MFE1, a Member of the Peroxisomal Hydroxyacyl Coenzyme A Dehydrogenase Family, Affects Fatty Acid Metabolism Necessary for Morphogenesis in Dictyostelium spp.

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β-Oxidation of long-chain fatty acids and branched-chain fatty acids is carried out in mammalian peroxisomes by a multifunctional enzyme (MFE) or α-bifunctional protein, with separate domains for hydroxacyl coenzyme A (CoA) dehydrogenase, enoyl-CoA hydratase, and steroid carrier protein SCP2. We have found that Dictyostelium has a gene, mfeA, encoding MFE1 with homology to the hydroxyacyl-CoA dehydrogenase and SCP2 domains. A separate gene, mfeB, encodes MFE2 with homology to the enoyl-CoA hydratase domain. When grown on a diet of bacteria, Dictyostelium cells in which mfeA is disrupted accumulate excess cyclopropane fatty acids and are unable to develop beyond early aggregation. Axenically grown mutant cells, however, developed into normal fruiting bodies composed of spores and stalk cells. Comparative analysis of whole-cell lipid compositions revealed that bacterially grown mutant cells accumulated cyclopropane fatty acids that remained throughout the developmental stages. Such persistent accumulation was not detected in wild-type cells or axenically grown mutant cells. Bacterial phosphatidylethanolamine that contains abundant cyclopropane fatty acids inhibited the development of even axenically grown mutant cells, while dipalmitoyl phosphatidylethanolamine did not. These results suggest that MFE1 protects the cells from the increase of the harmful xenobiotic fatty acids do not remain long in Dictyostelium cells (12). Upon exhaustion of nutrients, Dictyostelium amoebae initiate multicellular development to form fruiting bodies. At this stage, CFAs are barely detectable (12), indicating that fatty acid composition is regulated during development.

In order to understand how lipid abnormalities affect cellular functions, we aimed to perturb fatty acid composition in Dictyostelium and examine the consequences. We first searched the Dictyostelium cDNA database (15) to find Dictyostelium counterparts of peroxisomal MFE2 or DBP (MFE2/DBP). We found two genes, mfeA (GenBank accession number AB042104) and mfeB (accession number AB100096), encoding MFE1 and MFE2, respectively. In this study, we analyzed the in vivo functions of MFE1 in multicellular development of Dictyostelium by disrupting the mfeA gene and showed that mfeA plays a crucial role in optimization of cellular lipid composition necessary for multicellular development of bacterially grown Dictyostelium cells. We propose that MFE1 is essential for survival of Dictyostelium cells in nature. This enzyme might also be essential for the survival of other soil amoebae and animals such as Caenorhabditis elegans.

Membrane-mediated cellular functions are crucial for the life of the cell. For such functions, cellular lipid composition must be strictly regulated. The cellular lipid composition is under the control of the peroxisomal β-oxidation, which degrades very-long-chain fatty acids and branched-chain fatty acids (4). In fact, impaired β-oxidation in peroxisomes causes serious diseases with the accumulation of nonmetabolized fatty acids (4). In fact, impaired β-oxidation in peroxisomes causes serious diseases with the accumulation of nonmetabolized fatty acids (4). In fact, impaired β-oxidation in peroxisomes causes serious diseases with the accumulation of nonmetabolized fatty acids (4). In fact, impaired β-oxidation in peroxisomes causes serious diseases with the accumulation of nonmetabolized fatty acids (4).
Disruption and overexpression of the \textit{mfeA} gene. Clone SLA480 (accession no. AB042104 for DDBJ/GenBank) was obtained from the \textit{Dictyostelium} cDNA project (15). SLA480 contains the open reading frame (ORF) of \textit{mfeA} encoding MFE1 at the SalI and NdeI sites of pSPORT. In order to create a construct for the disruption of \textit{mfeA}, \textit{mfeA} was recloned into the SalI and NdeI sites of pBluescript II KS(+) after amplification by PCR using the primers GCGATCCAAAAATGGCACATGGCATTAAATTTTAAAG and GTCTAGATTATAATTTTGAACCTTGCAT. The BSR cassette that provides blasticidin S resistance (1, 24) after amplification by PCR using CGCGATCCAAATGGCATTTATTTGAACCTTGCAT and T7 promoter primer. After digestion with BamHI and SacI, the amplified \textit{mfeA} gene was cloned into the BamHI and SacI sites of pHBlbBam in order to fuse \textit{mfeA} at the C terminus of GFP. pHBlbBam was a generous gift from T. Ueda (National Institute of Advanced Industrial Science and Technology of Japan).

