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Appearance of Smaller Lipid Bodies and Protein Kinase Activation in the Lipid Body Fraction Are Induced by an Increase in the Nitrogen Source in the *Mortierella* Fungus

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We studied the regulation of lipid body biogenesis in the oleaginous fungus *Mortierella ramanniana* var. *angulispora* by investigating culture conditions to modulate lipid body size, which we found was affected by the carbon-to-nitrogen ratio (C/N ratio) in the culture medium. Increasing the nitrogen source or decreasing the C/N ratio from 38 to 9 induced the appearance of lipid bodies with diameters less than 2–3 µm, which are usually found at a C/N ratio of 38 in this fungus. To determine factors regulating lipid body size, we compared lipid body fractions from fungal cells cultured at different C/N ratios. We found some differences in polypeptide profiles between lipid body fractions from fungal cells cultured at different C/N ratios for 2 days when the lipid bodies were enlarged at a C/N ratio of 38. We then compared the phosphorylation of lipid body proteins, since protein phosphorylation plays a pivotal role in various aspects of signal transduction. *In vitro* phosphorylation in the lipid body fraction indicated that protein kinase activity toward endogenous and exogenous substrates such as histone IIIS, VIIS, and myelin basic protein increased in the lipid body fraction at a C/N ratio of 9. Further analysis by in-gel protein kinase assay indicated the presence of at least three activated protein kinases with molecular masses of 75, 72, and 42 kDa, which were also autophosphorylated. These results indicate the presence of nutrient-regulated protein kinases and increased phosphorylation in lipid bodies, which correlate with the appearance of smaller lipid bodies in this fungus. Further studies to characterize these protein kinases at the molecular level should provide new insights into the link between nutrient sensing and lipid storage.

Key words: carbon to nitrogen ratio, lipid body, *Mortierella ramanniana* var. *angulispora*, phosphorylation, protein kinase.

Abbreviations: C/N ratio, carbon-to-nitrogen ratio; MBP, myelin basic protein; TG, triacylglycerol.

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Lipid bodies (droplets, particles *etc.*) are the intracellular site of storage lipids such as triacylglycerol (TG). Although the size and number of lipid bodies in cells vary with the organism and tissue, their structure is basically the same. Although their molecular architecture is becoming clearer and many proteins associated with lipid bodies have been reported (*1–3*), the biogenesis of lipid bodies and the transport of proteins and lipids into lipid bodies remain unclear.

Since lipid bodies form as energy reservoirs in cells, their biogenesis is assumed to be closely related to intracellular energy balance or nutrient conditions. The study of nutrient sensing signal transduction has revealed that several pathways, including nutrient sensors, several types of protein kinases, and transcription factors, exist in *Saccharomyces cerevisiae* (*4–7*). It is unknown, however, how nutrient conditions affect storage lipids and lipid body biogenesis in this yeast.

In studies of lipid accumulation mechanisms in the oleaginous fungus *Mortierella ramanniana* var. *angulispora*, we found that the final step of TG biosynthesis catalyzed by diacylglycerol acyltransferase was more enriched in the lipid body fraction (*8*). Using fluorescent lipid analogs to study lipid transport into lipid bodies in this fungus, we found different transport pathways, one *via* phosphatidic acid and another *via* phosphatidylcholine in endoplasmic reticulum membranes (*9*, *10*). We report here on factors that affect lipid body formation. We found that the carbon-to-nitrogen ratio (C/N ratio) affects lipid body size as well as lipid accumulation. Further studies focused on lipid body proteins that are expressed and modified specifically when the C/N ratio was altered. The results indicated that the phosphorylation of lipid body proteins increased greatly with a decrease in lipid body size at lower C/N ratios, which may play an important role in signal transduction of nutrient conditions into lipid bodies.

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MATERIALS AND METHODS

Materials—Histone IIIS, histone VIIS, myelin basic protein (MBP), and cAMP were purchased from Sigma (St. Louis, MO, USA). Nile red was purchased from Aldrich Chemical (Milwaukee, WI, USA). [γ-^{32}P]ATP (3,000 Ci/mmol) was purchased from PerkinElmer Life Sciences Japan (Tokyo). ATP, AMP, glucose assay kits (glucose CII-test wako), and ammonia assay kits (ammonia-test wako) were purchased from Wako Pure Chemical Industries (Osaka). Sodium deoxycholate, and Triton X-100 were purchased from Nacalai Tesque (Kyoto). Silica gel 60 TLC plates were purchased from Merck (Darmstadt, Germany). All other reagents were of analytical grade.

Strains, Cultures, and Preparation of Subcellular Fractions—M. ramanniana var. *angulispora* (IFO 8187) was obtained from the culture collection of the Institute for Fermentation (Osaka). Liquid culture was conducted as described elsewhere (*9*, *11*). The C/N ratio of the medium was changed by varying the amount of $(NH_4)_2SO_4$, with 1.5 g per 1 liter added for a C/N ratio of 38 and 6 g per 1 liter for a C/N ratio of 9. The lipid body fraction and membrane fraction from fungal cells were obtained as described elsewhere (*8*), and stored at –80°C until use.

