An in vitro system was established to examine the targeting of proteins to maturing seed oil bodies. Oleosin, the most abundant structural protein, and caleosin, a newly identified minor constituent in seed oil bodies, were translated in a reticulocyte lysate system and simultaneously incubated with artificial oil emulsions composed of triacylglycerol and phospholipid. The results suggest that oil bodies proteins could spontaneously target to artificial oil emulsions in a co-translational mode. Incorporation of oleosin to artificial oil emulsions extensively protected a fragment of approximately 8 kDa from proteinase K digestion. In a competition experiment, in vitro translated caleosin and oleosin preferentially target to artificial oil emulsions instead of microsomal membranes. In oil emulsions with neutral phospholipids, relatively low protein targeting efficiency was observed. The targeting efficiency was substantially elevated when negatively charged phospholipids were supplemented to oil emulsions to mimic the native phospholipid composition of oil bodies. Mutated caleosin lacking various structural domains or subdomains was examined for its in vitro targeting efficiency. The results indicate that the subdomain comprising the proline knot motif is crucial for oleosin targeting to oil bodies. A model of direct targeting of oil-body proteins to maturing oil bodies is proposed.

Key words: Caleosin — Negatively charged phospholipids — Oil body targeting — Oleosin — Proline knot.

Abbreviations: EGTA, ethylene-glycol-bis(β-aminoethyl ether)-tetraacetic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL(s), phospholipid(s); PS, phosphatidylserine; SRP, signal-recognition particle, TAG(s), triacylglycerol(s).

Introduction

Plant seeds store triacylglycerols (TAGs) as energy sources for germination and post-germinative growth of seedlings. The storage TAGs are confined to discrete spherical organelles called oil bodies, lipid bodies, oleosomes or spherosomes (Yatsu and Jacks 1972, Murphy 1993, Huang 1996, Napier et al. 1996). Oil bodies remain as individual small organelles even after a long period of storage in plant seeds (Slack et al. 1980). This stability is a consequence of the steric hindrance and electronegative repulsion provided by structural proteins, i.e. oleosins, on the surface of oil bodies (Tzen et al. 1992). Oleosins seem to cover the entire surface of an oil body, such that the compressed oil bodies in the cells of a mature seed never coalesce or aggregate (Tzen and Huang 1992).

The TAG matrix of an oil body is surrounded by a monolayer of phospholipids (PLs) embedded with abundant oleosins and some minor proteins of higher molecular mass (Tzen et al. 1997). An oleosin molecule is proposed to comprise three distinct structural domains: an N-terminal amphipathic domain, a central hydrophobic anchoring domain, and a C-terminal amphipathic α-helical domain (Vance and Huang 1987). Sequence comparison among diverse species reveals that the central anchoring domain of oleosin is highly conserved, particularly in a relatively hydrophilic motif known as the proline knot (Tzen et al. 1992).

Targeting of oleosins to seed oil bodies has been investigated in either in vivo systems via transgenic plants (Lee et al. 1991, van Rooijen and Moloney 1995, Abell et al. 1997, Sarmiento et al. 1997) or in vitro systems using microsomal membranes for integration of translated proteins (Hills et al. 1993, Loer and Herman 1993, Thoytes et al. 1995). The proline knot motif seems to play a crucial role for oleosin targeting to oil bodies (Abell et al. 1997). Based on in vitro studies, oleosins are proposed to target to the ER under the assistance of the signal-recognition particle prior to their accumulation on oil bodies (Hills et al. 1993, Loer and Herman 1993).

Three minor proteins, temporarily termed Sops 1–3, have been identified exclusively in sesame oil bodies via immunofluorescence labeling (Chen et al. 1998). However, the biological functions of these three minor proteins remain unknown. Recently, a cDNA clone encoding sesame Sop1, named caleosin for its calcium-binding capacity, has been sequenced (Chen et al. 1999). Similar to oleosin structure, caleosin comprises three distinct structural domains: an N-terminal hydrophilic domain (including a calcium-binding motif), a central hydrophobic anchoring domain, and a C-terminal hydrophilic domain. In addition, a comparable proline knot motif is located in the central hydrophobic domain of caleosin. Whether this structural analogue in caleosin is responsible for its targeting to oil bodies remains to be elucidated.