**Southern blotting.** Genomic DNA was extracted according to the method of Nellen et al. (16). For Southern blotting, about 10 μg of DNA was digested with HindIII and NotI. The \textit{NdeI} and HindIII fragment obtained from \textit{mfeA} cDNA (SLA480) (Fig. 2A) was used as a probe after preparation according to the manufacturer's directions (digoxigenin labeling kit; Roche Diagnostics).

**Northern blotting.** In order to extract total RNA from cells at various developmental stages, both wild-type and \textit{mfeA}-null strains that had been cultivated for 2 days on an \textit{E. coli} lawn were allowed to develop at 2.5 × 10° C. For analysis of the simple lipid composition, the total lipids were separated by thin-layer chromatography (TLC) on a silica gel plate (silica gel 60; Merck, Darmstadt, Germany) with a solvent system of hexane-diethyl acetate (70:30:1 or 80:20:1 [vol/vol]). Authentic cholesterol, cholesterol oleate, triolein, oleic acid, and methyl oleate were purchased from Nu-Chek-Prep, Inc. (Elysian, Minn.). Lipids were visualized by heating the plate at 110°C for 10 min after spraying with 15% H2SO4. For fatty acid analysis, the plate was sprayed with acetic acid (4:1 [vol/vol]) containing 0.01% primuline. Each spot was separated by thin-layer chromatography (TLC) on a silica gel plate and separated by thin-layer chromatography (TLC) on a silica gel plate. Integration of the fatty acid moiety of each lipid was quantified by gas chromatography (GC) as described below, and methyl heicosaconic acid was used as an internal standard.
FIG. 2. Generation of the mfeA-null mutant. (A) A schematic view of the disruption construct and the parental gene. The disruption construct was made by the insertion of a BSR cassette into mfeA at the NdeI site. The NdeI and HindIII fragment of mfeA cDNA was used as the probe for both Southern and Northern blotting. The shaded boxes indicate the regions undetected by the probe. (B) Southern blotting was performed. Genomic DNA of wild-type and mfeA-null cells and mfeA cDNA and the disruption construct were analyzed after digestion with NsiI and HindIII. A 0.3-kb shift in band size occurred in mfeA-null cells. (C) Northern blotting was performed. Total RNA samples were extracted from wild-type (wt), mfeA-null (mfeA−), and mfeA-null cells expressing mfeA under the actin 15 promoter (A15:mfeA in mfeA−) at the time points indicated during the starvation. In the wild type, a single 1.45-kb transcript was constitutively expressed during development. In mfeA-null cells, this transcript was missing. In mfeA-null cells expressing mfeA under the actin 15 promoter, a slightly larger transcript (1.85 kb) was expressed depending on the actin 15 promoter activity. Ten micrograms of genomic DNA and total RNA was analyzed for each sample.

Nile red staining. Intracellular lipid droplets were detected by staining cells with Nile red according to the method described previously (7). The cells were observed with an Olympus microscope model AX70 and were photographed with a PM-C35DX camera equipped with U-PHOTO, a fully automatic exposure photo tube (Olympus, Tokyo, Japan).

RESULTS

Structure of peroxisomal MFE1 of Dictyostelium. By searching the Dictyostelium cDNA database, we found that Dictyostelium has two genes, mfeA and mfeB, with homology to the mammalian enzyme termed MFE or DBP, which consists of separate domains for hydroxyacyl coenzyme A (CoA) dehydrogenase (HCD), enoyl-CoA hydratase (ECH), and steroid carrier protein SCP2 (Fig. 1A). mfeA encodes MFE1, a protein with homology to the HCD and SCP2 domains, while mfeB encodes MFE2, a protein with homology to the ECH (Fig. 1A). The N-terminal and C-terminal domains of MFE1 show about 57 and 41% identity to the HCD and SCP2 domains, while mfeB encodes MFE2, a protein with homology to the ECH (data not shown). A tripeptide sequence SKL, consensus peroxisomal targeting signal 1 (PTS1), exists at the C terminus of MFE1 (Fig. 1C, asterisks). We also found that C. elegans has two enzymes with homology to Dictyostelium MFE1 and MFE2. The worm counterpart of Dictyostelium MFE1 is 54.6% identical to the Dictyostelium enzyme (Fig. 1B and C).