In Vitro Phosphorylation—In vitro phosphorylation of the lipid body fraction (5 μg protein) was measured in 20 μl of reaction buffer containing 50 mM PIPES buffer (pH 7.0), 10 mM $MnCl_2$, and 0.5 μM [γ-^{32}P]ATP (2 μCi/assay). Cofactors or exogenous phosphorylation substrates were added as indicated for each experiment. The reaction was conducted at 30°C for 15 min and stopped by adding 8 μl of SDS sample buffer containing 0.2 M Tris-HCl buffer (pH 6.8), 8% (w/v) SDS, 20% (v/v) 2-mercaptoethanol, 40% (v/v) glycerol, and 0.02% (w/v) bromophenol blue. The reaction mixture was boiled for 5 min and subjected to SDS-PAGE in 12.5% or 15% gels. Radioisotope images of the gels were obtained with a radioisotope image analyzer (Molecular Imager FX, Bio-Rad).

In-Gel Protein Kinase Assay—In-gel protein kinase assay was conducted as described elsewhere (*12*). The lipid body or membrane fraction (10 or 20 μg protein) was subjected to SDS-PAGE using a 10% gel with or without 0.5 mg/ml histone VIIS. The gel was then incubated in 20% 2-propanol, 50 mM Tris-HCl (pH 8.0) twice for 30 min, in 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol twice for 30 min, and in 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 6 M guanidine-HCl twice for 30 min. The protein kinase was renatured in 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 0.04% (v/v) Tween 40 at 4°C overnight with three changes of solution. The gel was then incubated in 50 mM PIPES buffer (pH 7.0), 10 mM $MnCl_2$ for 1 h at room temperature and in 50 mM PIPES buffer (pH 7.0), 10 mM $MnCl_2$, 0.1 μM [γ-^{32}P]ATP (10 μCi/gel) for 1 h at 30°C. The gel was washed ten times in 5% (w/v) trichloroacetic acid, and 1% (w/v) sodium pyrophosphate. A radioisotope image of the gel was obtained with a radioisotope image analyzer (Molecular Imager FX, Bio-Rad). In some experiments, protein kinase bands on the renatured gel were cut out, crushed with a pestle, and incubated in the *in vitro* phosphorylation assay mixture described above for 30 min at 30°C as described elsewhere (*13*). Radiolabeled proteins were analyzed by SDS-PAGE as described above.

Microscopy—A laser scanning confocal microscope (LSM 410, Zeiss) with a 63× oil plan-apochromat objective lens (N.A. 1.4, Zeiss) was used to obtain Nile red staining images of fungal cells as described elsewhere (*9*).

Lipid Analysis—Lipids in subcellular fractions were extracted by the method of Bligh and Dyer (*14*) and separated by TLC on silica gel 60 plates with the following solvents (*9*). TLC plates were developed first with chloroform/acetone/methanol/acetic acid/H_2O (50:20:10:10:5, by volume) to a height of about 8 cm above the origin. After drying, plates were developed with hexane/diethylether/acetic acid (80:40:1, by volume) to about 18 cm above the origin. Spots on TLC plates were visualized by spraying 13% (v/v) sulfuric acid solution containing 2% (w/v) $CuSO_4$ in the plate and heating the plate at 110°C for 1 h (*15*).

Other Analytical Methods—Dry cells and total cellular lipids were measured in weight units as described (*11*). Protein was measured with Coomassie Blue dye reagent (Bio Rad) (*16*) in the presence of 0.05 N NaOH. SDS-PAGE was conducted based on the protocol of Laemmli (*17*). Protein bands on SDS-polyacrylamide gels were detected with a silver staining kit (Amersham Biosciences). Glucose concentration in the culture medium was measured with a glucose assay kit (glucose CII-test wako) and ammonia concentration with an ammonia assay kit (ammonia-test wako).

RESULTS

Effects of the C/N Ratio on Fungal Culture and Lipid Body Size—To determine factors regulating lipid body biogenesis, we studied culture conditions affecting the size of lipid bodies in *M. ramanniana* var. *angulispora*. Since the C/N ratio of the culture medium is known to affect lipid content in fungi and yeasts (*18*), we examined the effect of the C/N ratio on lipid body size in this fungus. Previous results indicated that fungal cells initially had smaller lipid bodies with a diameter of about 1 μm and had larger lipid bodies with a diameter of about 2–3 μm after 36 h cultivation when cultured in medium at a C/N ratio of 38 (*9*). We also found previously that decreasing the C/N ratio to 9 reduced the lipid content by about half after 5 days of culture in this fungus (*11*). We then compared cell growth, total lipids, and glucose and ammonia concentrations in the culture medium when fungal cells were cultured in medium at C/N ratios of 9 or 38 (Fig. 1). The fungi grew somewhat more slowly at a C/N ratio of 9, consistent with slower glucose consumption in a 2-day culture. Nevertheless, the glucose in the medium was completely consumed after 3 days of culture and the fungi grew similarly after 4 days of culture in both cases. The ammonia in the medium was completely consumed after 2 days of culture at a C/N ratio of 38 (more precise analysis indicated that the ammonia concentration reached zero after 30 h of culture), whereas ammonia still remained after 4 days of culture at a C/N ratio of 9. Ammonia depletion in the medium correlated well with lipid body enlargement.

