Cells store lipids in droplets. Studies addressing how mammals control lipid-based energy homeostasis have implicated proteins of the PAT domain family, such as perilipin that surrounds the lipid droplets. Perilipin knock-out mice are lean and resistant to obesity. Factors that mediate lipid storage in fungi are still unknown. Here we describe a gene (Mpl1) in the economically important insect fungal pathogen *Metarhizium anisopliae* that has structural similarities to mammalian perilipins. Consistent with a role in lipid storage, *Mpl1* is predominantly expressed when *M. anisopliae* is engaged in accumulating lipids and ectopically expressed green fluorescent protein-tagged Mpl1 (*Metarhizium* perilipin-like protein) localized to lipid droplets. Mutant *M. anisopliae* lacking Mpl1 have thinner hyphae, fewer lipid droplets, particularly in appressoria (specialized infection structures at the end of germ tubes), and a decrease in total lipids. *Mpl1* therefore acts in a perilipin-like manner suggesting an evolutionary conserved function in lipid metabolism. However, reflecting general differences between animal and fungal lineages, these proteins have also been selected to cope with different tasks. Thus, turgor generation by Δ*Mpl1* appressoria is dramatically reduced indicating that lipid droplets are required for solute accumulation. This was linked with the reduced ability to breach insect cuticle so that *Mpl1* is a pathogenicity determinant. Blast searches of fungal genomes revealed that perilipin homologs are found only in pezizomycotinal ascomycetes and occur as single copy genes. Expression of *Mpl1* in yeast cells, a fungus that lacks a perilipin-like gene, blocked their ability to mobilize lipids during starvation conditions.

Eukaryotic cells contain droplets of triglycerides encased in phospholipid membranes. These lipid droplets (LDs) were once considered to be just inert storage vessels for energy-rich fat, but recent studies have shown they have additional roles in maintaining membranes and moving components around cells. The lipid droplets have also been implicated in lipid diseases, inflammation, diabetes, cardiovascular disease, and liver disease (1). Consistent with fat droplets being metabolically active, the membranes encasing them have proteins with wide ranging biochemical activities. The best studied are mammalian proteins of the perilipin family (also called the PAT family) such as perilipin, adipocyte differentiation-related protein, and TIP47. These proteins have a characteristic series of hydrophobic sequences (the PAT domain) that facilitate their localization to the surface of lipid droplets. By coating droplets, perilipin forms a barrier that restricts the access of cytosolic lipases. During food deprivation, perilipin is phosphorylated by protein kinase A; the barrier function of perilipin is attenuated, and lipolysis increases (2). In comparison with normal mice, perilipin-deficient mice have less fat, more muscle, a higher metabolic rate, and are resistant to diet-induced and genetic obesity (3–5). The effects of perilipin have also been studied in *Drosophila* where the lack of the perilipin homolog results in flies with less fat (6). For perilipin-free animals, fat storage is a losing battle because hormone-sensitive lipase metabolizes fat as soon as it is made. Not surprisingly therefore, human perilipin variants are also associated with obesity or leanness (7).

The proteins that mediate lipid storage in fungi are still unknown. In this study we show that the ascomycete *Metarhizium anisopliae*, a ubiquitous insect pathogen and biocontrol agent (8), produces a single mammalian perilipin homolog we designated as *Mpl1* for *Metarhizium* perilipin-like protein. To demonstrate possible conserved functions, we characterized the *Mpl1* gene, asked whether its product participates in the regulation of lipid storage, and investigated its influence on fungal processes such as pathogenicity. Our data suggest that *M. anisopliae* represents a tractable new model system to study the functions of LDs and identify the components and mechanisms of energy homeostasis.

