Oleosin of Plant Seed Oil Bodies Is Correctly Targeted to the Lipid Bodies in Transformed Yeast*

(Received for publication, September 18, 1996)

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Yeast (Saccharomyces cerevisiae) has been used extensively as a heterologous eukaryotic system to study the intracellular targeting of proteins to different organelles. The lipid bodies in yeast have not been previously subjected to such studies. These organelles are functionally equivalent to the subcellular storage oil bodies in plant seeds. A plant oil body has a matrix of oils (triacylglycerols) surrounded by a layer of phospholipids embedded with abundant structural proteins called oleosins. We tested whether plant oleosin could be correctly targeted to the lipid bodies in transformed yeast. The coding region of a maize (Zea mays L.) oleosin gene was incorporated into yeast high copy and low copy number plasmids in which its expression was under the control of GAL1 promoter. Yeast strains transformed with these plasmids produced oleosin when grown in a medium containing galactose but not glucose. The oleosin produced in yeast had a molecular mass slightly higher than that of the native protein in maize. Oleosin accumulated concomitantly with the storage lipids during growth of the transformed yeast, and it was not secreted. Subcellular fractionation of the cell extracts obtained by two different cell breakage procedures revealed that the oleosin was largely restricted to the lipid bodies. Oleosin apparently did not affect the lipid contents and composition of the transformed yeast lipid bodies but replaced some of the native proteins associated with the organelles. Immunocytochemistry of the transformed yeast cells showed that the oleosin was present mostly on the periphery of the lipid bodies. Oleosin isolated from maize or transformed yeast strain, alone or in the presence of phospholipids or SDS, did not bind to the yeast lipid bodies in vitro. We conclude that plant oleosin is correctly targeted to the lipid bodies in transformed yeast and that yeast may be used as a heterologous system to dissect the intracellular targeting signals in the oleosin.

Diverse organisms store lipids in subcellular particles as food reserves that will be mobilized during a forthcoming period of active metabolism. These lipid particles can be found in seeds, pollens, spores, and vegetative organs of plants (1). They are also present in the brown adipose (2) and other tissues of mammals (3), the eggs of some nematodes (4), and unicellular organisms such as yeast (5, 6), Euglena (7), and algae (8). Of all these subcellular storage lipid particles, those from plant seeds have been studied most extensively.

The seeds of many plant species store triacylglycerols (TAGs) as food reserves for germination and growth of the seedlings (1, 3). The TAGs constitute about 5–40% of the total seed dry weight. They are present in small discrete subcellular organelles called oil bodies (lipid bodies, oleosomes, and spherosomes). The spherical organelles have diameters of about 0.6–2.0 μm, depending on the plant species in which they occur. Each oil body contains a TAG matrix surrounded by a layer of phospholipids (PL) embedded with proteins termed oleosins (9). Oleosins essentially cover the whole surface of the oil body and represent 1–4% of the total mass of the oil body; this percentage is related to the size of the organelle. The $M_r$ of oleosins range from 15,000 to 26,000, depending on the isoforms and plant species in which they occur. Each oleosin molecule has the following three structural portions (10–13): (a) an N-terminal amphipathic stretch (20–60 residues) of undefined secondary structures residing on the organelle surface; (b) a central hydrophobic domain (72 residues) of long antiparallel β-structures penetrating into the matrix; and (c) a C-terminal amphipathic α-helix (30–40 residues) locating on the organelle surface, interacting with the PL layer. The three structural portions enable the protein to interact with other molecules on the surface of an oil body. Oleosins form a steric barrier on the surface of an oil body, preventing the PL layers of adjacent oil bodies from contacting and coalescing. Maintenance of the oil bodies as small entities provides a large surface area per unit TAG and would facilitate lipase binding and lipolysis during germination.

During seed maturation, oil bodies are synthesized on the rough ER by a budding process, which has been postulated on the basis of experimental evidence and consideration of the thermodynamics involved (1). TAGs are synthesized by enzymes in the ER and are sequestered, because of their hydrophobicity, between the two PL layers of the ER membrane, thereby forming a bud. PL synthesized by enzymes in the ER also diffuse to the surface of this budding TAG particle. Simultaneously, oleosin is synthesized on polyribosomes bound to the ER without appreciable co- or posttranslational processing. The newly synthesized oleosin moves to the budding TAG particle, presumably guided by the central hydrophobic domain. Alternatively, the oleosins being synthesized on polyribosomes are inserted directly into the budding particle. The budding particle, which has a TAG matrix surrounded by a layer of PL and oleosins, is released into the cytosol as a mature oil body.