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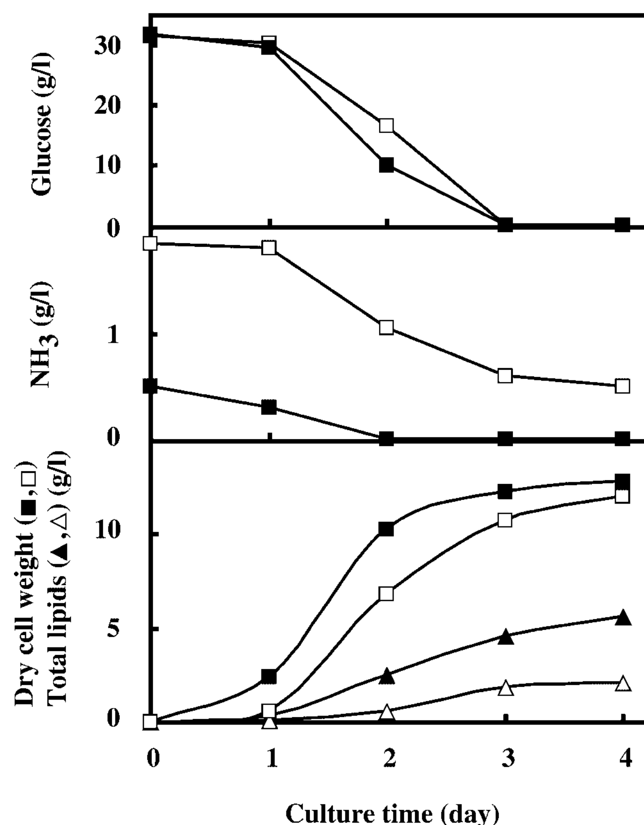

Fig. 1. **Glucose and NH_3 concentrations in the medium of fungal cells cultured at different C/N ratios.** Fungal cells were cultured at a C/N ratio of 9 or 38 as detailed in "MATERIALS AND METHODS." Glucose concentrations in the medium of fungal cells cultured at a C/N ratio of 9 (open squares) or 38 (solid squares) are shown in the upper panel. NH_3 concentrations in the medium of fungal cells cultured at a C/N ratio of 9 (open squares) or 38 (solid squares) are shown in the center panel. Dry cell weight of fungal cells cultured at a C/N ratio of 9 (open squares) or 38 (solid squares) and total lipids of fungal cells cultured at a C/N ratio of 9 (open triangles) or 38 (solid triangles) are shown in the lower panel. Data are presented as averages of duplicates.

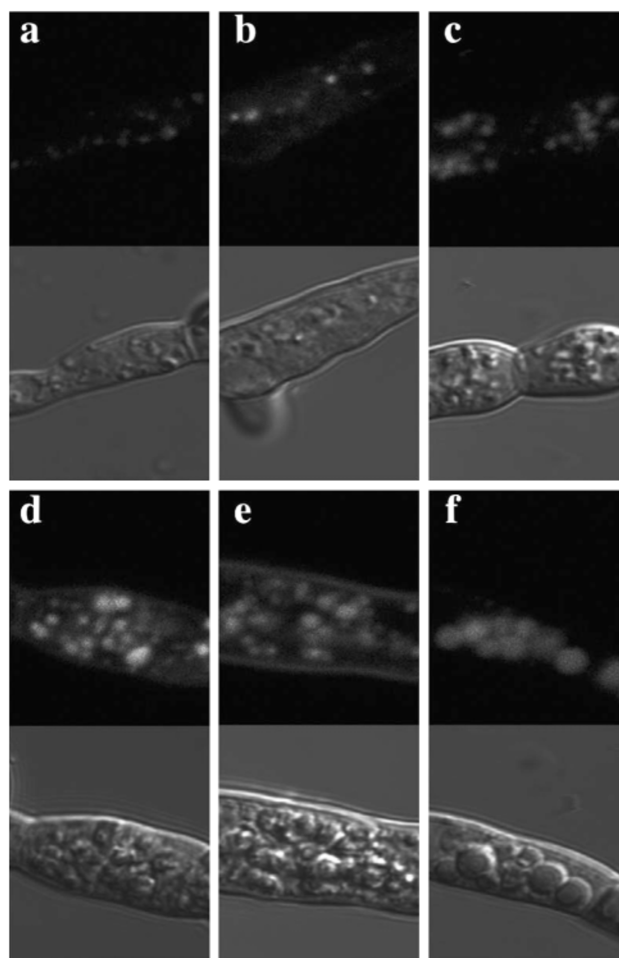

Fig. 2. **Time course of lipid body formation in fungal cells cultured at a C/N ratio of 9.** Fungal cells cultured at a C/N ratio of 9 for (a) 24 h, (b) 36 h, (c) 48 h, (d) 72 h, and (e) 96 h or at a C/N ratio of 38 for (f) 48 h were stained with Nile red. For (a)–(f), the top frame is a fluorescence image and the bottom frame is a differential interference contrast image. Bar = 3 µm.

