Dictyostelium discoideum Dgat2 Can Substitute for the Essential Function of Dgat1 in Triglyceride Production but Not in Ether Lipid Synthesis

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Triacylglycerol (TAG), the common energy storage molecule, is formed from diacylglycerol and a coenzyme A-activated fatty acid by the action of an acyl coenzyme A:diacylglycerol acyltransferase (DGAT). In order to conduct this step, most organisms rely on more than one enzyme. The two main candidates in Dictyostelium discoideum are Dgat1 and Dgat2. We show, by creating single and double knockout mutants, that the endoplasmic reticulum (ER)-localized Dgat1 enzyme provides the predominant activity, whereas the lipid droplet constituent Dgat2 contributes less activity. This situation may be opposite from what is seen in mammalian cells. Dictyostelium Dgat2 is specialized for the synthesis of TAG, as is the mammalian enzyme. In contrast, mammalian DGAT1 is more promiscuous regarding its substrates, producing diacylglycerol, retinyl esters, and waxes in addition to TAG. The Dictyostelium Dgat1, however, produces TAG, wax esters, and, most interestingly, also neutral ether lipids, which represent a significant constituent of lipid droplets. Ether lipids had also been found in mammalian lipid droplets, but the role of DGAT1 in their synthesis was unknown. The ability to form TAG through either Dgat1 or Dgat2 activity is essential for Dictyostelium to grow on bacteria, its natural food substrate.

Fat accumulation is not only a serious health problem in Western societies but also a desired trait in dairy cattle and crop plants. The biochemical pathway of fat synthesis is largely conserved in all organisms and governed by an enzyme activity that performs the last and committed step in fat production, namely, the conversion of diacylglycerol (DAG) to triacylglycerol (TAG) or triglycerides. These so-called acyl coenzyme A (CoA):diacylglycerol acyltransferases (DGATs) are therefore targets for therapeutic drug development and represent objects of intense genetic and biochemical studies (recently reviewed by reference 1).

Despite their relevance, the two enzymes mainly responsible for TAG production in mammals, DGAT1 and DGAT2, have been identified only about a decade ago. After the Dgat1 gene was cloned (2) and knocked out (3), the remaining enzyme activity suggested the existence of another enzyme. When the second Dgat gene was cloned (4), it became obvious that DGAT1 and DGAT2 are divergent in size (roughly 55 versus 43 kDa), differ in the number of transmembrane domains (6 or more versus only 2), and are largely unrelated in sequence (aligned in reference 1).

Although both proteins are expressed in virtually all tissues in mammals, their distinguishing features strongly influence their fate in the cell. Whereas DGAT1 is a resident protein of the endoplasmic reticulum (ER) (5), DGAT2 localizes to the ER (6) and to lipid droplets (7). The protein targeting of mammalian DGAT2 to both subcellular locations is even separable by mutations in the primary sequence (8) and thus appears to occur independently.

In newborn humans, a homozygous mutation of DGAT1 causes a severe diarrheal disorder that may even be fatal (9). In contrast, transgenic mice lacking Dgat1 are phenotypically normal, possibly due to strong expression of Dgat2 in the small intestine (9). Those mice do not become obese on a high-fat diet (3), indicating that DGAT1 is mainly responsible for assimilating fatty acids (FAs) liberated from nutrients. Homozygous DGAT2-deficient mice die soon after birth and do not contain fat deposits in liver or other tissues (10), suggesting that DGAT2 contributes the predominant enzyme activity in fat production of mammals. Moreover, DGAT2 appears to preferentially incorporate newly synthesized fatty acids into TAG, rather than utilizing fatty acids from the diet (11).

Double knockout mice, lacking both enzymes, DGAT1 and DGAT2, were produced in appropriate crosses, and only fetal cells could be used for biochemical analyses, because the embryos did not develop any further. Fibroblasts that were stimulated to differentiate into adipocytes were unable to produce TAG from added oleic acid and therefore also failed to develop lipid droplets as the fat storage organelle. If, however, fetal macrophages were challenged with oleate, this fatty acid was incorporated into steryl esters, which were stored in normal-looking lipid droplets (12).

The unicellular model system Saccharomyces cerevisiae also possesses a set of two enzymes producing TAG. Lro1p uses phospholipids as acyl donors to form TAG from DAG (13) and is thus unrelated to mammalian DGAT1, which employs coenzyme A-activated fatty acids as a substrate. Confusingly, yeast Dgat1p is the homologue of mammalian DGAT2. Various combinations of deletions of Lro1, Dgat1, and other candidate genes indicate that Dgat1p contributes the main activity for TAG synthesis in yeast (14, 15), similar to the role that DGAT2 plays in mammals.
ther extending this relationship, Dgalp also associates with lipid droplets in yeast (16), as DGAT2 does in mammalian cells.