Although it is well documented that oleosin is synthesized by

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*The work was supported by the United States Department of Agriculture, Grant 96-00531. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

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1 The abbreviations used are: TAG, triacylglycerol; PL, phospholipids; SCM, synthetic complete medium; TPS, Tris-HCl/phospholymethylsulfonyl fluoride MSF/sorbitol buffer; TS, Tris-HCl/sorbitol buffer; ER, endoplasmic reticulum; Tricine, N,N'-2-hydroxy-1,1-bis(hydroxymethyl)ethylglycine; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin.
polyribosomes bound to the ER, no cleavable N-terminal signal sequence in the protein has been identified (1). Apparently, oleosin does not enter the lumen of the ER, otherwise budding would occur at the luminal side of the ER and the newly formed oil body would enter the intracellular secretory pathway. The latter scenario occurs in the secretion of lipoproteins in mammals (14). The targeting signal in oleosin for the ER or budding oil body is unclear. At the N-terminal region of oleosin where a targeting signal could occur, there are no appreciable similarities in the length, the amino acid residues, and the charge distribution among oleosins of diverse plant species. Oleosin has a conserved central hydrophobic domain that could act as the targeting signal for the hydrophobic matrix of the oil bodies. This targeting signal would be equivalent to the internal hydrophobic targeting sequences in many membrane proteins of diverse organisms (15, 16). Whatever the targeting signal in the oleosin is, it appears to be universal, since maize (a monocotyledonous species) oleosin is correctly targeted to seed oil bodies in transformed Brassica (a dicotyledonous species) (17).

Yeast has been used extensively as a heterologous system to study the intracellular targeting of proteins (review, Ref. 18). Proteins from other eukaryotes have been tested to see whether they are correctly targeted to the appropriate subcellular locations in transformed yeast. These locations include the nuclei, mitochondria, peroxisomes, vacuoles, plasma membranes, and extracellular medium, etc. An obvious omission from this list of subcellular locations is the cytoplasmic lipid bodies. In yeast, the lipid bodies, of about 0.2–0.5 μm in diameter, contain about 50% TAGs and 50% sterol esters, which are accumulated as food reserves during the log phase and the late stage of growth (5, 6). These lipid bodies are similar to the seed oil bodies in function; whether they are also similar in structure is unknown. In this report, we show that plant seed oleosin is correctly targeted to the lipid bodies in yeast transformed with the plant oleosin gene.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—Saccharomyces cerevisiae FY1-1A (MATa, ura3-52, Lys2) and the yeast/Schizosaccharomyces pombe shuttle vectors pQC5 and pQC6 (19) were kindly provided to us by Drs. Q. Chao and M. E. Etzler, Section of Molecular and Cellular Biology, University of California, Davis, CA. Plasmid pTT71 (20) was obtained from Dr. D. R. Gallie, Department of Biochemistry, University of California, Riverside, CA.

Construction of Yeast Vectors Containing Plant Oleosin Gene—A 594-base pair fragment in a cDNA clone, pL2: (21), containing the complete coding sequence and 30 base pairs of the 5′-untranslated sequence of maize 18-kDa oleosin, was amplified by polymerase chain reaction. Using primers containing sequences for creating specific restriction sites, EcoRI and BamHI sites were added to the 5′ and the 3′ end of this fragment, respectively. The blunt-ended EcoRI-BamHI fragment was then subcloned into Smal-cleaved plasmid pTT71 (20) to create plasmid pL2T02 (Fig. 1).

The maize oleosin gene from plasmid pL2T02 was cut with EcoRI and ligated into the EcoRI site of the low copy number centromeric plasmid pQC5 (19) and the high copy number 2-μm plasmid pQC6 (19). Yeast Culture Condition and Transformation—Yeast strain was maintained on a agar medium of 1% yeast extract, 2% peptone, and 2% glucose. Transformation was performed by the lithium acetate method (22). Transformants were selected for uracil auxotrophy on uracil-omitted synthetic complete medium (SCM ura−) (23). The induction of the GAL1 promoter in the transformed yeast strains, glucose (2%) in SCM ura− medium was replaced by galactose (2%).

Yeast growth was monitored by reading the A600 nm of the undiluted culture in a spectrophotometer. The reading reached a maximum of 2.2 after 24 h (data not shown). The maximum absorbance was an underestimated value, since the spectrophotometric reading became nearly linear beyond 1.0. When the 50-h culture was diluted with culture medium such that the reading of A600 nm was at the range of 0.5–1.0, the calculated A600 nm of the 50-h culture was about 4–6. All the A600 nm readings described in the subsequent sections of “Experimental Procedures” indicated those of the undiluted cultures.