**EXPERIMENTAL PROCEDURES**

*Gene Cloning and Deletion—*Our EST analysis identified a high frequency contig (CN808339, 1.5% of transcripts) when the fungus was grown in insect hemolymph (9). A BLAST
search showed that it has a high similarity with the virulence factor CAP20 of plant fungal pathogen Colletotrichum gloeosporioides (10). To study its potential function in M. anisopliae, a full cDNA sequence was cloned, and the genomic DNA with flanking sequences was obtained by primer walking (DNA Walking Speedup™ kit, Seegene Inc.). Gene deletion was conducted using the plasmid pGPS3Bar as described before (11). The detailed procedure is provided in supplemental Fig. S1.

**GFP Fusion Constructs and Expression**—Full-length cDNA of Mpl1 was amplified from a library of genes expressed by M. anisopliae in insect hemolymph (9). The primers PerEF (CTTGGATCCGGGAATGCCGTGCCTCAGGTTCA) and PerER (CTTGATCTTGGCATTGGCATA) were used to introduce BamHI sites at both ends as well as an additional SmaI site at the 5’ end. The product was digested with BamHI and then inserted into the BamHI site of pYes2Per (Clontech) using an in-fusion cloning kit (Clontech) to generate the plasmid pYes2Per after digestion with SmaI. The product was integrated into the SmaI site of plasmid pYes2Per using an in-fusion cloning kit (Clontech) to generate the plasmid pGfpPer.

To determine whether MPL1 localizes to LDs in a fungus lacking endogenous perilipin-like proteins, Saccharomyces cerevisiae strain INVSc1 (Invitrogen) was transformed with pGfpPer and the transformant grown overnight in SC-U medium plus 2% raffinose. The culture was adjusted to A600 = 0.4 in 2% galactose SC-U medium and incubated at 30 °C, 250 rpm for 12 h.

To ectopically express GFP-tagged Mpl1 in its endogenous M. anisopliae environment, the Gfp-Mpl1 fusion protein was excised from pGfpPer and inserted into the BamHI site of pBARGPE1 (20) under the control of a constitutive GpdA promoter. The construct was linearized with Scal and transformed into M. anisopliae protoplasts as described (11).

**RT-PCR Analysis of Gene Expression**—To monitor gene expression of Mpl1 mycelium from 36-h Sabouraud dextrose broth (SDB), cultures were collected by filtration and washed three times with sterile distilled water. Equal amounts (0.2 g, wet weight) of mycelia were incubated (6 h) in 10 ml of water supplemented with 0.1% bean root exudate (25) or 1% peptone, and 2% dextrose, YPD plus 600 μM OA, 2% raffinose SC-U, or 2% raffinose SC-U plus OA. Cells harvested from the different media were washed three times, adjusted to A600 = 1.0 with sterile water, and incubated for up to 20 h to induce starvation. The cell concentration was adjusted to A600 = 2.0 and 0.5 ml used for lipid assay. Differences in lipid content between treatments were compared using the Duncan’s analysis of variance (SPSS, 11.0.0).

**Appressorial Turgor Assay**—To examine the potential involvement of MPL1 in this process, appressoria were induced on locust hind wings (14). Appressorial turgor pressure was assayed using serial solutions of PEG-8000 (2–13 g in 10 ml of distilled water) (15). Individual wings were dipped in PEG solutions for 10 min, and the percentage of collapsed appressoria was determined from 300 cells per PEG solution.

**Insect Bioassay**—Virulence of the wild type and ΔMpl1 was assayed against newly emerged 5th instar larvae of M. sexta (16). Conidia were applied either topically by immersion of larvae in an aqueous suspension containing 2 × 10⁷ conidia/ml for 20 s or by injecting the second proleg with 10 μl of an aqueous suspension containing 5 × 10⁶ spores per ml. Each treatment had three replicates with 10 insects each, and the experiments were repeated twice. Mortality was recorded every 12 h.

