Cotranslational Integration of Soybean (Glycine max) Oil Body Membrane Protein Oleosin into Microsomal Membranes

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Storage triglycerides in oil seeds are sequestered in discrete organelles termed oil bodies. They are bounded by a monolayer of phospholipids in which few distinct proteins (oleosins) are embedded. Synthesis of soybean (Glycine max) 24-kD oleosin was analyzed by in vitro transcription and translation in reticulocyte lysate in the presence of canine microsomes. Our results show that 24-kD oleosin is cotranslationally integrated into microsomal membranes. We demonstrate that oleosin is integrated into a bilayer membrane in preference to the oil body monolayer membrane, indicating that oleosin is synthesized on the endoplasmic reticulum (ER). A new model of oil body assembly involving a conformational change through initial association with the ER membrane is proposed.

Soybean (Glycine max) seed oil is stored as droplets of TAG, which accumulate in the cytoplasm as discrete organelles termed oil bodies. The oil bodies have a simple structure consisting of a central TAG matrix that is surrounded by a monolayer of phospholipids. Embedded in this membrane are a distinct family of proteins called oleosins, which are unique to the oil body (reviewed in Huang, 1992). Oleosins are predominantly hydrophobic proteins ranging from 15 to 26 kD. Recent data (Tzen and Huang, 1992) suggest that the oleosins cover most of the surface area of the oil body and may prevent coalescence of the oil bodies.

Oleosin cDNAs and genomic clones have been characterized from maize, carrot, soybean, radish, rapeseed, and sunflower (Vance and Huang, 1986; Hatzopoulos et al., 1990; Qu and Huang, 1990; Kalinski et al., 1991; Lee and Huang, 1991; Murphy et al., 1991; Cummins and Murphy, 1992). A comparison of the predicted amino acid sequence of these clones has demonstrated that oleosin proteins share a common secondary structure, which consists of three domains. The amino- and carboxy-terminal domains have very little sequence identity or even similarity; however, the hydrophobic nature of these domains has been conserved. The amino terminus forms a hydrophilic α-helix, whereas the carboxy terminus forms an amphipathic α-helix. In contrast, there is an extremely high degree of sequence identity in the central domain of the protein. The central domain is strongly hydrophobic except for a few relatively hydrophilic Pro residues that are highly conserved. The central domain is a unique feature of the oleosins because it is over 70 amino acids long, which is the longest continuous hydrophobic stretch of any known protein.

It is of interest to study the ontogeny of oil bodies because of the organelle's importance to TAG accumulation and storage. Techniques that might be expected to illustrate the mechanisms of oil body assembly have instead rendered confusing and inconclusive results. The processes involved in oil body formation, especially the contribution of the ER, are still controversial (see Murphy, 1990, and Huang, 1992, for recent reviews). One hypothesis suggests that newly synthesized TAG accumulate in the hydrophobic interior of the ER membrane between the two phospholipid layers. Eventually, a droplet of TAG surrounded by a phospholipid monolayer disconnects and is released into the cytosol (Freymuller et al., 1963; Schwartzenbach, 1971; Wanner et al., 1981). The oleosin proteins, which could be synthesized on the ER or by cytosolic ribosomes, would then be inserted. An alternate theory proposes that naked oil droplets are formed in the cytosol, perhaps on a membrane component, and are encased with a proteinaceous membrane late in seed development (Bergfeld et al., 1978; Stobart et al., 1986; Murphy et al., 1990). In this scenario, the ER is either not involved at all or is involved in an indirect manner.

In the present study, we have focused on learning more about the role of the ER in oil body assembly. Using a soybean 24-kD oleosin cDNA clone (Kalinski et al., 1991), we have investigated the interaction of newly synthesized oleosin with the ER in vitro. Our results demonstrate that oleosin is cotranslationally associated with ER-derived canine microsomes. Oleosins remain associated with microsomes even after extraction with sodium carbonate, indicating that oleosin can integrate into a bilayer membrane as well as a monolayer membrane. We also demonstrate that oleosin is integrated into a bilayer membrane in preference to the oil body monolayer membrane, suggesting that oleosin is synthesized on rough ER and not by cytosolic ribosomes. A new model of oil body assembly involving conformational changes of the oleosin protein through initial association with the ER membrane is proposed.