Northern Hybridization—Yeast RNA was prepared from mid-log phase cells (A600 nm = 1.0) by a method using glass beads for cell breakage (25). Electrophoresis was carried out with a 1.5% (w/v) agarose, 2.2% formaldehyde gel (26), and 10 μg of RNA was applied to each lane. Following electrophoresis, the RNA was transferred to a nylon membrane and hybridized either with a maize 18-kDa oleosin gene probe or a solution containing 0.125 μM Tris-HCl, pH 7.5, 1 mM MgCl2, 1 mM EDTA, 2 mM dithiothreitol, 0.25 μM sucrose. The 100,000 × g pellet was resuspended in 100 μl of Tricine buffer (0.15 M Tricine-KOH, pH 7.5, 1.5 mM MgCl2, 10 mM KCl, 1 mM EDTA, 2 mM dithiothreitol, 0.25 μM sucrose), mixed with an equal volume of ethylic ether to remove the neutral lipids. The ether extraction was repeated twice. The remaining suspension of oleosin and PL was sonicated and used (as preparation c and d, respectively). Sonication of each of the above suspensions was performed with a 4-mm diameter probe in a Braun-Sonic 2,000 ultrasonic generator (Freeport, IL) with a digital meter reading of 100 for three 20-s periods.

Preparation of Yeast Crude Extract—Yeast cells in 20 ml of culture medium were grown to an A600 nm of 2.0 and harvested by centrifugation at 3000 × g for 5 min. Cells were resuspended in TS buffer (50 mM Tris-HCl, pH 7.5, 1 mM sorbitol) and heated at 100°C for 10 min. The heated cells were centrifuged again and resuspended in new TS buffer. Acid-washed glass beads (425–600 microns) were added to the cell suspension. The cells were lysed by vortexing the suspension at a maximum speed for 10 min. The supernatant was collected as crude extract after the beads settled.

SDS-PAGE and Immunoblotting—Urea (12.5%) SDS-PAGE and the pretreatment of samples were described (26). Immunoblotting and the preparation of chicken antibod-

ies raised against maize 18-kDa oleosin were as described (27).

Subcellular Fractionation—Yeast cells harboring plasmid pQQ6-oleosin were grown in SCM ura− medium containing 2% galactose at 30°C to an A600 nm of 2.0 (at the late log phase). Cells were collected by centrifugation (3000 × g for 5 min), and the left over medium was saved for further analysis. The pelleted cells were resuspended in 60 ml of TS buffer containing 3 mg/ml lyticase (ICN, Costa Mesa, CA) and incubated at 30°C for 30 min. The resulting spheroplasts were harvested by centrifugation (3000 × g for 5 min), washed in TS buffer, and resuspended in 60 ml of 50 mM Tris-HCl, pH 7.5, 2 mM PMSF. The resuspension in a 250-ml flask was shaken vigorously by hand and homogenized in an ice-cold glass tissue homogenizer with 25 strokes, using a tight-fitting pestle. The preparation was centrifuged at 1000 × g for 5 min to remove cell debris. The supernatant was designated as the crude homogenate. An aliquot of the crude homogenate was saved for further use. Sorbitol was added to the crude homogenate to give a final concentration of 1 M. The homogenate (15 ml each in two tubes) was overlaid with 15 ml of TPS buffer (50 mM Tris-HCl, pH 7.5, 2 mM PMSF, 0.5 M sorbitol), and centrifuged at 100,000 × g for 2 h. A floating layer consisting of lipid bodies was collected from the top of the tube with a pipette. The lower culture medium, the 100,000 × g supernatant, and the crude homogenate were subjected to 80% ammonium sulfate precipitation to concentrate the fractions in the floating layer. A precipitated sample was resuspended in 300 μl of Tricine buffer (0.15 M Tricine-KOH, pH 7.5, 1.5 mM MgCl2, 10 mM KCl, 1 mM EDTA, 2 mM dithiothreitol, 0.25 μM sucrose). The 100,000 × g pellet was resuspended in 100 μl of Tricine buffer. The lipid body preparation was microcentrifuged at 14,000 × g for 15 min. The floating layer was left undisturbed, and the solution below the layer was removed with a fine needle. The microcentrifugation was performed several times to concentrate the lipid bodies to a final volume of 100 μl.