**RESULTS**

**Protein Structure and Characteristics**—The mammalian PAT proteins are characterized by conservation through ~350 amino acids of the N-terminal sequence where the lipid targeting functions reside. Distal to the N-terminal conservations, the proteins diverge to varying degrees (17). The full open reading frame of Mpl1 encodes a protein of 183 amino acids (20.3 kDa with a predicted pI of 8.96). MPL1 is therefore only 35% the size of mouse perilipin A (Per A), but it has a similar overall structure and several conserved regions with its N-terminal sequence (overall sequence similarity E = 1 × 10⁻²⁸) (Fig. 1A). In particular, like the N terminus of Per A (17, 18), the Mpl1 protein contains N-terminal β-strands, three moderately hydrophobic regions (H1, H2, and H3), and an acidic region (129–136 in Mpl1) before H2. Also like Per A, MPL1 has mul-
A Fungal Perilipin

A

MPL1

H1

H2

H3

183 aa

Per A

NH2

H1

H2

H3

517 aa

B

MPL1

MA-VFVNGDVPFRNSALQHLLAYPLSDSITHVANEYQASIQLGDSAYQTPAFVYLP 61

CAP20

MSMQAVGQDG-PAVNMTQHILLIDFVKHDGVAFRNFPLGSKSIAQDSAYPTFAAPVLP 61

MPL1

WLAKPYEVVSKVARADELSGDKTLDDEREPVKKTPSTLNDTSIILFNYKSIERGDH 123

CAP20

YLARPWGYLRFYAEKDALGQTLKTVEERVYPVIRKPTTELYAGKIALFIRPETESKH 123

MPL1

IFDVYASEAAXKIEQKGLVGQKAAVSTAPVESIETGLWLSFLLAAKKAETTVKKEVKQ 183

CAP20

VFKTYAQEKKVQGENLVTGYKAIVSTTLTTSEIIWGVDMYKKEAKDIVNEKVN 183

FIGURE 1. Schematic structure of MPL1 for comparison with mouse Per A (A) and the alignment of MPL1 with CAP20 protein (B). A, structural motifs include the following: β-strands (gray blocks), hydrophobic regions (H1, H2, and H3), and an acidic amino acid (aa) region (black) preceding H2. The symbols ∆, A, and ▲ indicate phosphorylation sites for cAMP-dependent protein kinase, tyrosine kinase, and protein kinase C, respectively. B, consensus sites between MPL1 and CAP20 are indicated by asterisks.

FIGURE 2. Mpl1 gene expression and protein localization. A, RT-PCR analysis of Mpl1 expression in mycelia cultured for 6 h in water, minimal medium (MM), Sabouraud dextrose broth (SDB), bean root exudates (RE), 1% (w/v) Manduca cuticle (Cut), or hemolymph (HE). B, time course study of Mpl1 expression in 36-h MM culture supplemented with oleic acid (600 μM). C, time course study of Mpl1 expression in conidia germinating in SDB. D and E, co-localization of neutral lipids and MPL1 demonstrated by NR staining of GFP-MPL1 expressing cells in conidia (D) and mycelium (E) of M. anisopliae, and a budding yeast (S. cerevisiae) cell (F), respectively. BF, bright field microscopy. Bar = 5 μm.

MPL1 shows homology to a reputed CAP20 protein (Fig. 1B) expressed during appressorial formation by the plant pathogen C. gloeosporioides and critical for virulence (10). However, our blast searches (Blastp or tBlastn) revealed no similarities between CAP proteins and either MPL1 or the protein from C. gloeosporioides. No evidence is provided by Hwang et al. (10) as to why the Colletotrichum sequence is a CAP protein. Aside from the animal perilipins, homologs (E < 10−5) of MPL1 are only present in pezizomycotinal fungi of the order Ascomycota, and are absent in yeasts and other fungi. Each pezizomycotinal fungus has only a single perilipin-like gene in contrast to animals encoding three perilipin genes (A, B, and C) (19, 20).