We then monitored lipid bodies in cells cultured at a C/N ratio of 9 (Fig. 2). In the early stage of culture, the fungal cells had small lipid bodies with a diameter of about 1 µm, as observed at a C/N ratio of 38. However, the lipid bodies did not enlarge after 36 h of culture, unlike the case at a C/N ratio of 38. Even after culture for 4 days, the lipid bodies did not enlarge to the extent as in cells grown at a C/N ratio of 38, with most remaining smaller.

Protein and Lipid Profiles in Lipid Body Fractions from Fungal Cells Cultured at Different C/N Ratios— We studied lipid body proteins involved in lipid body biogenesis that changed their existence with the alteration in lipid body size. First, we confirmed that the lipid body fraction from fungal cells cultured at a C/N ratio of 9 had less TG than at a C/N ratio of 38 (Fig. 3). Interestingly, the membrane fraction from fungal cells cultured at a C/N ratio of 9 had slightly higher levels of TG than cells cultured at a C/N ratio of 38, indicating that TG distribution in intracellular membranes is increased in fungal cells cultured at a C/N ratio of 9.

Lipid body proteins in fungal cells cultured at different C/N ratios were analyzed by SDS-PAGE followed by silver staining (Fig. 4). The polypeptide profiles of the lipid body fraction from fungal cells after culture for 2 days at different C/N ratios were mostly similar, although different proteins were candidates for involvement in size changes in lipid bodies. The polypeptide profiles from fungal cells cultured for 4 days differed more significantly and major peptide bands typified individual culture conditions. The results suggest that only a few lipid body proteins differed after 2 days of culture at either C/N ratio, but more drastic differences appeared to be dependent on the C/N ratio in a 4-day culture.

Protein Kinase Activities in the Lipid Body Fractions from Fungal Cells Cultured at Different C/N Ratios— We studied post-translational modifications in lipid body proteins, comparing protein phosphorylation in the lipid body fraction from fungal cells cultured at different C/N ratios, since protein phosphorylation plays a pivotal role in intracellular signal transduction and may contribute

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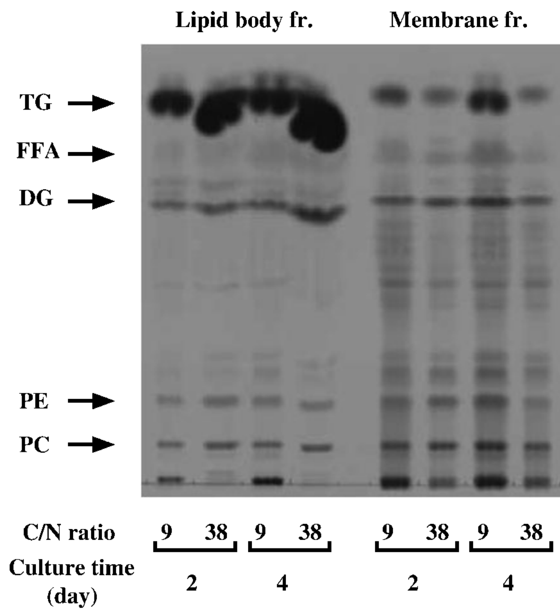

Fig. 3. **Lipid profiles of subcellular fractions from fungal cells cultured at different C/N ratios.** The lipid body and membrane fractions were from fungal cells cultured at a C/N ratio of 9 or 38 for 2 or 4 days. Extracted lipids were separated by TLC on silica gel 60 plates as detailed in "MATERIALS AND METHODS." The amounts of applied lipids were normalized by the fungal culture volume. Applied lipids in each lane were derived from 1 ml of fungal culture in a 1 liter flask (300 ml fungal culture). FFA, free fatty acid; DG, diacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

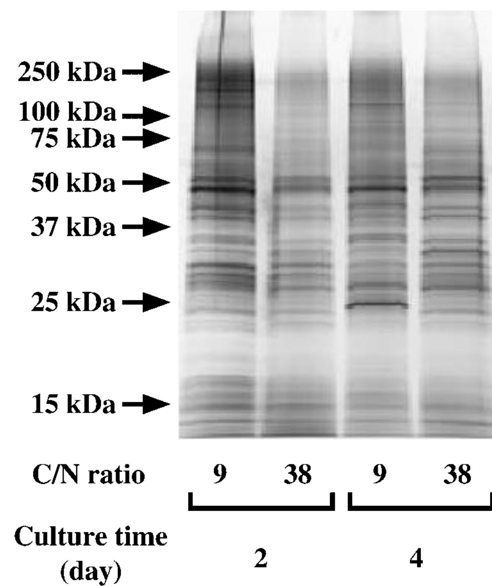

Fig. 4. **SDS-PAGE profiles of lipid body fractions from fungal cells cultured at different C/N ratios.** The lipid body fraction was from fungal cells cultured at a C/N ratio of 9 or 38 for 2 or 4 days. Each fraction (3 μg protein) was subjected to SDS-PAGE under reducing conditions in a 12.5% gel, followed by silver staining. Molecular mass standards (Bio-Rad) are indicated by arrows at the left.

to the transduction of nutrient conditions to lipid body size regulation. The *in vitro* phosphorylation in the lipid body fraction indicated that the lipid body fraction at a C/N ratio of 9 had increased phosphorylation as compared with that at a C/N ratio of 38 in the presence of Mn^{2+} (Fig. 5A). However, phosphorylation in the presence of Mg^{2+} was much lower in lipid body fractions at both C/N ratios. We found that deoxycholate activated *in vitro* phosphorylation in the lipid body fraction, an effect that was not