An alternative method of subcellular fractionation was carried out. Yeast cells collected by centrifugation (3000 × g for 5 min) were mixed with cold, acid-washed glass beads and homogenized with a mortar and pestle in a cold room. TPS buffer was added to make a suspension. The suspension was centrifuged (1,000 × g for 5 min) to yield a crude homogenate. The crude homogenate was subjected to a similar procedure of subcellular fractionation by centrifugation, as described in the preceding paragraph.

Preparation of Oleosin for in Vitro Binding Test—Maize oleosin, in a 1:12 (w/w) mixture of three isofoms of 18, 17, and 16 kDa, was prepared from oil bodies isolated from maize kernel (9). The oil body fraction, in 0.15 M Tricine-KOH, pH 7.5, 1 mM MgCl2, 10 mM KCl, 1 mM EDTA, 2 mM dithiothreitol, 0.25 μM sucrose, was mixed with an equal volume of ethyl ether to remove the neutral lipids. The ether fraction was repeated twice. The remaining suspension of oleosin and PL was sonicated and used (as preparations a and b under “Results”). An aliquot of the suspension was subjected to a procedure for PL removal using chloroform and methanol as described earlier (9). After removal of the phospholipids, the remaining oleosin was resuspended in water or a solution containing 0.125 μM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.1% SDS, and the water suspension was sonicated and used (as preparations c and d, respectively, under “Results”).
Oleosin synthesized in yeast strain transformed with pQC6-ole was prepared as follows. The transformed yeast cells were homogenized with glass beads, and the lipid bodies were isolated as described in the preceding section. The lipid body fraction was treated with diethyl ether to remove the neutral lipids as mentioned in the preceding paragraph. The resulting suspension of the 18-kDa oleosin, other proteins, and PL was sonicated and used (as preparation e under "Results"). An aliquot of the lipid body fraction was subjected to SDS-PAGE. After electrophoresis, the gel containing the oleosin was cut, and the protein in the gel was eluted into a solution containing 0.125 M Tris-HCl, pH 8.0, 1 mM EDTA, and 0.1% SDS. This suspension was used (as preparation f under "Results").

In Vitro Binding Test—The oleosin preparations were assayed for their protein contents using the Bradford method (28). The amounts of 18-kDa oleosin in the preparations were estimated by the amount of this oleosin in comparison with those of other proteins of the preparations in an SDS-PAGE gel. Each oleosin preparation was incubated with the homogenate (1.50 v/v) of nontransformed yeast strain (prepared as described under "Subcellular Fractionation") by shaking in a horizontal shaker at 200 rpm for 30 min in a cold room. The mixture was then subjected to subcellular fractionation (described under "Subcellular Fractionation"). In each mixture, the proportion of 18-kDa oleosin to homogenate was similar to that in the homogenate of yeast strain transformed with pQC6-ole (to be described in Fig. 5), except in oleosin preparation b, in which the oleosin:homogenate ratio was five times higher.

Determination of Lipids—Yeast cells were collected by centrifugation from the culture at different stages of growth. The volume of the pelleted cells was considered as 1 volume. The sample was mixed with 5 volumes of TS buffer containing 2 μg/μl lyticase and incubated at 30 °C for 2 h. The resulting spheroplasts were harvested and lysed by osmotic breakage as described in the preceding section. The lysate was extracted immediately with an equal volume of diethyl ether. The ether fraction was collected, and the ether was evaporated under a stream of nitrogen gas. The acyl esters in the residual lipids were quantitated (29).

Isolated yeast lipid bodies were extracted with diethyl ether three times. The acyl esters of the extracted lipids were quantitated similarly (29). The lipids were resolved by TLC, using a plate coated with silica gel 60A (Whatman, Maidstone, United Kingdom). The plate was developed in hexane/diethyl ether/acetic acid (80:20:2, v/v/v), dried, and allowed to react with iodine for color development.

Immunocytochemistry—Yeast strains nontransformed and transformed with pQC5-ole and pQC6-ole were grown to an A600 nm of 1.9. The culture was centrifuged at 3,000 × g for 5 min to pellet the cells. The pelleted cells were rinsed twice with water and resuspended in half-strength Karnovsky’s solution at 4 °C (30). After 0.5 h and mild agitation, the solution was replaced with half-strength Karnovsky’s solution, and the mixture was incubated for 12 h at 4 °C. The cells were washed twice with cold, 50 mM NaP buffer (pH 6.8). The preparation was rapidly dehydrated (in 1 h) in a series of ethanol solutions to 95% ethanol. The cells were infiltrated with LR. White resin over 12 h and allowed to polymerize at 60 °C for 30 h.