Expression Profile and Localization of MPL1—We analyzed Mpl1 expression in time course studies of M. anisopliae grown in different media. RT-PCR analysis demonstrated strong expression of Mpl1 when the fungus was grown in nutrient-rich media (insect hemolymph or SDB) as compared with nutrient-poor media (Fig. 2A). The addition of fatty acids to mammalian cells stimulates lipid accumulation and increases intracellular levels of perilipin (21). Likewise, transcription of Mpl1 by M. anisopliae germlings was up-regulated within 30 min following the addition of oleic acid to minimal medium (MM) (Fig. 2B). Conidia contained many large LDs (Fig. 3A) and demonstrated a strong enrichment of Mpl1 transcripts. Transcription levels decreased during germination (Fig. 2C), and LD numbers dropped by 40% between 6 and 12 h (Fig. S2) as lipid stores are mobilized during germ tube elongation (Fig. 3C). Collectively, these results suggest that Mpl1 regulation parallels lipid storage.

To visualize the intracellular targeting of MPL1 in vivo, we expressed an MPL1-GFP fusion protein in M. anisopliae using the Aspergillus GpdA promoter. We also determined whether LD localization is a general quality of MPL1 by expressing it with the Gal1-inducible promoter in the yeast S. cerevisiae, which is a system that lacks multiple phosphorylation sites, including a consensus cAMP-dependent protein kinase phosphorylation site at position 96 in a region conserved with Per A (Fig. 1, A and B).

The expression profile of per A (Fig. 1B) during appressorial formation by the plant pathogen C. gloeosporioides and critical for virulence (10). However, our blast searches (Blastp or tBlastn) revealed no similarities between CAP proteins and either MPL1 or the protein from C. gloeosporioides. No evidence is provided by Hwang et al. (10) as to why the Colletotrichum sequence is a CAP protein. Aside from the animal perilipins, homologs (E < 10−5) of MPL1 are only present in pezizomycotinal fungi of the order Ascomycota, and are absent in yeasts and other fungi. Each pezizomycotinal endogenous perilipin-like proteins. Consistent with GFP fusions of mammalian PAT proteins, i.e. perilipin A (22), TIP47 (23), and adipocyte differentiation-related protein (24), N-terminal fusion of MPL1 with GFP did not disrupt the ability of the protein to localize to lipid vesicles. Transformed yeast and Metarhizium cells treated with the neutral lipid stain NR co-localized with the GFP signal confirming that MPL1 is binding to LDs (Fig. 2, D–F). No additional diffuse cytoplasmic signal was seen with either GFP or NR. The expression patterns and the intracellular localization of MPL1 are therefore consistent with the proposal that the protein plays a regulatory role in global triacylglycerol (TAG) storage by acting at the level of LDs.
**A Fungal Perilipin**

**FIGURE 3.** MPL1 affects the number of lipid droplets and germinating morphology. Wild type and ΔMpl1 cells were stained with Bodipy to demonstrate the reduced numbers of lipid droplets in the mutant. A, wild type conidia; B, ΔMpl1 conidia; C, wild type germings; D, comparatively thin ΔMpl1 germings from 20-h MM cultures (Note, arrows show the transportation of lipid droplets to germ tube tips); E, wild type and F, ΔMpl1 appressoria produced 20-h post-inoculation on locust wings; G, wild type; and H, ΔMpl1 hyphal bodies harvested from the hemocoels of infected insects. Bar = 5 μm.