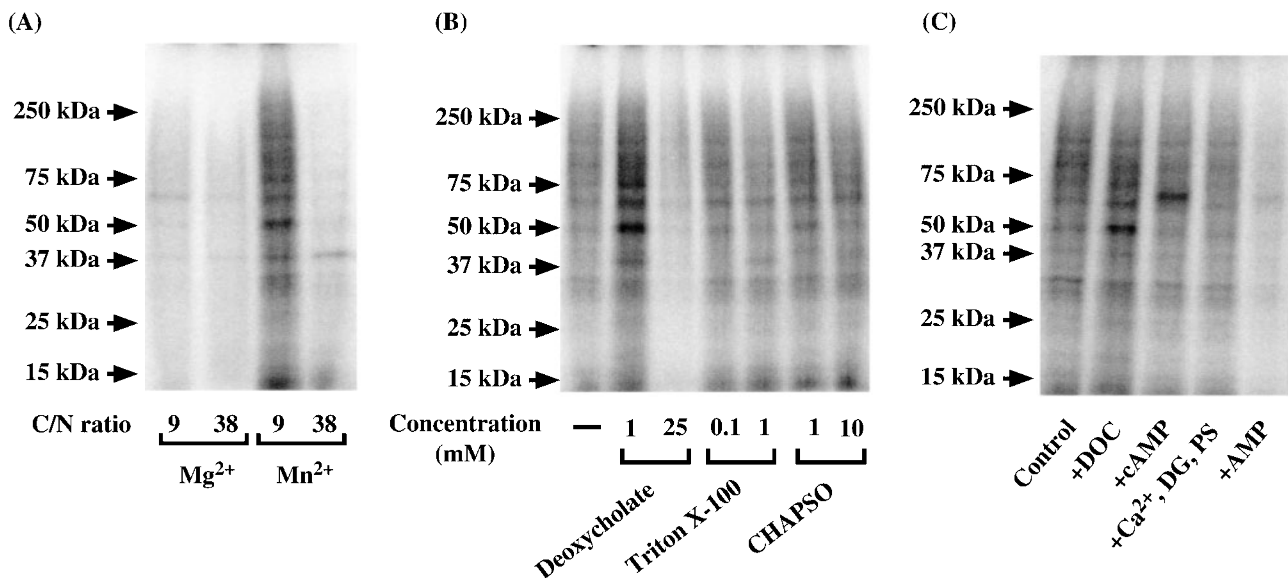

Fig. 5. ***In vitro* phosphorylation by lipid body fractions from fungal cells cultured at different C/N ratios.** The lipid body fraction was from fungal cells cultured at a C/N ratio of 9 or 38 for 2 days. *In vitro* phosphorylation by the lipid body fraction (5μg protein) was measured as described in "MATERIALS AND METHODS." ^{32}P-Labeled proteins were separated by SDS-PAGE under reducing conditions in 12.5% gels. (A) The effects of 10 mM Mg^{2+} and Mn^{2+} were studied in the presence of 1 mM deoxycholate. (B) The effects of detergents at the indicated concentrations were studied using the lipid body fraction (C/N ratio of 9) in the presence of 10 mM Mn^{2+}. (C) Effects of different cofactors for protein kinases [1 mM deoxycholate (DOC), 2 μM cAMP, 1 mM Ca^{2+}, 2.5 μg/ml 1,2-diolein (DG), 50 μg/ml phosphatidylserine (PS), 100 μM AMP] were studied using the lipid body fraction (C/N ratio of 9) in the presence of 10 mM Mn^{2+}. Molecular mass standards (Bio-Rad) are indicated by arrows at the left.

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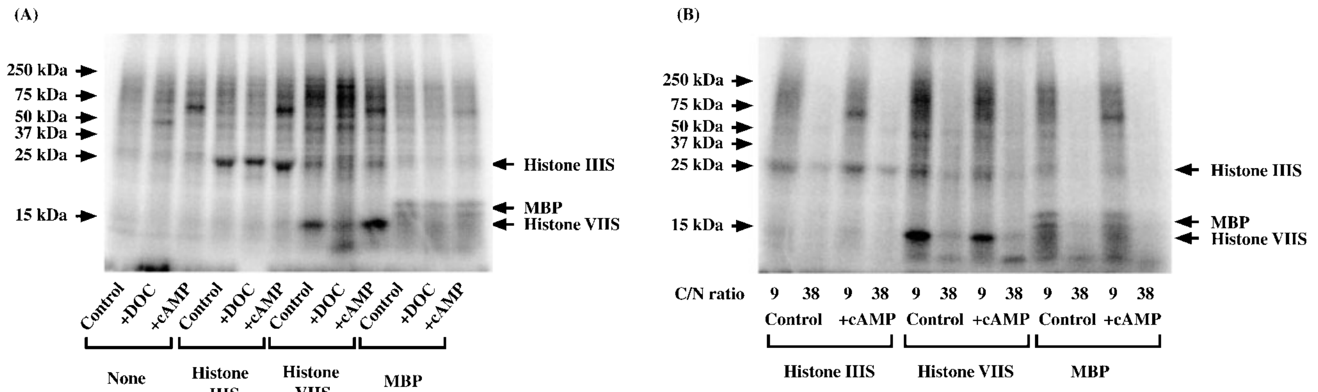