In the present work, we set out to identify the DGAT homologues of Dictyostelium discoideum, a eukaryotic amoeba. Two candidates were identified as homologues for mammalian DGAT1 and DGAT2. The Dgal1 enzyme localizes to the ER, as in mammals, and the Dgal2 protein is found on lipid droplets, as known from mammals and yeast. In Dictyostelium, however, Dgal1 provides the dominant activity for TAG synthesis, whereas deletion of Dgal2 alone is without effect, which is opposite from what is found in mammals and yeast, where DGAT2 is more prominent. However, overexpressing Dgal2 rescued TAG synthesis in a dgal1-deficient strain or a dgal1”/dgal2” double knockout but failed to produce ether lipids, which can be synthesized only by Dgal1.

MATERIALS AND METHODS

Tagging Dgal1 with GFP. Using reverse-transcribed total mRNA from Dictyostelium as a template, primers 500 (CGTATGGATCCAAATGGGAACCAATTCACCATC) and 501 (CCGGAGATCCATTTAATAGGGATCCATTACAGTTG) further amplified the complete coding sequence of the dgal1 gene, now flanked by BamHI restriction sites. This fragment was inserted into the unique BamHI site of plasmid 68 pDNeoGFP (17), generating vector 751, which expresses green fluorescent protein (GFP)-Dgal1. The opposite construct, Dgal1-GFP (752), was produced similarly, except that primer 500 was combined with primer 502 (CGCTACTTAAATGGATCCATTTAATAGGGATCCATTACAGTTG), which eliminated the stop codon, so that translation could proceed into the GFP coding region provided by plasmid 48 pDd-A15-GFP without ATG (described in reference 18 as modified in reference 19).

Disruption of dgal1 by homologous recombination. First, the 5’ region of the dgal1 gene was amplified from Dictyostelium genomic DNA using primers 500 and 590 (CCGGATCCCTGCGCATCTATGATATTC), and the product was maintained in pGEM-T Easy (Promega) as vector 816. Next, from a customized pGEM-T Easy derivative that contained a single BamHI site (761), the SpeI site was eliminated by blunting with T4 polymerase, yielding vector 817, which subsequently received the dgal1 fragment from 816 into its BamHI site. The product, plasmid 818, was digested with SpeI within the first exon of Dgal1, where the Small-flanked blasticidin resistance cassette (Bs’) from pLPBLP (20) was integrated. The product, in which the cassette was transcribed in the same direction with respect to dgal1, was numbered 840. Digestion with BamHI produced a fragment that was electroporated into Dictyostelium. The resulting clones were screened by PCR on their genomic DNA using a primer, 585 (CAGTACGGCAATGGGATTC), which was not part of the targeting construct, in combination with either primer 57 (CGTACTATTACATATTACTAGA), binding within the resistance gene, or alternatively primer 590. Clones 1-17 and 2-10, derived from two independent transformation events, were further investigated in this work.

Construction of GFP-tagged Dgal2 proteins. The dgal2 gene was amplified from Dictyostelium genomic DNA using primers 290 (CCGGATCCCTGCGCATCTATGATATTC) and cloned into the pGEM-T Easy vector (Promega) to yield plasmid 619. After sequencing, the entire fragment was excised at EcoRI recognition sites provided by the primers and ligated into the EcoRI site residing behind the GFP gene of 68, resulting in plasmid 620 GFP-Dgal2. In order to tag the C-terminal end of Dgal2, primer 290 was combined with primer 371 (CCGGATCCATTTTATTTTAAAATTTCCAGTTG), which served to delete the stop codon from dgal2. After intermediate cloning into pGEM-T Easy (product 621), the EcoRI-flanked insert was transferred into vector 48, resulting in plasmid 622 DGAT2-GFP.