Loss of Function Mutants Confirm That MPL1 Plays an Important Role in Lipid Homeostasis—Mpl1 null mutants were generated by homologous replacement (Fig. S1). The conidia of wild type (WT) *M. anisopliae* have multiple LDs (mean per cell = 19.4 ± 3.76) that mostly cluster at the poles of the cell (Fig. 3A). ΔMpl1 conidia have ~2.4-fold fewer LDs than WT (*p* < 0.05) indicating that knock-out of Mpl1 impairs, but does not abolish, the ability of *M. anisopliae* to store lipid (Fig. 3B). During germination of WT conidia in minimal medium, the total number of LDs diminishes by 62% indicative of lipid degradation, and the remaining LDs migrate into the germ tube apexes (mean per apex = 3.6 ± 1.1). This distribution of LDs is explainable by fungi being tip growers; the tip is the area of greatest metabolism and where new membranes are being laid down. Relative to the WT, the ΔMpl1 germ tubes are thinner and apparently analogous to the “lean” phenotype of perilipin-deficient mice (3) (Fig. 3, C and D). Approximately 50% of ΔMpl1 germ tubes lack visible LDs. The remaining germ tubes have up to three LDs. The aggregated, albeit small, clusters of LDs at the poles of ΔMpl1 conidia (Fig. 3B) and the successful transportation of residual LDs to their hyphal tips (Fig. 3D) indicate that Mpl1 is not crucially involved in mediating the positioning of droplets in *Metarhizium*. This contrasts with *Drosophila* where perilipin regulates transportation of LDs (25). The pattern of LD distribution was also studied during formation of appressoria on locust hind wings. LDs moved into the differentiating hyphal tip so the WT appressoria were enriched in LDs. ΔMpl1 appressoria frequently contained no LDs at all (Fig. 3, E and F).

After breaching the insect cuticle, *Metarhizium* produces variably shaped hyphal bodies for dispersal in the hemocoel (11). The distribution of LDs in these was also examined. Unlike conidia, LDs are dispersed throughout hyphal body cells, but as with other cell types, the ΔMpl1 mutants have fewer (Fig. 3, G and H).

Because ΔMpl1 cell types contain relatively few, if any, LDs, the regulation of lipid homeostasis by MPL1 in *Metarhizium* was also studied by assaying total levels of intracellular lipids (Fig. 4A). Consistent with an elevated number of LDs, there was a more dramatic difference in lipid levels (>7-fold) of WT and ΔMpl1 conidia in a minimal medium (Fig. 4A), indicative of rapid depletion of residual stored lipid in the mutant. As OA up-regulates Mpl1 expression (Fig. 2B), Mpl1 activity might...
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FIGURE 5. MPL1 affects appressorial differentiation and turgor pressure. The appressoria produced by conidia germinating on locust hind wings were visualized by staining with Calcofluor white to show the formation of septa (indicated by an arrow) between the appressoria (AP) and conidia (CO) in wild type (A) but not ΔMpl1 germlings (B). Relatively higher levels of ΔMpl1 appressoria collapse when infected locust wings are immersed in serial solutions of PEG-8000 (PEG) for 10 min (C). The insets show collapsed appressoria. Bar = 5 μm.

adjust storage of lipids when nutrients are available to ensure extended survival when food supply is limiting. However, the 8% increase in lipid content in conidia harvested from PDA plus OA as compared with PDA alone was not statistically significantly (p > 0.05).

We also quantified lipids in yeast transformed with Mpl1 cDNA under the control of the Gal1 promoter (Fig. 4B). No significant differences in lipid content were observed between cells grown in noninductive medium (YPD and 2% raffinose) or inductive medium (2% galactose) with or without the addition of OA. However, yeast cells that had not produced MPL1 rapidly reduced their lipid content during starvation. In contrast, cells previously induced to produce MPL1 failed to utilize their stored lipid when deprived of nutrients showing that MPL1 blocks the access (Fig. 4B).

MPL1 Affects Appressorial Differentiation and Fungal Virulence—The "Cap20" transcript of the Mpl1 homolog in the plant pathogen C. gloeosporioides is present in conidia, expressed during formation of appressoria, and null mutants are not pathogenic to tomatoes (10). To investigate the role of Mpl1 on appressorium formation/function in M. anisopliae, we induced appressorial differentiation on locust hind wings. WT appressorial differentiation typically occurs after nuclear division with a septum being formed between the appressorium and conidial mother cell (Fig. 5A). However, this compartmentalization of germ tubes occurred in only 10% of the mutant germlings (Fig. 5B).