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Abbreviation: TAG, triglycerides.
MATERIALS AND METHODS

Plasmid Construction

Plasmid p2491, in which an 867-bp cDNA encoding a 24-kD soybean (Glycine max) oleosin is cloned into the EcoRI site of Bluescript SK− (Stratagene) downstream of the T3 promoter, has been described (Kalinski et al., 1991). Due to a cloning artifact, the 24-kD oleosin cDNA is truncated at the 3′ end and is missing a portion of its 3′ untranslated sequence including a polyadenylation signal. To add the necessary 3′ untranslated sequence (see “Results and Discussion”), a 650-bp ClaI/HindIII fragment from plasmid pKYLX71 containing the transcription terminator of the pea rbcS-E9 gene (Schardl et al., 1987) was isolated and purified. The p2491 plasmid was digested with ClaI and HindIII (these sites are located immediately downstream of the EcoRI site). The ClaI/HindIII fragment from the pKYLX71 plasmid was then ligated into the ClaI and HindIII sites on the p2491 plasmid. The new plasmid, which contains the coding region of 24-kD soybean oleosin and a transcription terminator from the E-9 gene, is referred to as p2491.1.

In Vitro Transcription and Translation

Plasmid p2491.1 was used as a DNA template to synthesize capped oleosin mRNA using T3 RNA polymerase and the mCAP mRNA Capping Kit (Stratagene) according to the manufacturer’s instructions. The oleosin mRNA was translated in a rabbit reticulocyte lysate system (Promega) with the addition of 1 unit of RNase Block I1 (Stratagene) and 0.5 mCi/mL [3H]Leu (100-200 Ci/mmol at 5 mCi/mL; Amer sham) in the presence or absence of canine pancreatic microsomal membranes (Promega) according to the manufacturer’s instructions.

After translation, microsomes were separated from lysates by centrifugation on a Suc gradient as described in Paul and Goodenough (1983) with the following modifications. Lysates were diluted to 300 μL with a 0.25 M Suc solution (0.1 M KCl, 3 mM MgCl2, 2 mM DTT, 20 mM Hepes, pH 7.5) and layered on top of a 0.5 M/2.0 M Suc solution step gradient constructed in a 11 x 34 mm polycarbonate centrifuge tube (Beckman). Gradients were centrifuged at 100,000g for 30 min at 4°C in a Beckman TLS-55 rotor. The microsomal membranes banded at the 0.5 M/2.0 M interface.

For the posttranslational assays, microsomes were added after translation had occurred for 60 min. Further elongation was inhibited by the simultaneous addition of cyclohexamide to a final concentration of 10 μg/mL. Incubation was continued for another 60 min and then the sample was fractionated on a Suc gradient as described.

To assay whether oleosins were resistant to carbonate extraction, oleosin mRNA was translated in vitro in the presence of microsomes. Sodium carbonate (pH 11.0) was added to the translation reaction to a final concentration of 0.1 M (Fujiki et al., 1982). The sample was placed on ice for 30 min and then fractionated over a gradient as described.

To study translation in the presence of oil bodies, purified soybean oil bodies (see below) at a concentration of approximately 1 mg/mL were added to translation reactions in various amounts (see legends to Figs. 3 and 4). To compare oleosin association with oil body membranes versus microsomal membranes, translation reactions were carried out either with oil bodies (0.04 μg/μL) alone or with the addition of canine microsomes. After translation, the samples were washed with carbonate as described above. The samples were then diluted to 300 μL with 0.25 M Suc solution, layered over a 0.5 M/2.0 M step gradient, overlaid with 400 μL of buffer without Suc and centrifuged as described above. The oil bodies floated to the top of the buffer overlay and were removed with a wide bore pipette tip. The 0.25 M Suc layer was separated from the rest of the gradient and recentrifuged for 15 min in a microfuge to allow any contaminating oil bodies to float to the top, where they could be easily removed. Examination of Nile red (Fowler and Greenspan, 1985) stained fractions by fluorescence microscopy showed little or no contamination of oil bodies in the 0.25 M Suc layer containing the soluble components of the lysate.

Translation products were analyzed by SDS-PAGE according to protocols supplied by Promega and visualized by fluorography with Enhance (Dupont-NEN) according to the manufacturer’s instructions, and subsequent autoradiography was performed on Kodak XAR-5 film at -70°C for 5 d. To increase sensitivity and for accurate quantitation of fluorographic results (Laskey and Mills, 1975), the x-ray film was preflashed with a Sensitize flashgun (Amersham) according to the protocol supplied by the manufacturer.