Construction of a dgal2 knockout strain. Plasmid 619 was cut with EcoRV, leaving a 5’ region of dgal2 with 749 bp and a 3’ end of 443 nucleotides, between which a Smal-flanked blasticidin resistance cassette (Bs’) from pLPBLP (20) was inserted. In addition, a BsmI site in the middle of the 5’ region was blunted and religated to interrupt the reading frame. The two products carrying the cassette in the same or in reverse orientation with respect to the direction of DGAT2 transcription were numbered 623 and 624, respectively. From both vectors, an EcoRI digest released the fragment, subsequently used for gene disruption by homologous recombination. Dictyostelium clones were screened by PCR with various combinations of primers on the genomic level, of which only 361 (GGTGCAATATGGTGTATATGATAC), binding upstream of the 5’ end of the sequence used for targeting, and 453 (CCATTGAAATGGGTGTTGCG), binding within the 3’ end of the disruption construct, are shown here. The absence of dgal2 mRNA was demonstrated by reverse transcription-PCR (RT-PCR) using the primer pair 290 and 291. Clone 1-7, derived from plasmid 623, and clone 2-5, originating from recombination with 624, are used within this publication.

Construction of a dgal1”/dgal2” double knockout. The dgal1 knockout clone 1-17 was transiently transformed with plasmid pDexRH-NLS-Cre (20) encoding the Cre recombinase, and clones now sensitive to blasticidin were screened for the deletion of the resistance cassette. PCR with the primer combination 585 and 590 identified successful events. The dgal1 gene remained inactive due to the presence of one loxP site within its first exon. Two strains were then used to disrupt the dgal2 gene with a fragment of plasmid 623 as well as 624 and again verified by PCR as described above. We continued the analysis of two independent double knockout clones, named 1-19 and 2-15.

In general, plasmids expressing GFP-tagged Dgal enzymes were transformed into Dictyostelium AX2 cells referred to as wild type or, if specified as such, into dgal1 knockout mutants by electroporation. In this case, transformants were selected by virtue of G418 resistance provided by the GFP-encoding plasmids, but individual clones were also derived by spreading dilutions on bacterial lawns. Two or more clones originating from separate transformation events that behaved the same were conserved. Plaque diameters of mutant cells were measured in a similar fashion.

The localization of GFP-tagged proteins was confirmed by indirect immunofluorescence (21) using a mouse monoclonal antibody (MAb), 221-64-1, raised against the protein disulfide isomerase (PDI) as a marker for the endoplasmic reticulum (22), or the lipid droplet-specific dye LD540 (23), if lipid droplet formation was induced by adding palmitic acid as described previously (24). Western blot assays employed antibodies directed against the mitochondrial protein porin, MAb 70-100-1 (25), or those recognizing GFP, MAb 264-449-2 (available from Millipore).

Thin-layer chromatography (TLC) for qualitative lipid analysis and an enzymatic assay to determine TAG levels were performed as described previously (24).

Identification of the unknown lipid. Purification of lipid droplets was performed by flotation as detailed in reference 24. For further analysis, the compound of unknown identity was scraped from TLC plates and reextracted twice with 1 ml hexane. After acidic methanolysis (26), the gas chromatography–flame ionization detection (GC-FID) analysis was performed as described in reference 24. For the identification of the dimethyl acetal, the GC-mass spectrometry (MS) analysis was arranged as published previously (27). For accurate mass measurement, the solved compound after reextraction was dried under a stream of nitrogen and resolved in 100 μl chloroform-methanol-water (65:33:8, vol/vol/vol). Aliquots of 2 μl have been used for ultra-performance liquid chromatography–electrospray ionization (UPLC-ESI) time of flight (TOF) MS analysis. The analysis was performed by UPLC (Acquity UPLC system; Waters Corporation, Milford, MA, USA) coupled with a photodiode array (PDA) detector (UPLC eLambda, 800 nm; Waters Corporation, Milford, MA, USA) and with an orthogonal time of flight mass spectrometer (TOF-MS; LCT Premier; Waters Corporation, Milford, MA, USA). For LC, an Acquity UPLC BEH Shield RP18 column (1 by 100 mm, 1.7-μm particle size; Waters Corporation, Milford, MA, USA) was used at
a temperature of 50°C, at a flow rate of 0.2 ml/min, and with a binary gradient of solvent A (water/formic acid, 100:0.1, vol/vol) and solvent B (acetonitrile/formic acid, 100:0.1, vol/vol). The following gradient was applied: 0 to 0.5 min of 80% solvent B, 0.5 to 7 min of 80% up to 100% solvent B, 7 to 18 min of 100% solvent B, and 18 to 21 min of 80% solvent B. The TOF-MS was operated in negative as well as positive electrospray ionization (ESI) mode in W optics and with a mass resolution larger than 10,000. Data were acquired by Masslynx 4.1 software (Waters Corporation, Milford, MA, USA) in centroided format within the dynamic range enhancement mode over a mass range of m/z 500 to 1,200 with a scan duration of 0.5 s and an interscan delay of 0.1 s. The capillary and the cone voltage were maintained at 2,700 V and 30 V and the desolvation and source temperature were maintained at 350°C and 80°C, respectively. Nitrogen was used as the cone (30 liters/h) and desolvation gas (800 liters/h). All analyses were monitored by using leucine-enkephalin ([M + H]⁺ 557.2771 or [M + H]⁺ 556.2799 as well as its 13C isotopomer [M + H]⁺ 555.2615; Sigma-Aldrich, Deisenheim, Germany) as the lock spray reference compound. The raw mass spectrometry data were analyzed using Masslynx software (Waters Corporation, Milford, MA, USA).