Sections of 100 nm thickness were cut on a MT6000 Ultramicrotome (RMC Inc. Tucson, AZ) and picked up on nickel (300 mesh) grids. All subsequent procedures were carried out in 75-μl droplets in ceramic wells. Grids were submersed for 30 min in a solution of 150 mM NaP buffer, pH 6.8 (PBS), 0.1% Triton X-100, and 0.1% BSA. Grids were incubated for 1 h in anti-oleosin polyclonal chicken antibodies (27) diluted 1:500 with PBS, 0.1% Triton X-100, and 0.1% BSA. Grids were washed five times, each for 1 min, in PBS, 0.1% Triton X-100, and 0.1% BSA, and then incubated for 1.5 h in rabbit anti-chicken IgG conjugated with 12-nm colloidal gold particles (Jackson Immuno Research Laboratories, West Grove, PA) diluted 1:40 with PBS, 0.1% Triton X-100, and 0.1% BSA.

Grids were poststained for 15 min in 1% aqueous uranyl acetate and for 2 min in Reynolds’s lead citrate. Photographs were taken using Kodak 4489 Electron Microscope film (Eastman Kodak Co.) on a Philips 400 transmission electron microscope at 100 kV.

RESULTS
Maize Oleosin Gene Was Expressed in Transformed Yeast—The coding region of a maize seed oleosin gene was incorporated into two yeast plasmids in which its expression was under the regulation of GAL1 promoter (Fig. 1). A low copy number centromeric plasmid (pQC5-ole) and a high copy number 2-μm plasmid (pQC6-ole) were constructed.

Untransformed yeast strain and yeast strains transformed with the above two plasmids and with the same plasmids but harboring no maize oleosin gene were grown in a medium containing galactose. Total RNAs were extracted from these yeast strains at the mid-log phase of growth and subjected to Northern blot hybridization using a 32P-labeled maize oleosin cDNA probe (Fig. 2). Oleosin mRNA, of about 0.8 kilobase pairs, was present in yeast strains transformed with pQC5-ole and pQC6-ole and was substantially more abundant in the latter strain. Yeast strain that was not transformed or transformed with pQC5 or pQC6 (plasmid without oleosin gene) did not contain oleosin mRNA. When yeast strain transformed with pQC6-ole was grown in glucose instead of galactose, it did not contain oleosin mRNA. These findings indicate that the oleosin gene in yeast strain transformed with pQC6-ole was properly expressed under the control of the GAL1 promoter.

The crude extracts of the above yeast strains were analyzed for their contents of oleosin protein by SDS-PAGE and immunoblotting, using antibodies against the maize oleosin (Fig. 3). The preimmune IgY and antibody-containing IgY did not recognize any protein of the yeast strains not transformed or transformed with control plasmids (pQC5 or pQC6). The antibodies recognized a protein of 19 kDa in yeast strains transformed with pQC5-ole and pQC6-ole, but they did not recognize any other protein on the blot. This 19-kDa protein was not recognized by the preimmune IgY. Although the 19-kDa protein in yeast strain containing pQC6-ole was easily detectable, its presence in yeast strain containing pQC5-ole was barely observable by the eye but not observable after photography (Fig. 3). This 19-kDa protein should be the oleosin derived from the maize gene in the plasmids. Apparently, both oleosin mRNA and protein were stable in yeast strains transformed with pQC5-ole and pQC6-ole.
Oleosin present in the two transformed yeast strains had an $M_r$ of about 19,000, and no apparent breakdown products of oleosin of lower $M_r$ were detected by immunoblotting (Fig. 3). In the preparation of crude extracts (shown in Fig. 3), the yeast cells were heated to 100°C to prevent proteolysis and then homogenized. Breakdown products of oleosin were observed when the cells were treated with a commercial lyticase preparation and lysed by osmotic shock (to be described). Oleosin in transformed strains (no plasmid) or transformed with pQC5-ole (with maize oleosin gene), pQC5 (without maize oleosin gene), pQC6-ole (with maize oleosin gene), or pQC6 (without maize oleosin gene). Yeast strains were grown in a medium containing galactose (gal) or glucose (glu) as indicated. The lower panel shows an identical gel after electrophoresis (without blotting), and the gel was stained with ethidium bromide. This panel reveals that roughly equal amounts of intact 26S and 17S ribosomal RNA were present in each sample.

The yeast strain transformed with pQC6-ole produced a higher amount of oleosin, and we used this yeast strain to explore biochemically the expression of the oleosin gene and the subcellular location of the oleosin protein.