In this study, we established a novel in vitro system to examine protein targeting to seed oil bodies using artificial oil
emulsions constituted with TAG and PL. Our results suggest that oil-body proteins spontaneously target to artificial oil emulsions in a co-translational mode. Effects of negatively charged PLs on in vitro targeting of caleosin and oleosin to artificial oil emulsions were evaluated. The structural domain required for caleosin targeting to artificial oil emulsions was also investigated by deletion mutagenesis. Based on the experimental results, a direct targeting model of oil-body proteins to maturing oil bodies is proposed.

### Results

**Migration shift of caleosin induced by calcium binding in SDS-PAGE**

It has been demonstrated that caleosin migrates faster in SDS-PAGE when associated with Ca\(^{2+}\) (Chen et al. 1999). Caleosin purified from sesame oil bodies, over-expressed in *Escherichia coli*, or in vitro translated in the Promega reticulocyte lysate system was separately treated with 100 mM EGTA and then incubated with 100 mM Ca\(^{2+}\). The proteins were either stained with Coomassie Blue (for oil-body and *E. coli* proteins) or visualized by fluorography (for in vitro translated caleosin). Labels on the left indicate the molecular masses of proteins.

**Spontaneous and co-translational targeting of in vitro translated caleosin and oleosin to artificial oil emulsions**

An in vitro system was established to examine protein targeting to maturing seed oil bodies by incubating in vitro translated proteins with artificial oil emulsions composed of TAG and PL. For the two known oil body proteins, caleosin and oleosin, relatively high amounts of in vitro translated proteins were located in the oil fraction when artificial oil emulsions were supplied during protein translation, but found in supernatant and pellet fractions when artificial oil emulsions were incorporated after protein translation (Fig. 2). The recovered pellet in the fractionation was almost invisible and probably composed of aggregation of in vitro translated proteins. For the soluble 2S albumin precursor, relatively high amounts of in vitro translated proteins were consistently found in supernatant regardless the incorporation of artificial oil emulsions. For the hydrophobic membrane protein HrpW, relatively high amounts of in vitro translated proteins were consistently found in the pellet regardless of the incorporation of artificial oil emulsions. The results indicate that oil-body proteins spontaneously target to artificial oil emulsions in a co-translational mode without apparent assistance of other factors, and that the targeting does not result from non-specific hydrophobic interaction between translated proteins and artificial oil emulsions. In the presence or absence of Ca\(^{2+}\), similar efficiency was observed for caleosin targeting to artificial oil emulsions. It is evident that calcium...
binding is not essential for caleosin targeting to artificial oil emulsions. This judgment is also supported by the targeting analysis using mutated caleosin lacking N-terminal domain derived from deletion mutagenesis (see the following results).

Enhancement on resistance of oleosin to proteolytic digestion after targeting to artificial oil emulsions

To examine the topology of oleosin in the above in vitro targeting system, oleosin was translated before or after inclusion of artificial oil emulsions and subjected to proteinase K digestion. In vitro translated oleosin was completely degraded within 20 min when it was translated before inclusion of artificial oil emulsions (Fig. 3A). The degradation could be significantly retarded when oleosin was translated in the presence of artificial oil emulsions prior to proteinase K digestion (Fig. 3B). After exhaustive digestion, a polypeptide of approximately 8 kDa, equivalent to the mass of oleosin central hydrophobic domain, was observed. Similar protein profile, particularly a protected polypeptide of approximately 8 kDa, could be observed when seed-purified oil bodies were subjected to proteolytic digestion (Tzen et al. 1992). The results suggest that oleosin co-translationally targeting to artificial oil emulsions apparently elevated its resistance to proteinase K digestion and its central hydrophobic domain is probably protected from the proteolytic digestion.