**Transformation of yeast and induction of expression.** In order to examine *Dictyostelium* Dgat1 function in yeast, the coding region was excised from plasmid 751 by BamHI digestion and transferred into pYES2 (Invitrogen) cut with the same enzyme, resulting in plasmid 1065. Alternatively, plasmid 751 was first digested with Pael, blunted by the action of T4 polymerase, and then cut with XhoI to release the GFP-Dgat1 fusion and transferred into a pYES2 vector cut with Ecl136II and XhoI, producing vector 1066. Similarly, the reverse construct Dgat1-GFP from plasmid 752, flanked by a T4-blunted PstI site and an overhang originating from XhoI digestion, was ligated into the Ecl136II and XhoI sites of pYES2, yielding plasmid 1067. Transformation, expression, and analysis of TAG formation were performed as described previously (28).

**RESULTS**

Dgat1 localizes to the ER and is essential for TAG synthesis. The *Dictyostelium* homologue of the human DGAT1 enzyme was identified by a BLAST search of the sequence UniProt ID O75990 against the *Dictyostelium* genome. The hit with the best score, DDB_G0271342, already annotated as *dgat1* (29), had a total of 26.4% identical residues and 30.8% conserved amino acids, which is high if one considers that the *Dictyostelium* protein is composed of 617 amino acids while the human protein is only 488 residues in length. To check the localization of the Dgat1 enzyme, it was fused to the N terminus of green fluorescent protein (Dgat1-GFP) and expressed in *Dictyostelium* wild-type cells. A strong ring outlining the nucleus and a thin network throughout the cell coincided with the labeling of a known ER marker (Fig. 1A). The same distribution was also obtained from GFP-Dgat1, i.e., when the order of the proteins in the hybrid was reversed (Fig. 1B). The localization of Dgat1 did not change when the formation of lipid droplets was induced by adding fatty acid (Fig. 1C and D).

To address the function of Dgat1 in triglyceride production, a knockout mutant was generated that carried a blastidicin resistance (B’s) cassette within the coding sequence of *dgat1*. Integration of this construct into the genome allows the amplification of a fragment using a primer binding within the resistance cassette and a second primer located upstream, just outside the region present in the targeting construct (Fig. 2A). Because this construct can be generated only in knockout cells, we replaced the B’s primer with an oligonucleotide binding downstream of the cassette, within the *dgat1* coding region. This combination yielded a short product from the wild-type copy of the gene and longer products from the mutants, signifying the integration of the 1.5-kb resistance cassette (Fig. 2B). Whereas cells of the wild type strongly accumulated triglycerides upon addition of fatty acid to the culture, the band for this substance was rather faint in the *dgat1* mutant clones, when analyzed by thin-layer chromatography (Fig. 2C). The same result was obtained when TAG levels were quantified using an enzymatic assay (Fig. 2D), indicating that Dgat1 is the major enzyme responsible for TAG production in *Dictyostelium* cells.

**Dgat1 produces alky and alkenyl containing ether bonds in triglycerides as well as wax esters.** Fatty acid stimulation of wild-type cells also leads to the accumulation of a band migrating slightly above TAG that disappears in the extract of cells where the *dgat1* gene is inactivated (Fig. 2C). In order to identify this unknown lipid (UKL), the TLC spot resulting from wild-type cells was scraped out and reextracted from the silica gel. After acidic methanalysis, a dimethyl acetal was identified in addition to fatty acid methyl esters (Fig. 3A). This identified the UKL as a lipid molecule that harbors alkenyl moieties (http://lipidlibrary.aocs.org/Lipids/ethers/index.htm). The lipid identity was further substantiated by accurate mass measurement, and this revealed the assigned molecular species profile (Fig. 3B). The relative abun-
dance (summing up to about 30%) of these moieties relative to the total number of side chains confirmed that most UKL molecules are monoalk(en)yldiacylglycerols (subsequently abbreviated as MDG). Thus, Dictyostelium lipid droplets store a significant amount (13% [24]) of MDG in addition to TAG, similar to what is known from mammalian cells (30).