**Oleosin Accumulated Concomitantly with Lipids in Transformed Yeast during Growth**—Yeast accumulates lipids (TAGs and steroid esters) as food reserves during growth. In the current study, yeast strains nontransformed and transformed with pQC6-ole or pQC6 grew in galactose and accumulated lipids by an indistinguishable pattern (data not shown). The accumulation of lipids in these strains lagged behind the increase in cell culture density (Fig. 4); this delay is expected for the accumulation of a food reserve. In the yeast strain transformed with pQC6-ole, the amount of oleosin per culture volume increased with time and appeared to follow that of the lipids (Fig. 4); the low sensitivity of the immunoblot assays prevents us from making a definite statement. Supporting evidence comes from the finding that the amount of oleosin per equal amounts of total cell proteins also increased with time (Fig. 4). One possibility is that oleosin was produced constitutively but did not accumulate (i.e. was degraded) in the absence of lipids. Further studies are required to examine this possibility. In all of the above yeast strains, cell growth was faster in a medium containing glucose than in one containing galactose (data not shown).

**Subcellular Fractionation Revealed That Oleosin in Transformed Yeast Was Present Mostly in the Lipid Bodies**—We explored the subcellular location of oleosin in the yeast strain transformed with pQC6-ole by fractionating the cell homogenate into various subcellular fractions and analyzing their oleosin contents. Cells at the late log phase (45 h, see Fig. 4) were harvested by low speed centrifugation. They were lysed by treatments with a lyticase preparation and followed by an osmotic shock. After cell breakage, the homogenate was centrifuged to yield fractions of 100,000 $\times$ g supernatant, 100,000 $\times$ g pellet, and floated lipid bodies. The crude homogenate, the culture medium (containing excreted proteins), and the various subcellular fractions were subjected to SDS-PAGE and immuno-
We also studied the subcellular location of oleosin in the transformed yeast cells using an alternative procedure of cell breakage in which the oleosin was maintained intact. After being harvested by a low speed centrifugation, the cells were homogenized with glass beads using a mortar and pestle. The homogenate was subjected to a similar procedure of subcellular fractionation by centrifugation. As much as 80–90% of the oleosin could be recovered in the lipid body fraction, and the remaining oleosin was present in the supernatant and the pellet (Fig. 5). In this procedure of cell breakage, the percentage of oleosin recovered in the lipid body fraction varied, from 50 to 90%, apparently depending on the severity of the homogenization with the pestle. The advantage of using this procedure was that it generated no degraded oleosin.

The results obtained from subcellular fractionation using the two different procedures of cell breakage clearly show that oleosin in the cells of the yeast strain transformed with pQC6-ole was localized in the lipid bodies. Collaborative evidence comes from immunocytochemistry results (to be described).

Oleosin Did Not Alter the Lipids of the Transformed Yeast Lipid Bodies but Replaced Some of the Native Proteins Associated with the Organelles—Lipid bodies isolated from yeast strains nottransformed and transformed with pQC6-ole were subjected to lipid extraction, and their lipid constituents were separated by TLC (Fig. 6). Two major neutral lipid constituents were resolved; they were tentatively identified to be steroid esters and TAGs, as reported earlier (5, 6). The lipid composition of the lipid bodies from both yeast strains as resolved by TLC were indistinguishable (Fig. 6). This finding, together with the identical patterns of cell growth and lipid accumulation during culturing of the two yeast strains, shows that the presence of oleosin in the yeast cell did not affect appreciably the synthesis and accumulation of lipids in the lipid bodies. Nevertheless, oleosin did affect the native yeast proteins in the lipid bodies. The proteins in the lipid body fractions from the two yeast strains were resolved by SDS-PAGE (Fig. 6). Equal amounts (acyl ester bonds) of lipid bodies from the two yeast strains were loaded onto the gel for a direct comparison. The
proteins from these two samples resolved in the gel would represent those from the same number of lipid bodies, since the lipid bodies in both yeast strains appeared to be of a similar size (next section). The organelles from the nontransformed yeast strain contained several proteins, of Mr in the range of 30,000–75,000 (Fig. 6). This pattern of proteins associated with the lipid body fraction of yeast was similar to that reported earlier (6). In the transformed yeast strain, although the native proteins were still present, their amounts were reduced to about half of those in the nontransformed yeast strain. The loss of the native proteins apparently was compensated by the gain of a similar amount of oleosin, and the protein-to-lipid ratios in the lipid bodies from the two yeast strains remained about the same. Thus, replacement of half of the native proteins with an equal amount of oleosin did not affect appreciably the integrity and synthesis of the organelles.