Analysis of appressorial collapse rates in M. anisopliae using serial concentrations of PEG-8000 shows that the loss of Mpl1 results in a dramatic reduction of turgor pressure (Fig. 5C). This suggests that lipolysis of LDs and consequent production of solutes produce turgor in the wild type. Virulence tests using 5th instar larvae of M. sexta revealed a significant reduction of mortality and speed of kill by ΔMpl1 as compared with the WT. The mutant failed to achieve 50% mortality before the insects pupated (Fig. 6A). However, when the cuticle was bypassed by injecting spores directly into the hemocoele, the speed of kill by the WT (LT 50 = 4.42 days) and the mutant (LT 50 = 4.72 days) were not significantly different (p = 1.58, t = 0.13) (Fig. 6B). The results indicate that the lack of Mpl1 affects fungal virulence by reducing mechanical penetration of the host cuticle.

DISCUSSION

Perilipins were the first identified mammalian lipid-associated proteins. In this study we demonstrate that the fungus M. anisopliae produces a protein MPL1 that has structural elements in common with mammalian perilipin. These include the N-terminal β-strands and central three hydrophobic regions that target and anchor Per A to LDs and are critical for TAG storage (17, 18). Phosphorylation of perilipin by cAMP-dependent protein kinase results in the conformational change that initiates lipolysis by hormone-sensitive lipase (29). Consistent with this, MPL1 has multiple phosphorylation sites. The studies we report here demonstrate that MPL1 operates in a perilipin-like manner by localizing to lipid droplets and modulating the rate of hydrolysis. Similar to the lean Per A knock-out mouse under diet control (3), the germ tubes of ΔMpl1 are slimmer than those of the wild type (Fig. 3, C and D). This demonstrates that perilipin-like proteins in the most diverse eukaryotes share an ancestral function. It also opens up new perspectives in this field by providing insights into the original functions of these proteins and will facilitate tracing...
when in the course of evolution the different forms and roles of the perilipin family proteins diverged.

It is interesting that among fungi, homologs ($E \geq 10^{-5}$) of MPL1 are only present in pezizomycotinal ascomycetes. The pezizomycotinal fungi however include many of the most important and well known animal and plant pathogens. Different lipid-associated proteins have been identified in yeast (21), but the yeast genome does not have a perilipin homolog. Yeast cells expressing Mpl1 were unable to make use of their stored lipids during starvation. It suggests that the yeast has lost, or never acquired, the phosphorylation mechanism for removing perilipin–like proteins to access LDs. The pathways responsible for lipid metabolism in non-perilipin-producing majority of fungi are clearly worth exploring.

Reflecting general differences between animal and fungal lineages, perilipin-like proteins have also been selected to cope with different tasks as the absence of MPL1 produced unique phenotypes, including reduced appressorial turgor pressure (27) with different tasks as the absence of MPL1 produced unique phenotypes. This may be a candidate for regulating these processes. This may be a general phenomenon among pathogenic ascomycetes as Cap20 mutants of the plant pathogen C. gloeosporioides (26, 27) also take up water to generate turgor for mechanical penetration of the host surface (27). Turgor is generated by accumulation of solutes, particularly the lipid-breakdown product glycerol (27, 28). Besides the accumulation of glycerol, cell compartmentalization is also required in rice blast fungus Magnaporthe grisea (26, 27) to generate appressorial turgor (27). Our data suggest that the single MPL1 homolog in M. grisea (MGG_11916.5, $E = 1.42 \times 10^{-43}$) could be a fungal perilipin.

The functional demonstration of a role for Mpl1 in virulence to insects is important for understanding the molecular and biochemical basis of pathogenicity and should be relevant to innovating new measures of pest control. In addition, our previous microarray analyses indicated that Mpl1 is up-regulated (>2-fold) in aging fungal sectors, and the degenerated fungus demonstrates decreased levels of cAMP and variable rates of lipid metabolism (30). Collectively, the data suggest that M. anisopliae represents a tractable new model system to study the functions of LDs and identify the components and mechanisms of energy homeostasis and how that relates to other physiological processes, including aging.

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