Role of MFE1 in multicellular development of Dictyostelium. In order to understand the role of MFE1 in Dictyostelium development, mfeA, the gene which encodes MFE1, was disrupted by homologous recombination (Fig. 2A). Eight independent transfectants were obtained; six were arrested at the preggregation stage on bacterial lawns (Fig. 3A), and the other two transfectants developed normally (data not shown). The plaque sizes of the wild-type cells and the mutant cells on E. coli HB101 plates and the wild-type cells and the mutant cells on SV40 plates were examined by incubating axenically grown mutant cells for 6 h. The plaque sizes of the wild-type cells and the mutant cells on 5LP plates according to the method of Bligh and Dyer (2) and was subjected to TLC with a solvent system of chloroform-methanol-water (65:25:4 [vol/vol/vol]). After visualization with primuline, the spot corresponding to phosphatidylethanolamine (PE) was scraped from the plate and reextracted by the method of Bligh and Dyer (2). The effect of bacterial PE was examined by incubating axenically grown mutant cells for 20 h in HL5 supplemented with bacterial PE at 0.16 mg/ml. For an experimental control, dipalmitoyl PE (Sigma, St. Louis, Mo.) was used at the same concentration. After harvesting, cells were washed, resuspended in PB, and allowed to develop on the nitrocellulose filter (Millipore Co., Bedford, Mass.) at a density of 2.5 × 10⁶ cells/cm². The developmental process was photographed with an HC-300ZOL digital camera equipped for an Olympus SZX12 (Olympus).

In situ hybridization. Whole-mount in situ hybridization analysis was performed according to the method described previously (5, 14, 24). Digoxigenin-labeled RNA probe was prepared by in vitro transcription by using SP6 RNA polymerase. SLA480 linearized with SalI digestion was used as a template.

Fluorescent microscopic observation. To determine the intracellular localization of MFE1, cells expressing GFP-MFE1 were observed under an agar overlay (6) after fixation with 100% ethanol containing 1% (vol/vol) formaldehyde at −10°C. Bonner’s salt solution (10 mM NaCl, 10 mM KCl, and 3 mM CaCl₂) (3) was used in this experiment instead of PB, and phosphate-buffered saline containing 0.2% Triton X-100 was used to remove the fixative. Images were photographed with an Olympus AX70.
revealed that a single 1.45-kb transcript of mfeA was constitutively expressed throughout development in the wild type. In situ hybridization indicates that mfeA was expressed in all cell types (data not shown).

Surprisingly, mfeA-null cells developed into fruiting bodies with normal stalk cells and spores when axenically cultured and were then allowed to develop on nonnutrient agar (Fig. 3B). This suggests that certain bacterial substances exert deleterious effects on development. In order to test this possibility, development of mfeA− cells was monitored on nonnutrient agar after cultivation for various periods with bacteria. Development was severely affected depending on the period of bacterial cultivation. mfeA-null cells that had been cultured for 8 or more hours with E. coli failed to aggregate properly on nonnutrient agar (Fig. 3B). When cultivated with E. coli for 3 h and then transferred to nonnutrient agar, they formed a few fruiting body-like structures. Such structures contained neither intact stalk cells nor intact spores (Fig. 3B). These results suggest that certain bacterial substances inhibit both multicellular development and cell differentiation of the mutant.

**HCD domain and PTS1 have essential roles in Dictyostelium development.** The intracellular localization of MFE1 was examined by expressing GFP-conjugated MFE1 in mfeA-null cells. When GFP was fused to the C terminus of MFE1, MFE1-GFP was distributed throughout the cytoplasm and transformed cells remained developmentally arrested (data not shown), perhaps as a consequence of GFP masking PTS1 at the C terminus. In contrast, GFP-MFE1 in which GFP was fused to the N terminus of MFE1 localized to particulate structures (Fig. 4), as seen in cultured human fibroblasts (10). This fusion protein rescued the mfeA-null phenotype. These results suggest that MFE1 is a peroxisomal enzyme.

As mentioned previously, MFE1 consists of the N-terminal HCD and C-terminal SCP2 domains and has a tripeptide SKL.
as PTS1 at the C terminus (Fig. 1A). We next examined which domain is responsible for the MFE1 function. In order to do this, each domain was expressed under the control of the actin 15 promoter in mfeA-null cells and their complementation ability of the mutant phenotype was examined (Fig. 5). Developmental defects of the bacterially grown mutant were rescued by expressing either full-length MFE1 (Fig. 2C and 5) or the SKL-tagged HCD domain (Fig. 5). Therefore, we conclude that MFE1 plays an indispensable role within peroxisomes for development of bacterially grown Dictyostelium cells, which is mediated by the HCD domain.