Fig. 6. ***In vitro* phosphorylation of exogenous substrates by lipid body fractions from fungal cells cultured at different C/N ratios.** The lipid body fraction was from fungal cells cultured at a C/N ratio of 9 or 38 for 2 days. *In vitro* phosphorylation of 250 μg/ml histone IIIS, histone VIIS, and MBP by the lipid body fraction (5 μg protein) was measured as described in "MATERIALS AND METHODS." ^{32}P-Labeled proteins were separated by SDS-PAGE under reducing conditions in 15% gels. Molecular mass standards (Bio-Rad) are indicated by arrows at the left. (A) The lipid body fraction (C/N ratio of 9) was assayed with exogenous substrates in the presence and absence of 1 mM deoxycholate (DOC) or 2 μM cAMP. (B) The lipid body fraction (C/N ratio of 9 or 38) was assayed with exogenous substrates in the presence and absence of 2 μM cAMP.

likely to be due to its effect as a detergent since a concentration above its critical micelle concentration inhibited phosphorylation and other detergents did not have similar effects (Fig. 5B). Effects of several cofactors for protein kinases on phosphorylation in the lipid body fraction suggested that phosphorylation was not due to protein kinase C (Fig. 5C). AMP-activated protein kinase, which plays a role in energy metabolism (*19*), was not responsible for phosphorylation in the lipid body fraction. AMP rather inhibited the phosphorylation, and may serve as a negative regulator. cAMP increased the phosphorylation of the 63 kDa protein, indicating that a cAMP-dependent protein kinase contributed to phosphorylation in the lipid body fraction. We assumed, however, that cAMP-independent protein kinases were responsible for most protein phosphorylation in the lipid body fraction, especially the phosphorylation of the 48 kDa protein increased upon the addition of deoxycholate.

Protein kinases in the lipid body fraction at a C/N ratio of 9 also phosphorylated exogenous substrates such as histone IIIS (H1), histone VIIS (H2B), and MBP (Fig. 6A). Interestingly, deoxycholate, which increased the phosphorylation of endogenous lipid body proteins, did not increase the phosphorylation of exogenous sub-

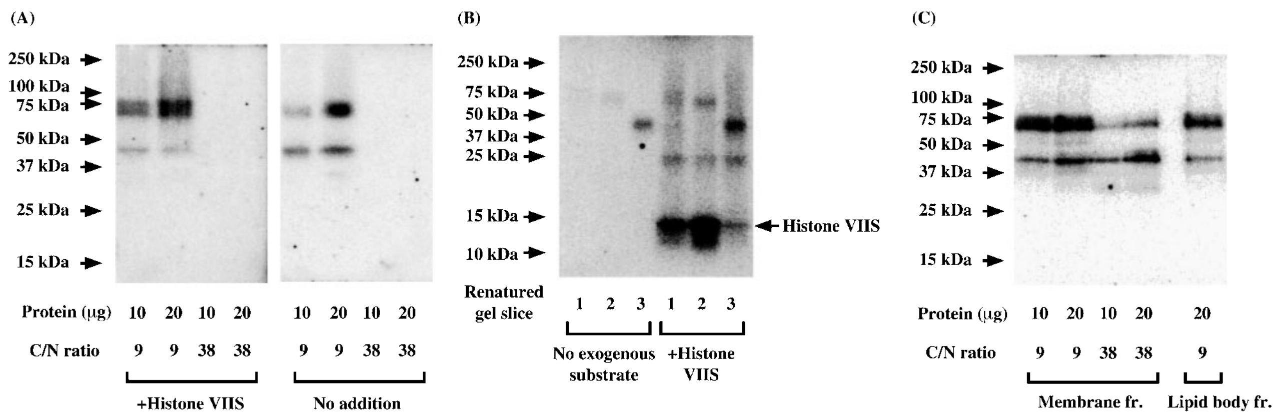

Fig. 7. **In-gel protein kinase assay of lipid body and membrane fractions from fungal cells cultured at different C/N ratios.** The lipid body and membrane fractions were from fungal cells cultured at a C/N ratio of 9 or 38 for 2 days. (A) The lipid body fraction (10 or 20 μg protein) was subjected to SDS-PAGE under reducing conditions using a 10% gel with or without 0.5 mg/ml histone VIIS, and in-gel protein kinase assay was conducted as described in "MATERIALS AND METHODS." Molecular mass standards (Bio-Rad) are indicated by arrows at the left. (B) The lipid body fraction (C/N ratio of 9, 20 μg protein) was subjected to SDS-PAGE under reducing conditions using a 10% gel without 0.5 mg/ml histone VIIS. After the gel was renatured as described in "MATERIALS AND METHODS," the gel was sliced so as to isolate the protein kinase bands described above (1: 75 kDa band, 2: 72 kDa band, 3: 42 kDa band). *In vitro* phosphorylation by these gel slices was measured for 30 min at 30°C in the presence and absence of 250 μg/ml histone VIIS as described in "MATERIALS AND METHODS." ^{32}P-Labeled proteins were separated by SDS-PAGE under reducing conditions in 15% gels. Molecular mass standards (Bio-Rad) are indicated by arrows at the left. (C) The membrane fraction (10 or 20 μg protein) or the lipid body fraction (20 μg protein) was subjected to SDS-PAGE under reducing conditions in 10% gels with 0.5 mg/ml histone VIIS, and the in-gel protein kinase assay was conducted as described in "MATERIALS AND METHODS." Molecular mass standards (Bio-Rad) are indicated by arrows at the left.