Immunocytochemistry was performed to locate oleosin in the yeast strain transformed with pQC5-ole and pQC6-ole, and the nontransformed yeast strain was used as a control. The lipid bodies in the cells of these yeast strains were electron transparent, of diameters about 0.2–0.5 μm (Fig. 7). They were usually present in clusters and apparently did not coalesce. There were interfacial materials on the periphery of the lipid bodies. In the immunodetection of oleosin in the yeast strains, the nontransformed strain showed no immunogold particles in the cytoplasm including the lipid bodies, whereas yeast strains transformed with pQC5-ole, and abundant in yeast transformed with pQC6-ole. In the transformed yeast, the immunogold particles were present mostly on the periphery of the lipid bodies.

**Fig. 6.** TLC of lipids and SDS-PAGE of proteins from lipid bodies of yeast strains nontransformed and transformed with pQC6-ole and from oil bodies of maize seed. Equal amounts (acyl ester bonds, 1 [TIMES]) of lipid bodies from the two yeast strains and oil bodies from maize (1, 3, or 12.5 [TIMES]) were applied to the chromatograph and the gel. The upper panel shows the thin layer chromatograph on which the lipids were allowed to react with iodine. The positions of the steroid esters, TAGs, and origin are indicated on the right. The lower panel is the SDS-PAGE gel in which the proteins were stained with Coomassie Blue. Maize oil bodies were larger than yeast lipid bodies and therefore contained a lesser amount of proteins (surface) per unit of lipids (matrix); only the 12.5 [TIMES] sample showed the oleosins clearly. Positions of the three oleosins (shown with their molecular mass), as well as position of the oleosin synthesized in the yeast strain (arrow), are shown on the right.

**Fig. 7.** Electron micrographs of portions of yeast cells immunolabeled with antibodies against the maize oleosin. Yeast strains nontransformed (uppermost photo) and transformed with pQC5-ole (middle photo) and pQC6-ole (lowest photo) were allowed to grow to late log phase (45 h, see Fig. 4) and subjected to electron microscopy and immunolabeling. Lipid bodies are seen as electron-transparent particles of about 0.2–0.5 μm in diameter. Immunogold particles were absent in the nontransformed yeast, scarce in yeast transformed with pQC5-ole, and abundant in yeast transformed with pQC6-ole. In the transformed yeast, the immunogold particles were present mostly on the periphery of the lipid bodies.
the lipid bodies and, specifically, was present on the periphery of the organelles.

By SDS-PAGE and immunoblotting (Fig. 3), we barely detected the oleosin in yeast strain transformed with the low copy number plasmid pQC5-ole. By immunocytochemistry, we were able to observe in this yeast strain numerous immunogold particles that were concentrated on the lipid bodies (Fig. 8). Apparently, immunocytochemistry was a more sensitive method of detection.

Oleosins Did Not Bind to Yeast Lipid Bodies in Vitro—Whether oleosin synthesized by transformed yeast strains bound to the lipid bodies in vivo merely because of its hydrophobicity was investigated. Maize oleosin was incubated with the homogenate of nontransformed yeast strain, and the mixture was subjected to subcellular fractionation. Six different preparations of oleosin were used. (a) Oleosins in three isoforms, of 16, 17, and 18-kDa, together with the native PL, were obtained from isolated maize oil bodies and sonicated into a suspension; (b) same as a except the amounts of all components were 5 times higher; (c) same as a except the PL were removed by chloroform/methanol; (d) same as c except the oleosins were not sonicated but resuspended in 0.1% SDS; (e) yeast-synthesized oleosin, together with other proteins and PL, was obtained from lipid bodies isolated from pQC6-ole transformed yeast strain and sonicated into a suspension; and (f) yeast-synthesized oleosin was obtained from lipid bodies isolated from pQC6-ole-transformed yeast strain by SDS-PAGE and resuspended in 0.1% SDS.

In each of the mixtures, the proportion of 18-kDa oleosin to homogenate was similar to that in the homogenate of yeast strain transformed with pQC6-ole (see Fig. 5), except for oleosin preparation b, in which the proportion of oleosin to homogenate was 5 times higher. Fig. 9 shows results of the subcellular fractionation. In each of the six mixtures, the in vitro applied oleosin was not found in the isolated lipid bodies. Instead, it was present in the pellet fraction and, in most mixtures, also in the supernatants. Oleosin in this form was present in the mixture during its incubation with the homogenate and did not bind to the lipid bodies in vitro. This observation reiterates that the oleosin present on the lipid bodies in transformed yeast represents the consequence of a specific in vivo targeting event.