**Altered lipid composition in mfeA-null cells.** The amino acid sequence of MFE1 indicates that it is likely to be involved in β-oxidation of fatty acids. Therefore, intracellular lipid content could be altered in mfeA-null cells. To test this possibility, cells were stained with Nile red, an excellent vital stain for lipid droplets (7). When bacterially grown cells were stained, brilliant green granular structures were detectable in mfeA-null but not in wild-type cells (Fig. 6A), suggesting that nonmetabolized fatty acids might be sequestered in lipid droplets in the mutant. Lipid droplets were not observed in either axenically grown mfeA-null or wild-type cells (data not shown).

Total lipids were extracted from bacterially grown Ax2 and mfeA-null cells and were subjected to TLC. Although the content of total lipids varied depending on culture periods and experiments, it was roughly twofold higher in the mutant than in wild-type cells after 9 h of cultivation with bacteria. The most remarkable change was an increase in triacylglycerides (TGs) and sterolesters (SEs) in the mutant, and this change was dependent on the length of time that the cells were feeding on bacteria (Fig. 6B and C). These results suggest that certain fatty acids might have been sequestered in these neutral lipids due to the loss of MFE1.

**Altered fatty acid composition in mfeA-null cells.** In order to understand the function of MFE1 in diet lipid metabolism, fatty acid composition was analyzed in E. coli and Dictyostelium...
In addition to these major fatty acids, C\textsubscript{14:0}, C\textsubscript{16:1}(5), and C\textsubscript{18:0} were detected as polyunsaturated fatty acids increased to 6.1\% in axenically grown cells. Also, it was notable that the ratio of C\textsubscript{18:2} to total lipid was about 2.5-fold lower in bacterially grown mutant cells than in wild-type cells. C\textsubscript{18:2} was the major fatty acid constituent of TGs, SEs, and PLs in wild-type cells. C\textsubscript{18:2} gradually increased, but C\textsubscript{16:1}, cy\textsubscript{17:0}, and cy\textsubscript{19:0} decreased during starvation. On the other hand, both cy\textsubscript{17:0} and cy\textsubscript{19:0} were barely detectable in axenically grown mutant cells. However, a substantial amount of these fatty acids became detectable in bacterially grown cells. The most remarkable difference between the mutant and wild-type cells was observed in cy\textsubscript{17:0} and cy\textsubscript{19:0}. CFAs, which were more enriched in the mutant than in wild-type cells. These CFAs were the major fatty acids in TGs, SEs, and phospholipids (PLs) in the mutant cells. It is noteworthy that these CFAs decreased in wild-type cells during starvation but not in the mutant cells (Table 3), indicating that MFE1 is involved in CFA metabolism.

### TABLE 1. Fatty acid composition in total lipids of Ax2, mfeA-null cells, and E. coli<sup>a</sup>

| Fatty acid | E. coli | Wild type | mfeA<sup>a</sup> |
|------------|---------|-----------|-----------------|
| C\textsubscript{16:0} | 32.7 | 5.1 | 6.3 |
| C\textsubscript{16:1}(9) | 4.2 | 2.3 | 4.0 |
| C\textsubscript{16:2}(5,9) | 5.4 | 1.7 | 4.9 |
| C\textsubscript{18:1}(11) | 23.8 | 21.8 | 31.8 |
| C\textsubscript{18:2}(5,9) | 41.4 | 32.7 | 17.2 |
| cy\textsubscript{17:0} | 19.5 | 7.6 | 20.7 |
| cy\textsubscript{19:0} | 13.0 | 8.9 | 15.0 |

<sup>a</sup> Fatty acid composition was analyzed for the total lipids extracted from E. coli and Dictyostelium cells that had been grown for 24 h on an E. coli lawn as described in Materials and Methods.

### TABLE 2. Major fatty acid composition in TGs, SEs, and PLs of bacterially grown Dictyostelium cells<sup>a</sup>

| Fatty acid | TGs (%) | SEs (%) | PLs (%) |
|------------|---------|---------|---------|
| C\textsubscript{14:0} | 2.5 | 3.3 | Wild type |
| C\textsubscript{16:0} | 4.0 | 2.1 | mfeA-null |
| C\textsubscript{16:1}(9) | 20.7 | 13.5 | 8.0 |
| C\textsubscript{18:2}(5,9) | 22.6 | 10.0 | 33.7 |
| cy\textsubscript{17:0} | 18.4 | 31.8 | 5.8 |
| cy\textsubscript{19:0} | 9.5 | 21.7 | 3.2 |

<sup>a</sup> After separation of total lipids by TLC, the plate was visualized by using primuline. Each spot was scraped off from the plate and was then transmethylated. FAMEs were analyzed by GC and GC/MS as described in Materials and Methods.