The mammalian DGAT1 enzyme is known to produce a variety of lipids other than TAG, including diacylglycerols, retinyl esters, and waxes (31). For further analysis, the Dictyostelium enzyme was expressed in untagged form as well as in both GFP con-

![FIG 2 Dgat1 is essential for TAG production. (A and B) PCR products from genomic DNA isolated from the wild-type strain (AX2) and two independently derived dgat1- mutants (1-17 and 2-10). (A) Combining one primer binding upstream of the dgat1 coding region (585) and one specific for the resistance cassette (57), the disrupted copy of the dgat1 gene (*) can be amplified only in the mutants but not in the wild type; an unrelated gene, thioredoxin (trx), was included to demonstrate the integrity of the template DNA. (B) The primer pair 585 and 590, the latter being complementary to a sequence within exon 2, reveals the insertion of the roughly 1.5-kb-sized Bsr cassette in exon 1 of dgat1*. The relevant sizes of the DNA marker (M) are given in base pairs. (C) Thin-layer chromatography resolving triacylglycerol (TAG), free fatty acids (FFA), and cholesterol (CHL) (as an endogenous control), as well as an unknown lipid (UKL), from lipid extracts of AX2 and dgat1- mutants incubated with fatty acid (± FA) or in normal medium (− FA). (D) TAG levels measured through an indirect enzymatic assay of three replicates with mean ± standard deviation shown in the bar diagram. The value of 1.0 in the wild type is considered the background level (horizontal line) because of the absence of detectable TAG in the TLC (see panel C) and serves as a reference for the relative units (r.u.) of TAG (for more details, see reference 24). Enzymatic assays are vertically aligned with images of the corresponding TLC plate throughout this work.

![FIG 3 Alkyl and alkenyl containing ether lipids are produced in wild-type cells after fatty acid stimulation. (A) Identification of a dimethyl acetal after acidic methanolysis of the unknown lipid (UKL) by GC-FID analysis. The following signals were identified and confirmed by GC-MS spectra: dimethyl acetal (hexadecanal dimethyl acetal), 16:0 (hexadecanoic acid), 16:1(n-7) (9-hexadecenoic acid), IS (internal standard, heptadecanoic acid), 18:0 (octadecanoic acid), 18:1(n-9) (9-octadecenoic acid), 18:1(n-7) (11-octadecenoic acid), 18:2(n-9) (5,9-octadecadienoic acid), 18:2(n-7) (5,11-octadecadienoic acid), 18:2(n-7)* (7,11-octadecadienoic acid), 20:0 (eicosanoic acid), 22:0 (docosanoic acid). (B) The molecular species of the alkyl and alkenyl ether lipids were revealed by UPLC-ESI TOF MS analysis. The accurate mass measurement shows the molecular species profile of the putative ether lipids based on the total number of double bonds (DB) per molecule and the number of the carbon atoms (CA), respectively, resulting from the alkyl/alkenyl and the two acyl side chains. (C) TLC of yeast lipid extracts prepared from the quadruple knockout H1246, expressing either empty plasmid (pYES2), untagged Dgat1 from Dictyostelium, or its N- or C-terminally GFP-tagged version. Cells remained untreated (−) or received 1 mM octadecenoic acid (+ FA) or 1 mM corresponding 18:1 long-chain alcohol (+ LCA). TAG and wax esters (WE) were identified by comigration of standard substances (not shown).
structs in a yeast quadruple mutant, H1246 (15), which is unable to synthesize neutral storage lipids. Upon expression of the Dictyostelium Dgat1 enzyme, TAG formation was observed irrespective of added tags (Fig. 3C). It used endogenous fatty acid (FA) or added FA as a source. Moreover, wax ester production was elevated by GFP-Dgat1 or the untagged enzyme, if a long-chain alcohol was added to the yeast culture medium (Fig. 3C), suggesting that the Dictyostelium Dgat1 has broad substrate specificity, similar to the mammalian enzyme. The production of ether lipids, however, was observed only in Dictyostelium cells carrying an intact Dgat1 enzyme and not in yeast.