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strates. Deoxycholate may affect enzyme-substrate interactions in lipid bodies rather than activating protein kinases. We then found that the lipid body fraction at a C/N ratio of 9 had much higher protein kinase activity toward exogenous substrates than at a C/N ratio of 38 (Fig. 6B), which was consistent with protein kinase activity toward endogenous substrates. The phosphorylation of exogenous substrates in the presence of Mg^{2+} was also much less efficient than that in the presence of Mn^{2+}, as in the case of endogenous substrates (data not shown).

We conducted in-gel protein kinase assays to identify protein kinases responsible for increased phosphorylation in the lipid body fraction at a C/N ratio of 9. In-gel protein kinase assay indicated that three kinase bands with molecular masses of 75, 72, and 42 kDa appeared toward histone VIIS in the lipid body fraction at a C/N ratio of 9, whereas no protein kinase bands appeared in the lipid body fraction at a C/N ratio of 38 (Fig. 7A). These protein kinase bands were also detected toward MBP (data not shown), and detected even in the absence of exogenous substrates (Fig. 7A), indicating that these protein kinases were autophosphorylated. To determine whether the detected protein kinases really had activity toward exogenous substrates in addition to autophosphorylation, we cut the gel into slices corresponding to each protein kinase band, and measured its kinase activity toward histone VIIS (Fig. 7B). The results confirmed that each protein kinase phosphorylated histone in addition to itself, and that these protein kinases increased phosphorylation in the lipid body fraction at a C/N ratio of 9.

To examine the localization of these protein kinases, we conducted in-gel protein kinase assays in the membrane fraction. The results indicated that the three major protein kinase bands in the lipid body fraction were also detected in the membrane fraction (Fig. 7C). The three protein kinases in the membrane fraction had higher activities and their response to different C/N ratios was different from that in the lipid body fraction. The 75-kDa and 72-kDa protein kinases showed higher activity in the membrane fraction at a C/N ratio of 9, although the same protein kinase bands were also detected with lower activity at a C/N ratio of 38. The 42-kDa protein kinase was equally detected in the membrane fractions at C/N ratios of 9 and 38. These results suggest that the regulation of protein kinases in lipid bodies in response to the C/N ratio is influenced by the dynamic status of these protein kinases in lipid bodies and intracellular membranes.

DISCUSSION

Lipid bodies are observed ubiquitously in cells as sites of accumulated lipids. Although their names differ with the organism and tissue, their structures are assumed to be mainly the same, *i.e.*, a phospholipid monolayer and lipid body proteins surround hydrophobic neutral lipids (*1–3*). Recent observations have indicated that the surface of lipid droplets (lipid bodies) in animal cells is covered with a phospholipid monolayer (*20*). Lipid bodies vary greatly in size from about 0.1 μm to about 200 μm in diameter (*3*), and this difference is not necessarily proportional to the amount of accumulated lipids. It appears that lipid body size is regulated by mechanisms associated with, but distinct from, TG biosynthetic enzymes. Lipid body size in plant seeds is affected by the relative amounts of oleosins, major plant lipid body proteins, which may prevent lipid body coalescence (*21–23*). Although oleosins are present in lipid bodies of many plant oil seeds, there exist some plant lipid bodies that lack oleosins and the existence of oleosins in oleaginous microorganisms has not been reported (*2*, *3*). These results suggest that other mechanisms regulate lipid body size, at least in oleaginous microorganisms.

We demonstrated that the C/N ratio of the culture medium affected lipid body size and TG localization in subcellular fractions, accompanied by altered protein phosphorylation in the lipid body fraction of the oleaginous fungus, *M. ramanniana* var. *angulispora*. In relation to the appearance of smaller lipid bodies at a C/N ratio of 9, TG in the lipid body fraction decreased, while that in the membrane fraction increased, which suggests two possibilities. First, TG biosynthesis in the lipid body fraction is affected by changing the C/N ratio, while that in the membrane fraction is not. This is consistent with our previous observation that diacylglycerol acyltransferase, which catalyzes the final step of TG biosynthesis, is localized in the lipid body fraction, as well as in the membrane fraction (*8*). Secondly, TG transport from intracellular membranes to lipid bodies is affected by changing the C/N ratio, which decreases TG in the lipid body fraction, but rather increases TG in the membrane fraction.