### FIG. 7. Effect of bacterial PE on the morphogenesis of mfeA-null cells

The pictures were taken at 16 (a and c) and 22 (b and d) h of starvation. Scale bar, 0.5 mm.

The most remarkable difference between the mutant and wild-type cells was observed in cy\textsubscript{17:0} and cy\textsubscript{19:0}. CFAs, which were more enriched in the mutant than in wild-type cells. These CFAs were the major fatty acids in TGs, SEs, and phospholipids (PLs) in the mutant cells. It is noteworthy that these CFAs decreased in wild-type cells during starvation but not in the mutant cells (Table 3), indicating that MFE1 is involved in CFA metabolism. On the other hand, the proportion of C\textsubscript{18:2} to total lipid was about 2.5-fold lower in bacterially grown mutant cells than in wild-type cells (Table 1). C\textsubscript{18:2} was the major fatty acid constituent of TGs, SEs, and PLs in wild-type cells but not in mfeA mutant cells (Table 2). C\textsubscript{18:2} continued to rise in the wild type during starvation but not in the mutant (Table 3). These results suggest that the mutant phenotype might have resulted either from an increase in CFAs or a decrease in C\textsubscript{18:2} or both.
that bacterial PE but not dipalmitoyl PE significantly suppressed the multicellular development of the axenically grown mutant cells (Fig. 7). After being cultivated for 20 h in HL5 supplemented with dipalmitoyl PE and then being starved, the mutant cells developed almost normally into fruiting bodies (Fig. 7). However, when cultured in the presence of bacterial PE, mfeA-null cells did not develop beyond the tipped aggregate stage after starvation (Fig. 7). It appears that the developmental arrest of bacterially grown mfeA-null cells results from abnormal accumulation of CFAs.

DISCUSSION

In the present study, we showed that peroxisomal MFE1 plays an essential role in the survival strategy of Dictyostelium grown on bacteria. Bacterially grown mfeA-null cells cannot develop beyond the aggregation stage, and spores are not made. The amino acid sequence of MFE1 suggests that it is involved in β-oxidation of fatty acids, such as CFAs, that are the major components of bacterial lipids (9, 17). In fact, there was a remarkable increase in TGs and SEs with CFAs in bacterially grown Dictyostelium cells lacking MFE1. Concomitantly, there was a decrease in the proportion of dienoic acids such as C18:2 in bacterially grown mutant cells, suggesting that inhibition of Δ5-desaturases occurred in these cells (13, 20, 21). Thus, either an increase in CFAs or a decrease in these dienoic acids might have led to the mutant phenotype. However, a decrease in the proportion of C18:2 to total lipid also occurred in axenically grown mutant cells, which develop normally. Moreover, we found that development of mfeA-null cells was inhibited when cultured in the presence of bacterial PE, which abundantly contained CFAs. Therefore, it appears that the problems result from abnormal accumulation of CFAs.

How might accumulation of CFAs inhibit multicellular development of Dictyostelium? Since PLs and probably also SEs are the major components of the cell membrane, the accumulation of CFAs in these lipids might cause a reduction of membrane fluidity and/or an alteration of membrane microdomain structures, which might perturb membrane physiology and membrane protein functions. This notion is supported by our preliminary results demonstrating that both cell-cell contact mediated by gp80 and cyclic AMP signaling are impaired in bacterially grown mfeA-null cells (S. Matsuoka et al., unpublished data). We have also noticed that the mutant cells could not grow under shaking culture conditions in PB supplemented with washed bacteria (H. Kuwayama, unpublished data). These observations suggest that the unusual accumulation of CFAs seen in bacterially grown mutant cells interferes with normal membrane functions.

The present study demonstrates that the optimization of the cellular fatty acid composition is essential for the multicellular development of Dictyostelium. MFE1 plays an irreplaceable role in this process. Although the mechanism by which excess CFAs inhibit multicellular development is still unsolved, our study suggests that critical regulation of lipid and fatty acid composition is absolutely necessary for the survival of Dictyostelium in nature.

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