Dgat2 is a lipid droplet constituent that synthesizes TAG in a dose-dependent manner. The second class of enzymes capable of TAG synthesis, Dgat2, was represented in the proteome of Dictyostelium lipid droplets (24). Its expression level in vegetative cells is about 16-fold less than that of dgat1 as measured in microarrays (http://dictyexpress.biolab.si/). The Dgat2 enzyme is just 330 amino acids in length, and 47% of its sequence is identical to the human enzyme. Again, its full coding sequence was fused to either the N terminus (Fig. 4A) or the C terminus (Fig. 4B) of GFP. Already in the absence of added fatty acid, a punctate distribution of the proteins was visible. By provision of the cells with fatty acid, lipid droplets increased in size and number and both proteins, GFP-Dgat2 and Dgat2-GFP, formed small rings outlining the fat-containing core of lipid droplets (Fig. 4C and D).

Because Dictyostelium cells mostly lack lipid droplets in unsupplemented medium or have only a few, we suspected that overexpression of Dgat2 induced the formation of fat stores by rerouting metabolic pathways. Accordingly, we selected three clones expressing widely disparate levels of GFP-Dgat2 (Fig. 4E, inset) and measured their TAG levels enzymatically (Fig. 4E). While the weakly expressing clone 1-5 yielded values almost identical to those of wild-type cells, stronger expression, as in clone 1-1 or 2-3, resulted in elevated concentrations of TAG. This was true not only in cells stimulated with palmitic acid but seen even in cells lacking fatty acids in their diet (Fig. 4E). This observation is also supported by the TLC analysis shown in Fig. 4F. Together, these data suggest that also the DGAT2 protein has the ability to synthesize TAG in Dictyostelium cells.

Because not much TAG seems to remain when the dgat1 gene is knocked out, we wanted to investigate again the degree to which Dgat2 contributes to total TAG synthesis through gene disruption. As described for dgat1 above, one primer located upstream of the region present in the targeting construct was combined with a primer downstream of the resistance cassette to demonstrate the gain of 1.5 kb in the dgat2 locus (Fig. 5A). As a further control, we chose to perform RT-PCR, which succeeded in detecting the expression of dgat2 mRNA in wild-type cells but failed to do so in the mutants created by homologous recombination (Fig. 5B). Although TLC analysis did not reveal any differences between the TAG content of wild-type cells and that of dgat2^- mutants to 5 μm. (E) TAG levels (measured in relative units [r.u.]) in the enzymatic assay (n = 3) are increased in two strains (1-1 and 2-3) expressing GFP-Dgat2 in the wild-type background at levels detectable via Western blotting using an anti-GFP antibody (αGFP, inset). An antibody directed against mitochondrial porin serves as loading control. A faintly expressing cell line (1-5) behaves like the wild-type (AX2). (F) The intensity of the TAG band, as identified by comigration with a marker (M) in TLC, corresponds to the bars from panel E aligned above. Further abbreviations are as in Fig. 2.
(Fig. 5C), we repeated the enzymatic test for TAG concentration but failed to detect a phenotypic alteration in the ability to synthesize fat (Fig. 5D). These results seem to indicate that the Dgat2 enzyme plays a minor, if any, role in TAG synthesis in Dictyostelium.

To address the possibility that a small but important function of Dgat2 is obscured by the presence of the dominant Dgat1 enzyme, we decided to construct a double mutant lacking both genes. To this end, the blasticidin resistance cassette of the dgat1/H11002 mutant was excised by transient expression of Cre recombinase, leaving only a loxP site in the genome. This is reflected by a PCR product across the dgat1 locus that is only 85 bp larger than the gene in the wild type (Fig. 6A). A set of engineered stop codons in all reading frames ensures that the dgat1 gene remains useless.