Translated caleosin and oleosin preferentially targeting to artificial oil emulsions instead of microsomal membranes

Compared with the post-translational results in Fig. 2, the relative content of co-translated caleosin or oleosin increased in the pellet fraction when incubated with microsomal membranes (Fig. 4, middle panel), in accord with the previous reports (Hills et al. 1993, Loer and Herman 1993). To evaluate the preferential targeting of oil-body proteins to the ER and budding oil bodies in maturing seeds, microsomal membranes and artificial oil emulsions were allowed to compete for translated caleosin or oleosin in the in vitro targeting system. In our experimental conditions, both caleosin and oleosin preferred targeting to artificial oil emulsions instead of microsomal membranes (Fig. 4, right panel), even though the PL content (accessible surface area for protein targeting) of microsomal membranes was approximately 250 times more than that of artificial oil emulsions in the co-existent competition. The results suggest that oil-body proteins are capable of targeting to maturing oil bodies directly without ER transportation.

To examine if the preferential targeting to artificial oil emulsions is specific for oil-body proteins, the soluble 2S albumin precursor and hydrophobic membrane protein HrpW were in vitro translated accordingly for targeting analysis. A relatively high amount of the soluble 2S albumin precursor was detected in the microsomal fraction regardless the presence of artificial oil emulsions, presumably resulting from its ER targeting via the signal-recognition particle (SRP) by recognizing the N-terminal signal sequence. The majority of the hydrophobic HrpW was consistently found in the pellet fraction regard-
In vitro protein targeting to oil bodies

Caleosin comprises three structural domains including an N-terminal hydrophilic domain, a central hydrophobic anchor-

Discussion

An in vitro system using microsomal membranes as targets for integration of translated proteins has been employed to investigate oleosin targeting to seed oil bodies (Hills et al. 1993, Loer and Herman 1993, Thoytes et al. 1995). However, detection of oil-body protein targeting in this in vitro system raises the concern whether the detected polypeptides in the microsomal fraction are translated proteins truly integrated into the membranes or merely co-precipitated contaminant proteins aggregated due to their hydrophobic property. To investigate specific targeting of oil-body proteins, we established a novel in vitro system by constituting artificial oil emulsions mimicking maturing seed oil bodies for integration of translated proteins. Under our experimental conditions, valid targeting to artificial oil emulsions was observed only for co-translated oil-body proteins, but not for post-translated polypeptides (Fig. 2). Impediment on post-translational targeting to artificial oil emulsions presumably resulted from hydrophobic aggregation of the oil-body proteins following translation. Our results suggest that oil-body proteins are capable of spontaneously targeting to oil bodies in a co-translational mode without apparent assistance of other factors, such as SRP complex, ER membrane proteins, or molecular chaperones. In fact, neither caleosin nor oleosin possesses cleavable signal sequence required
for the SRP-dependent pathway of targeting to the ER (Chen et al. 1997, Chen et al. 1999).

The success of our in vitro targeting system also suggests that oil-body proteins are capable of integrating into maturing oil bodies whose surface areas presumably are not widely covered by proteins. In contrast, in vitro translated proteins including oil-body specific caleosin and oleosin could hardly target to mature oil bodies whose surface areas are entirely covered by proteins (data not shown). This observation is in accord with the result using in vitro translated soybean oleosin reported by Loer and Herman (1993). Meanwhile, in vitro translated proteins were unable to integrate into oil emulsion constituted with pure TAG (data not shown). A similar phenomenon was observed using in vitro translated rapeseed oleosins (Hills et al. 1993). Indeed, the unstable pure TAG emulsions coalesced to form an oil layer on top of the aqueous reaction solution not long after emulsification, and thus segregated from the translated proteins.

It has been proposed that oleosins are transported via the ER prior to their accumulation on oil bodies (Loer and Herman 1993, Hills et al. 1993). However, our results (Fig. 2, 4) suggest another possible (probably the major) route of direct targeting of oil-body proteins to maturing (or budding) oil bodies (Fig. 7). The model of direct targeting proposes that oil-body proteins spontaneously target to maturing oil bodies in a co-translational mode without packaging and transportation via the ER while TAG, PC, PS, and PI are first synthesized in the smooth ER, directed toward maturing oil bodies, and assembled into mature lipid-storage organelles.