Isolation of Oil Bodies

Oil bodies were isolated from soybean seeds that had been imbibed for 24 h by homogenization in 0.1 M Tris-HCl (pH 8.6)/3 mM MgCl2 using a Brinkman Polytron. The homog enate was filtered through several layers of cheesecloth and then centrifuged for 20 min at 19,000 rpm in a Beckman SW-28 rotor. The oil body pad was resuspended in the homogenization buffer and centrifuged as before. The oil body pad was then resuspended in 0.5 M NaCl/0.1 M Tris-HCl (pH 8.6)/3 mM MgCl2 and centrifuged. Next, the oil body pad was resuspended in 0.1 M Na2CO3 and incubated for 30 min on ice to remove proteins not embedded in the membrane (Fujiki et al., 1982). The Na2CO3 extracted oil pad was recovered by centrifugation as before. The oil body pad was resuspended in 0.1 M KCl/3 mM MgCl2/2 mM Hepes (pH 7.5) and recentrifuged two times to lower the pH. Finally, the oil body pad was resuspended in 0.1 M KCl/3 mM MgCl2/2 mM Hepes (pH 7.5)/2 mM DTT. Typical yields were approximately 3 to 3.5 g of purified oil bodies from 35 g of dry seeds. The oil body suspension was either used immediately or stored at -70°C.

RESULTS AND DISCUSSION

We used an in vitro translation lysate with the addition of canine microsomes as a model system to study the potential interaction of newly synthesized oleosin with the ER. To construct an appropriate cDNA template for in vitro tran-
Cell-Free Synthesis of an Oil Body Membrane Protein

Figure 1. Addition of microsomes enhances translation of 24-kD oleosin. The p2491.1 plasmid was used as a DNA template to transcribe 24-kD oleosin mRNA, which was then translated in a reticulocyte lysate system. Lane 1 contains a control translation reaction to which no oleosin mRNA was added; however, microsomes were added. Lane 2 contains oleosin mRNA translated without microsomes. Lane 3 shows the result of a translation reaction in the presence of microsomes. The arrows indicate molecular mass markers of 30 and 21.5 kD.

To determine whether oleosin associates with microsomes in a co- or posttranslational process, microsomes were added either before or after translation reactions were stopped by the addition of cyclohexamide. The microsomes were then separated from the soluble components of the lysate by centrifugation through a Suc step gradient, and the gradient fractions from both samples were analyzed by SDS-PAGE fluorography (Fig. 2, A and B). The posttranslational addition of microsomes to the translation reaction resulted in low-level synthesis of the full-length (23.5 kD) oleosin polypeptide and a relatively high proportion of truncated product (Fig. 2A, lane 1). Both polypeptides remain in the soluble supernatant fraction of the gradient and there is no detectable oleosin product found in the 0.5 M/2.0 M interface, where the microsomes band (Fig. 2A, lane 3). In contrast, when microsomes are added to the reaction cotranslationally, the great majority of the 23.5-kD oleosin product is found in the 0.5 M/2.0 M interface microsomal fraction (Fig. 2B, lane 3), and there is no detectable product found in the soluble supernatant fraction (lane 1). Translation in the presence of microsomes also appears to reduce the synthesis of truncated oleosin products (Fig. 2B, lanes 1 and 3).

These results show that oleosin exhibits a cotranslational requirement for microsomal membranes to be efficiently translated in an in vitro system. Oleosins do not appear to have a cleavable signal peptide. A comparison of oleosin synthesized both in vivo and in vitro has not shown a difference in mobility when analyzed by SDS-PAGE (Qu et al., 1986; Herman, 1987). Also, none of the oleosin genes characterized to date encode a recognizable cleavable signal sequence.

Extraction of membranes by carbonate (pH 11) is used to distinguish peripheral and secreted polypeptides from integral membrane proteins (Fuziki et al., 1982). We used this technique to demonstrate that newly synthesized oleosin is stably integrated into the microsomal membrane bilayer.

Figure 2. Cotranslational integration of 24-kD oleosin into microsomal membranes. Microsomes were added to reticulocyte lysate after translation was completed (A), cotranslationally (B), and cotranslationally with subsequent extraction with sodium carbonate (C). Microsomes were separated from soluble components of the lysate by centrifugation through a Suc step gradient, and gradient fractions were analyzed by SDS-PAGE fluorography. A and B, Lanes 1 contain the supernatant fraction (0.25 M Suc solution), lanes 2 contain the 0.5 M Suc solution fraction, lanes 3 contain the microsomal fraction (0.5 M/2.0 M interface), and lanes 4 contain the 2 M Suc pad. The arrows to the left of A indicate molecular mass markers of 30, 21.5, and 14.3 kD. C, Lane 1 contains the carbonate-resistant microsomal fraction and lane 2 contains the supernatant fraction.
Oleosin was synthesized in vitro in the presence of microsomes and the lysate was treated with 0.1 M sodium carbonate. The sample was then fractionated on a Suc step gradient, the supernatant and microsomal fractions were analyzed by SDS-PAGE fluorography, and the results are presented in Figure 2C. A comparison of lanes 1 and 2 shows that oleosin is localized exclusively in the carbonate-resistant microsomal fraction. The results of this experiment indicate that the cotranslational association of oleosin with microsome membranes allows stable integration into the microsomal membrane bilayer.