Two of these strains, 1-19 and 2-15, served as parents to further disrupt the DGAT2 gene (Fig. 6B), as verified by PCR using the same primer combination as shown in Fig. 5A, as well as 4 other.
primer sets supporting the predicted genomic structure of the
double knockouts (data not shown). Thin-layer chromatography
of lipid extracts derived from fatty acid-induced double mutant
cells revealed only a vague shadow at the position where a bit of
residual TAG is seen in the dgat1− single mutant (Fig. 6C). Quan-
titative enzymatic tests also confirmed the strongly reduced TAG
content of the double mutants (Fig. 6D). Thus, the results again
seem to suggest that Dgat2 has only a minor contribution to TAG
synthesis, which mainly depends on Dgat1 in Dictyostelium.
The dgat1−/dgat2− double mutants grew normally in axenic
medium and also tolerated fatty acid addition, indicating that
palmitic acid does not exert lipotoxic effects on Dictyostelium,
as known from mammalian cells. Furthermore, the double mutants
showed normal development into fruiting bodies. Subsequently,
their amoeba, however, took several days to fully emerge from the
protective spore capsule, a process that is completed in less than 12
h in the wild type. When cells (not spores) were seeded on a lawn
of bacteria, the dgat1−/dgat2− double mutant formed only very
small plaques (Fig. 6E). This is noteworthy, because each of the
single mutants affecting either dgat1 or dgat2 behaved like the wild
type in this respect (Fig. 6E). Because palmitic acid is tolerated
well, the basis of this defect must rather reflect the inability of the
dgat1−/dgat2− double mutant to successfully cope with the com-
plex lipids liberated from the bacterial diet.
Dgat2 can rescue defects originating from the lack of Dgat1.
In order to test whether Dgat2 can substitute for Dgat1 function,
GFP-Dgat2 was expressed in the double mutant cells. As a control,
we also tried to rescue the dgat1− single knockout. Most obvi-
ously, the small plaque diameter seen in the dgat1−/dgat2− double
mutant was reverted to normal (Fig. 7A). In the dgat1− single mu-
tant, the plaque diameter was wild type-like and showed only a
modest increase when GFP-Dgat2 was overexpressed in this mu-
tant background (Fig. 7A). The TAG level, as determined enzy-
masically, also reached or even exceeded wild-type levels for the
dgat1−/dgat2− double mutant or for the strain lacking dgat1
alone, respectively (Fig. 7B). These measurements were confirmed
by TLC (compare Fig. 7C to B). Despite this functional redun-
dancy between Dgat1 and Dgat2, the ether lipid was still lacking in
these cells (Fig. 7C, arrowheads). Comparing this finding to the
lipid pattern shown in Fig. 6C, we conclude that the synthesis of
the ether lipid strictly depends on Dgat1 but not on Dgat2 activity.

**DISCUSSION**

Subcellular localization of neutral lipid-producing enzymes.
The ER is the main site of neutral lipid production in cells (32).
Lipid molecules that are completely hydrophobic, rather than
amphiphilic, are thought to accumulate between the leaflets of the
ER membrane, from where they can bud off either into the cyto-
plasm to become a lipid droplet or into the lumen of the ER to
become a lipoprotein particle secreted from some specialized cells.
Whether the recently proposed dual topology of the DGAT1 en-
zyme (33) is the cause for this rerouting of lipid molecules is un-
known. Wherever tested, the DGAT1 protein is solely found in the
ER. Its localization there is unaffected by various sorts of tagging
(34), and irrespective of whether GFP was fused to the N-terminal
or to the C-terminal end of Dgat1, a perfect colocalization with a
known ER marker in Dictyostelium was obtained (Fig. 1).

The localization of DGAT2 is different. This protein is found in
the ER of plant cells, where it sorts away from DGAT1 into distinct
subdomains (35). In mammalian cells, localizations at two differ-
ent organelles were reported, namely, the ER and lipid droplets.
Moreover, if a cryptic localization signal is exposed, the protein
also associates with mitochondria (8, 36). The yeast protein (16)
and its Dictyostelium counterpart localize predominantly to lipid
droplets (Fig. 4). In addition, a faint labeling of the ER is observed
when GFP-tagged Dgat1 is expressed in dgat1− single mutants or
dgat1−/dgat2− double knockouts (data not shown), and upon cell
fractionation, also a small amount of the DGAT2 family member
Dga1 is detected in the yeast ER (16).

This dual localization is not uncommon. In a yeast strain un-
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able to form lipid droplets, all proteins typically associated with the lipid droplet localize to the ER instead (37). Also, a set of mammalian proteins shift their localization from the ER to lipid droplets as soon as their formation is induced (38–40). Furthermore, our previous studies have identified at least three Dictyostelium proteins, Smt1, Ldp, and Net4, which also undergo a relocalization from the ER to lipid droplets when fatty acids are supplied to produce these TAG stores (24).

**Broad versus narrow specificity in DGAT enzymes.** In an approach to gather DGAT sequences for evolutionary *in silico* studies, Turchetto-Zolet et al. (41) have searched the genome of *Dictyostelium purpureum*, a close relative of *Dictyostelium discoideum*, and identified four candidate genes. Two of them, called *DdiA* and *DdiB*, clearly correspond to *dgat1* and *dgat2* investigated here. The third gene, *DdiC*, may rather be a steryl ester-forming enzyme such as Are1p and Are2p from yeast, and the fourth protein is only barely integrated into the DGAT1 and DGAT2 families.