Regardless the lack of sequence homology, both caleosin and oleosin, the two known oil-body proteins, possess a similar proline knot motif (Frandsen et al. 2001). The proline knot motif in oleosin has been proposed to play an essential role for its targeting to oil bodies (Abell et al. 1997). In our in vitro targeting analysis, the crucial structural portion required for caleosin targeting to artificial oil emulsions is the central hydrophobic domain, particularly the subdomain including the proline knot motif. The helical subdomain preceding the proline knot subdomain of caleosin increases the hydrophobicity of the protein, and thus probably enhances its stability in seed oil bodies via hydrophobic interaction. Apparent reduction in targeting efficiency of caleosin lacking the helical subdomain (Fig. 6C) may not imply direct involvement of this subdomain in protein targeting to artificial oil emulsions since the targeting effi-
In vitro protein targeting to oil bodies efficiency could be mostly recovered when the hydrophilic N-terminal domain was also truncated. Instead, the apparent reduction may result from post-targeting structural instability of attenuated hydrophobic interaction between artificial oil emulsions and the central anchoring domain of the mutated caleosin lacking the helical subdomain.

Negatively charged PLs have been proposed to be involved in targeting of certain proteins to various membrane structures using in vitro systems, such as model phospholipid membranes (Kennedy et al. 1997) and vesicle bilayers (Leventis and Silvius 1998). Negatively charged PS and PI are present in a consistent amount (30–40%) in the PLs of oil bodies from diverse seeds (Tzen et al. 1993). Taking advantage of the in vitro targeting system established in this study, we found that inclusion of negatively charged PS and PI in artificial oil emulsions substantially enhanced the targeting efficiency of caleosin and oleosin to those artificial oil emulsions (Fig. 5). It is likely that the negatively charged PS and PI, presumably resided in the surface area of seed oil bodies, are involved in protein targeting to these lipid-storage organelles. Nevertheless, it remains to be studied what the structural requirements in oil-body proteins are that interact with the surface negatively charged PLs of oil bodies for their efficient targeting.

Materials and Methods

Purification of sesame oil bodies

Mature sesame (*Sesamum indicum* L.) seeds were gifts from the Crop Improvement Department, Tainan District Agricultural Improvement Station. The seeds were soaked in water for 10 min prior to purification of oil bodies. Extracted oil bodies were subjected to further purification including two-layer flotation by centrifugation, detergent washing, ionic elution, treatment of chaotropic agent, and integrity testing with hexane (Tzen et al. 1997).

Over-expression of sesame caleosin in *Escherichia coli*

A full-length cDNA clone of sesame caleosin (Accession no. AF109921) was constructed in the non-fusion expression vector, pET29a(+) (Novagen), using an *Nde*I site at the initial methionine position and a *Xho*I site in the polylinker of the vector. The recombinant plasmid, termed pECal, was used to transform *E. coli* strain NovaBlue (DE3). Over-expression was induced by 0.1 mM IPTG in a bacteriophage T7 RNA polymerase/promoter system. Three h after induction, the *E. coli* cells were harvested, lysed by sonication in 10 mM sodium phosphate buffer, pH 7.5, and then subjected to further analyses.