**DISCUSSION**

Yeast transformed with a plant oleosin gene synthesizes oleosin, which remains stable in the cell. In addition, the oleosin is correctly targeted to the lipid bodies. The targeting ap-
pears to be a specific in vivo event. In a transformed yeast strain that contains a high amount of oleosin, we have demonstrated by biochemical means that the oleosin is present mostly in the lipid bodies. This localization does not appear to be a fortuitous association of the abundant foreign hydrophobic protein with the lipid bodies because we have also shown by immunocytochemistry that a transformed yeast strain containing a minimal amount of oleosin has the oleosin present exclusively in the lipid bodies. Oleosin is present only on the amphipathic surface of the lipid bodies rather than on other amphipathic membranes. Also, oleosin prepared in different forms, including solitary molecules and those associated with PL and SDS, fails to bind to the yeast lipid bodies in vitro. The targeting signal in oleosin is unknown, although apparently it is not the C-terminal α-helix (31, 32). What is known is that oleosin is synthesized on bound polyribosomes without appreciable co- or posttranslational processing (11). Whether the targeting signal resides on the N terminus or the long central anti-parallel β-stranded hydrophobic domain, or both, and what role the ER plays in directing the oleosin to the budding oil body remain to be elucidated. It is possible that the targeting signal and the stability of the oleosin on the lipid body together contribute to its stable association with the organelles.

It is tedious to study the targeting signal in oleosin by transforming plants with numerous modified oleosin genes. The current study offers the opportunity of using yeast as a heterologous system to analyze in detail the targeting signal in oleosin via in vitro mutagenesis of an oleosin gene. Plants and yeast share many intracellular targeting signals in proteins, although yeast does not recognize the targeting signal in plant storage protein for the vacuoles (19, 33). One should analyze extensively the targeting signals in the oleosins for the yeast lipid bodies and then test the validity of selected results with the tedious plant transformation system.

In plants, oleosin exists in two isoforms, which apparently occur as a heterodimer or heteromultimer on the surface of the oil bodies (26, 27). The current study shows that one oleosin isoform can be present alone on the organelle surface (i.e., the maize isoforin 18-kDa oleosin used in the current study is present alone without its counterpart isoforin 16-kDa oleosin). Thus, dimerization does not appear to play a role in targeting the oleosin to the yeast lipid bodies.

The plant oil bodies and the yeast lipid bodies are similar in their apparent structure and function. Whether they possess some differences remains to be explored. A plant oil body has a matrix of TAGs surrounded by a layer of PL embedded with unique and abundant oleosins (1). This organization allows the stable association of all the molecules involved, such that numerous oil bodies of small sizes can be maintained in the cytosol. On the basis of this concept, we visualize a yeast lipid body to have the abundant hydrophobic TAGs and steroid esters located in the matrix and the amphipathic PL and proteins (of minimal but sufficient quantities (5, 6)) at the periphery. Unlike oleosins in plant oil bodies, the proteins in yeast lipid bodies are of numerous molecular species (Fig 6); whether these proteins serve as the structural proteins is unknown. Isolated yeast lipid body fractions contained diacylglycerol acyltransferase (34) and sterol Δ⁴⁴methyltransferase (6), which catalyze the last steps of TAG and steroid ester synthesis, respectively. The activities of these two enzymes in the lipid body fractions were high in terms of specific activities (on per mg of protein basis) but low in terms of percentage of total cellular activities (because there were minimal amounts of proteins in the lipid body fractions). Whether these enzymes are authentic proteins of the lipid bodies needs to be elucidated. In plants, diacylglycerol acyltransferase of a high specific activity but of a low percentage of total cellular activity was reported to be present in an isolated oil body fraction; it was subsequently shown to be a contaminant from the ER (1, 3).

Thus, we do not know whether in yeast TAGs and steroid esters are synthesized in the ER, as has been shown for the plant TAGs, or directly on the surface of existing lipid bodies, as has been suggested on the basis of the high enzyme specific activities. During utilization of the oil reserves in plants, lipase is newly synthesized and binds to the oil bodies (35). In yeast, steroid ester hydrolyase activity was detected in many subcellular fractions (36). Whether the activity belonged to one or more enzymes and which of these enzymes, if any, mobilized the storage steroid esters remain to be studied. Plant seeds contain several acyl hydrolases, which are known not to be responsible for the mobilization of the storage oils (1). Even less is known about the catabolism of TAGs in yeast lipid bodies. The abundant information on the structure, function, and ontogeny of plant oil bodies, both in concept and study techniques, should be utilized for detailed studies of yeast lipid bodies.

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