectedly added another item to the list of cellular activities involving (E)-sphing-4-enine derivatives. The field of research on Δ4-desaturated sphingolipids is now open to the use of molecular and genetic tools. This will contribute to an understanding of the increasing diversity of sphingolipid signaling.

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