In vitro transcription/translation of sesame caleosin

Caleosin was in vitro translated using a TnT® T7 coupled reticulocyte lysate system (Promega). Coupled transcription/translation was executed for 90 min at 30°C according to the supplier’s protocol using 0.5 μg pECal construct as a DNA template in a reaction mixture of 25 μl. The translated caleosin was radio-labeled with Amersham International Redivue i-[^35]S)methionine.
Effect of calcium binding on caleosin migration in SDS-PAGE

Oil bodies (50 mg) in 500 μl Tris-buffered-saline containing 10 mM Tris-HCl buffer, pH 7.5 were mixed with an equal volume of 200 mM ethylene-glycol-bis[(β-aminoethyl ether)-tetraacetic acid (EGTA) for 5 min at room temperature. The oil bodies were subjected to examination of caleosin migration in SDS-PAGE (Laemmli 1970) directly or after incubation with 100 mM CaCl₂. Similar treatments were also applied to caleosin over-expressed in E. coli or in vitro translated in the Promega reticulocyte lysate system. After electrophoresis, proteins extracted from oil bodies or E. coli cells were stained with Coomassie Blue R-250 while the in vitro translated caleosin in the SDS-PAGE gel was fixed with methanol: water: acetic acid (50:40:10, v/v/v), dried on a sheet of Whatman 3MM filter paper, and visualized by fluorography using a Kodak X-ray film exposed for 48 h.

Purification of TAG and PL

To separately extract neutral lipids (mainly TAG) and PLs, sesame oil bodies were initially mixed with an equal volume of diethyl ether (Peng and Tzen 1998). After centrifugation, the upper ether fraction was harvested and evaporated under nitrogen to collect TAG. The lower water fraction was further extracted with chloroform/methanol (2:1, v/v). After centrifugation, extracted PLs in the lower chloroform fraction were collected. The native TAG and PLs extracted from sesame oil bodies were subjected to the following preparation of artificial oil emulsions.

Preparation of artificial oil emulsions

For preparation of artificial oil emulsions, a 1-ml suspension containing 20 mg TAG and 200 μg PL was prepared in a 1.5-ml eppendorf tube. Native PLs purified from sesame oil bodies or various combinations of PLs (PC, PE, PS, and PL purchased from Sigma) dissolved in chloroform were placed at the bottom of the eppendorf tube, and the chloroform was allowed to evaporate under nitrogen. After evaporation, purified TAG was incorporated and then sonicated with a 3-mm-diameter probe in a Sonics & Materials VCX-400 ultrasonic processor with the amplitude at 30% for 15 s. The sample was cooled on ice for 5 min. The sonication was repeated two more times to generate artificial oil emulsions. The artificial oil emulsions were diluted one thousand times before they were supplied to in vitro translated proteins for targeting analysis.

Construction of plasmids for in vitro translation of oleosin, 2S albumin precursor, and HrpW

A recombinant plasmid termed pEoLe, was constructed with a full-length cDNA clone of rice oleosin (Accession no. Z49390) in the non-fusion expression vector, pET28a(+) (Novagen), using an Ncol site at the initial methionine position and a Xhol site in the polylinker of the vector. A recombinant plasmid termed pEAlb, was constructed with a full-length cDNA clone of sesame 2S albumin precursor (Accession no. AF391841) in the fusion expression vector, pET30c(+) (Novagen), using an EcoRI site and a Xhol site in the polylinker of the vector. Sesame 2S albumin precursor comprises an N-terminal cleavable signal sequence of 21 residues responsible for targeting to the ER via the SRP-dependent pathway, and its processed mature polypeptide is a soluble storage protein accumulated in seed protein bodies (Tai et al. 1999). A recombinant plasmid termed pEhrp, was constructed with a full-length cDNA clone of HrpW (Accession no. U25812) in the non-fusion expression vector, pET29a(+) (Novagen), using an Ndel site at the initial methionine position and a Xhol site in the polylinker of the vector. HrpW, composed of six predicted transmembrane helices, is one of the membrane-bound subunits consisting of a protein apparatus for secretion of elicitors responsible for hypersensitive response and pathogenicity (Huang et al. 1995).