Elevated phosphorylation and protein kinase activation in the lipid body fraction induced by lowering the C/N ratio or increasing nitrogen sources may act as a signal to lipid bodies about nutrient conditions. Nutrient-regulated protein kinases, which receive extracellular nutrient signals in cytosol and transmit these signals to nuclei, have been recently documented in *S. cerevisiae* (*4–7*). In addition to their roles in signal transduction to gene expression, some of these protein kinases also transmit signals to energy-related systems such as autophagy and carbohydrate storage (*7*). Protein kinases in the lipid body fraction of this fungus thus appear similar to nutrient-regulated protein kinases, but are involved in lipid storage in response to the C/N ratio in the medium. Our observations show that the activation of protein kinases and increased phosphorylation in lipid bodies are inversely correlated with lipid body size and lipid accumulation, which raises the possibility that the phosphorylation of lipid body proteins by activated protein kinase directly affects lipid body size and lipid accumulation. Although it is not clear how protein kinases in lipid bodies are regulated, the translocation of protein kinases from intracellular membranes to lipid bodies may take place in response to the C/N ratio. Further studies, including molecular characterization of protein kinases in lipid bodies, should clarify the regulatory mechanisms and physiological roles of increased phosphorylation in lipid bodies.

In vitro phosphorylation in the lipid body fraction indicated the existence of cAMP-dependent phosphorylation. Since the presence of sufficient glucose in medium increases intracellular cAMP in *S. cerevisiae* (*7*, *24*), activated cAMP-dependent phosphorylation in lipid bodies may be derived from glucose sensing in this fungus. It should be noted, however, that cAMP-dependent phos-

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phorylation only emerged in lipid bodies when nitrogen sources were increased in the medium. cAMP-dependent phosphorylation in the lipid body fraction at a C/N ratio of 38 was much lower than that at a C/N ratio of 9, indicating that the increase in cAMP-dependent protein kinase activity in lipid bodies was induced by sufficient amounts of nitrogen sources. The interpretation may therefore be more complex, and signals from carbon and nitrogen sources may interact.

Other phosphorylation reactions induced in the lipid body fraction were active in the absence of cofactors. Deoxycholate, which we unexpectedly found activated *in vitro* phosphorylation of lipid body proteins, is not a known cofactor for protein kinases. Our results suggest that deoxycholate affects the interaction of substrate proteins with enzymes rather than protein kinase activity. In addition, Mn^{2+} was much more effective in promoting protein kinase activity in the lipid body fraction than Mg^{2+}. Since these characteristics of *in vitro* phosphorylation do not coincide with those of known phosphorylation reactions, further molecular characterization of the protein kinases detected by in-gel protein kinase analysis is required to elucidate the phosphorylation reactions. One candidate protein kinase is mitogen-activated protein kinase, since the mitogen-activated protein kinase cascade responds to nutrient limitation (*25*, *26*). Another candidate protein kinase is AMP-activated protein kinase, which is involved in signal transduction in response to lower glucose (*7*, *27*). AMP inhibited *in vitro* phosphorylation in the lipid body fraction, however, suggesting that AMP-activated protein kinase is not responsible for phosphorylation induced in the lipid body fraction.

The phosphorylation of lipid body proteins studied in mammalian cells has shown that a major lipid droplet protein, perilipin, is highly phosphorylated by cAMP-dependent protein kinase (*28*, *29*). Although perilipin reportedly increases TG storage by decreasing TG hydrolysis (*30*), the physiological roles of its phosphorylation remain unclear. Some proteins such as caveolin (*31–33*) and Nir2 (*34*) are not constitutively located in lipid droplets, but transiently targeted to lipid droplets under appropriate conditions. Caveolin is phosphorylated by tyrosine kinases, but it is not known whether its phosphorylation affects its targeting to lipid droplets. Nir2, which has a phosphatidylinositol transfer protein-like domain, is phosphorylated at a specific threonine residue, and its phosphorylation regulates the targeting of Nir2 to lipid droplets (*34*). In contrast, little is known about the phosphorylation of lipid body proteins in microorganisms and plants. In plant oil seeds, caleosin, an oil body protein, has putative phosphorylation sites (*35*, *36*), although the significance of its phosphorylation has not been shown. In *S. cerevisiae*, major lipid particle proteins were identified that did not include protein kinases (*37*). Further studies should show whether nutrient-regulated phosphorylation in lipid bodies is characteristic in this oleaginous fungus or that similar events occur in other organisms.

In conclusion, we found that the C/N ratio affected lipid body size and that lowering the C/N ratio by increasing the nitrogen source activated protein kinases and elevated phosphorylation in lipid bodies accompanied by decreased lipid body size. Molecular characterization of the activated protein kinases and phosphorylated proteins in lipid bodies should provide new insights into the dynamics of lipid bodies. What induces phosphorylation in lipid bodies as a nutrient signal is another interesting question. The signal may be triggered by the total amounts of the nitrogen source or by the relative amounts of the nitrogen and carbon source. Alternatively, signals from carbon and nitrogen sources may affect each other, which determines overall cell responses such as phosphorylation in lipid bodies. Finally, it would be interesting to know how phosphorylation in lipid bodies relates to enzymes in energy-producing systems such as the glycolytic pathway and the citric acid cycle, which are coordinately regulated in response to nitrogen exhaustion in oleaginous microorganisms (*18*, *38*). Answers to these questions will clarify the relationship between nutrients and lipid storage.

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