Inactivation of *dgat1* by homologous recombination in *Dictyostelium discoideum* caused TAG levels to decline to roughly 10% of wild-type values (Fig. 2), while a *dgat2* mutant was not significantly affected (Fig. 5). The relative contributions of the two enzymes may be explained by the 16-fold difference in expression levels, but possible differences in catalytic activity cannot be excluded. In the double mutant lacking both *dgat1* and *dgat2*, there was only very little residual TAG left (Fig. 6), strongly suggesting that these proteins are the most important enzymes responsible for TAG synthesis. The residual amounts of TAG may have originated from the activity of the two additional DGAT-like proteins, encoded by *DdiC* and *DdiD*. In a similar way, residual lipid formation is still detectable in a yeast strain lacking both *LR1p* and *Dga1p* (14, 15). This is probably mediated by *Are1p* and *Are2p*, because only in a yeast quadruple mutant does TAG synthesis become undetectable (14, 15). *Are1p* and *Are2p* enzymes have substrate specificities that exceed the known formation of steryl esters (42), and mammalian DGAT1, besides synthesizing TAG, is also able to produce DAG, wax esters, and retinyl esters (31).

If expressed in yeast, the *Dictyostelium* Dgat1 enzyme also revealed its promiscuity by synthesizing wax esters (Fig. 3C). Moreover, deletion of *dgat1* either alone or in combination with *dgat2* also results in the disappearance of another band from TLC plates (Fig. 2 and 6), which was preliminarily designated UKL. Further analysis revealed that this species was a glycerol-based lipid (MDG) (30). Their precursors may be formed in peroxisomes by the acylation of dihydroxyacetone phosphate and the subsequent exchange of the acyl moiety by a long-chain alcohol to produce the ether linkage present in MDG (43, 44), but the enzymes mediating the final conversions, most likely in the ER (45), are still unknown. We show here that *Dictyostelium* Dgat1 is a good candidate for mediating this reaction (Fig. 2). Finally, Dgat2 is clearly unable to produce ether lipids (Fig. 7), and also mammalian DGAT2 shows only a very limited capacity for synthesizing lipids other than TAG (31, 46).

**Role of fatty acid metabolism for *Dictyostelium*.** If *Dictyostelium* is cultivated under the excess of fatty acids, lipid droplets grow not only in numbers but also in size (24). We considered the possibility that the Dgat1 enzyme could directly provide lipid droplets with TAG, synthesizing it on their surface. In a mutant lacking *dgat2*, however, not only were there normal numbers of lipid droplets but also their size was not detectably altered (data not shown). This was unexpected, because *in vitro* studies with purified mammalian lipid droplets had suggested that they were carrying DAG and were able to convert it to TAG locally (7). Furthermore, work in *Caenorhabditis elegans* suggests that DGAT2 acts in concert with FATP1 to expand the size of lipid droplets (47).

On the other hand, *Dictyostelium* seems to be different because not Dgat2, as in yeast and mice (10, 14) (for details, see the introduction), but rather Dgat1 plays the major role in TAG synthesis (Fig. 2). Nevertheless, the double knockout of *dgat1* and *dgat2* genes has an additional effect, which was not expected from the single mutants, as these cells are barely able to grow on bacteria (Fig. 6E). Two explanations for this deficiency are possible.

For one, it could be that accumulating fatty acids liberated from the bacterial food source could build up a lipotoxic effect as seen in fibroblasts (48) or yeast cells (49). Failure to efficiently degrade cyclopropane fatty acids from bacteria in peroxisomes in a *Dictyostelium mfeA* mutant also leads to the inability to grow on bacteria in suspension, but for unknown reasons, growth on a plate is unaffected (50). Second, it is also conceivable that cell growth, i.e., the concerted doubling of genome, proteome, and lipidome, which has to precede successful mitosis, becomes deregulated when cells are unable to intermediately build up lipid droplets, where fatty acids can be stored for later production of membrane lipids. Thus, although triglyceride synthesis is dispensable during axenic growth of *Dictyostelium*, it plays an important role in its natural life cycle in the wild, when feeding on bacteria.

**ACKNOWLEDGMENTS**

The lipid droplet-specific dye LD540 was kindly provided by Christoph Thielé (Bonn, Germany). We are grateful to Carmen Demme for producing monoclonal antibodies from hybridoma cell lines as well as Pia Meyer and Sabine Freitag for technical support.

S.K. is supported by the Fonds der Chemischen Industrie and by the Göttingen Graduate School for Neurosciences and Molecular Biosciences (GGNB).

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