Analysis of targeting efficiency of translated proteins to artificial oil emulsions

In the presence of 1 μl diluted artificial oil emulsions, caleosin, oleosin, 2S albumin precursor, or HrpW was in vitro translated as described previously using pECal, pEoLe, pEAlb, or pEhrp as the reaction template. After translation, the reaction mixture (25 μl) was added with 5 μl undiluted artificial oil emulsions (to assist formation of oil layer in later centrifugation) and 175 μl washing solution containing 0.5 M sucrose and 0.1 M NaClO, at pH 11.5. The sample was kept on ice for 30 min with occasional mixing, and then fractionated into oil layer, supernatant, and pellet by centrifugation at 14,000 rpm for 30 min at 4°C using an eppendorf centrifuge. The oil layer and the pellet were separately resuspended in 195 μl (equivalent to the volume of the supernatant) washing solution. Relative contents of in vitro translated proteins in the three fractions were resolved in SDS-PAGE and detected by fluorography as mentioned previously.

Proteolytic digestion of in vitro translated oleosin

Oleosin was in vitro translated in a reaction mixture of 25 μl before or after inclusion of artificial oil emulsions, and then subjected to proteolytic digestion using 200 ng proteinase K (Sigma). The digestion was executed in a buffer containing 10 mM Tris-HCl, pH 8.0 and 10 mM CaCl₂, at room temperature for 0, 20, 40, 60, 90, or 120 min. Proteolytic digestion was stopped at various periods of time by adding phenylmethysulfonyl fluoride to a final concentration of 0.1 mM. The degradation of translated oleosin was monitored in SDS-PAGE by fluorography as described previously.

Competition between microsomal membranes and artificial oil emulsions for in vitro translated caleosin, oleosin, 2S albumin precursor, and HrpW

In a competition experiment, in vitro targeting efficiency was analyzed as mentioned previously except using canine pancreatic microsomal membranes (Promega) and/or artificial oil emulsions as target sources for integration of translated proteins. In the 25 μl reaction mixture, 1.2 μl microsomal membranes (according to the supplier’s protocol) and/or 1 μl diluted artificial oil emulsions were supplied to translated caleosin, oleosin, 2S albumin precursor, or HrpW. The PL content (>50 μg) of microsomal membranes was approximately 250 times more than that (0.2 μg) of artificial oil emulsions in the co-existent competition.

Construction of plasmids for mutated caleosin

Constructs of mutated caleosin lacking various structural domains and/or subdomains were obtained by PCR-based mutagenesis. In each construct, two 5′-phosphorylated divergent primers were designed to anneal to the desired mutation site in the pECal plasmid. For those constructs encoding truncated caleosin polypeptides lacking the N-terminal domain, an initial methionine codon was introduced via one of the paired primers. The paired primers are 5′-cattatgcattctctteta-3′ and 5′-gtgtagtcctctctattagcttctc-3′ for pECal-N (lacking N-terminal domain), 5′-attgaaccaatttgaagattcagact-3′ and 5′-agttgctacttctctccttctctttc-3′ for pECal-C (lacking helical subdomain), 5′-catatggctactctcct-3′ and 5′-gtgctttgctttcct-3′ for pECal-N (lacking N-terminal domain and helical subdomain), 5′-caggaagcttgagcttttcgagcagcctctccttcgagttggc-3′ for pECal-P (lacking proline knot subdomain), or 5′-caggaagcttgagcttttcgagcagcctctccttcgagttggc-3′ for pECal-HP (lacking helical and proline knot subdomains). Amplification was carried out using pfu DNA polymerase (Stratagene) for 12 cycles at 94°C, 1 min; 50°C, 1 min; 72°C, 14 min.
In vitro protein targeting to oil bodies

The blunt-ended linear PCR product was resolved in an agarose gel, purified by ethanol precipitation, and self-ligated to form a circular plasmid. The mutated DNA was transformed and amplified in E. coli (DH10B). The mutations were confirmed by sequencing using the SequenceTM Version 2.0 DNA Sequence kit (USB). Furthermore, the mutated constructs were transformed and over-expressed in E. coli strain NovabluE (DE3), and the relative mobility of the over-expressed polypeptides was resolved in SDS-PAGE (data not shown) to ensure the accuracy of construct expression. All the mutated constructs were subjected to analysis of targeting efficiency of their translated products to artificial oil emulsions using the procedure described previously.

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References


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