To determine whether the cotranslational integration of oleosin is specific for microsomal membranes, in vitro translation reactions were performed either in the presence of isolated soybean oil bodies alone or in the presence of both soybean oil bodies and microsomes. Our initial experiments showed that in contrast to the enhanced translation of oleosin observed with the addition of microsomes, the addition of oil bodies greatly repressed translation. We titrated the concentration of added oil bodies to determine the amount that would permit a satisfactory level of oleosin to be synthesized (Fig. 3).

Oleosin was then synthesized in an in vitro translation lysate in the presence of oil bodies. After translation, the reactions were extracted with carbonate as described above. The samples were then diluted with 0.25 M Suc solution, layered over a 0.5 M/2.0 M step gradient, overlaid with buffer (without Suc), and centrifuged. The oil bodies float to the top of the gradient, leaving a generous amount of buffer between the oil bodies and the soluble components of the lysate in the 0.25 M Suc layer, which allows the oil bodies to be easily removed. The 0.25 M Suc fraction was then spun again for 15 min in a microfuge to allow any contaminating oil bodies to float to the top, where they were removed. To further ensure that the oil bodies were exclusively in the top buffer layer and not in the 0.25 M Suc layer, aliquots of the fractions were stained with Nile red, which acts as a fluorescent probe for lipids (Fowler and Greenspan, 1985). Examination of the stained fractions by fluorescence microscopy showed little or no contamination of oil bodies in the 0.25 M Suc layer containing the soluble components of the lysate. The gradient fractions were then analyzed by SDS-PAGE fluorography and the results are shown in Figure 4A. Examination of lane 2 shows that a small amount of labeled oleosin is found in the soluble supernatant (0.25 M Sucose) fraction; however, no labeled oleosin is detectable in lane 1, which contains the oil body fraction. (The faint bands at 30 and 21.5 kD in lane 1 are the result of leakage from an adjacent marker lane.) The level of synthesis seen in lane 2 is expected because there is a certain amount of background translation of oleosin message even in the absence of microsomes. The signal in

![Figure 3](image1.png)

**Figure 3.** Addition of oil bodies represses translation of 24-kD oleosin. Translation of oleosin mRNA was performed in the presence of increasing amounts of purified soybean oil bodies. Lanes 1 to 3, Translation reactions with the addition of 0.04, 0.1, and 0.2 μg/μL oil bodies, respectively. The arrows indicate molecular mass markers of 30 and 21.5 kD.

![Figure 4](image2.png)

**Figure 4.** Oleosin cotranslationally integrates specifically into microsomal membranes. Translation of oleosin mRNA was performed in the presence of approximately 0.04 μg/μL of purified soybean oil bodies alone (A) or in the presence of both oil bodies and canine microsomes (B). The samples were centrifuged through a Suc step gradient, and gradient fractions were analyzed by SDS-PAGE fluorography. A and B, Lanes 1 contain the oil body fraction (the faint bands at 30 and 21.5 kD in lane 1 are the result of leakage from an adjacent marker lane), lanes 2 contain the supernatant fraction (0.25 M Suc solution layer), lanes 3 contain the 0.5 M Suc solution fraction, lanes 4 contain the microsomal fraction (0.5 M/2.0 M interface), and lanes 5 contain the 2 M Suc pad. The arrows to the left of A indicate molecular mass markers of 30 and 21.5 kD.
lane 3 is also due to background translation of the oleosin message. The results of this experiment indicate that newly synthesized oleosin does not associate with the oil body membrane.

Figure 4B shows the result of a second translation performed in the presence of both oil bodies and microsomes. The sample was extracted with carbonate and put over the same type of gradient described above. Again, aliquots of the top buffer layer and the 0.25 M layer were stained with Nile red, and fluorescence microscopy was used to determine that the oil bodies were located exclusively in the top buffer layer and not in the 0.25 M layer. The majority of the labeled oleosin product preferentially partitions with the 0.5 M/2 M interface microsomal fraction (lane 4), and there is no detectable accumulation of oleosin in the oil body fraction (lane 1).

We are not certain why there is some labeled oleosin product in the supernatant layers (lanes 2 and 3). Previous work has shown that oleosin is not carbonate extractable from oil bodies (Herman, 1987). Therefore, we speculate that oleosin bodies may reduce the efficiency of cotranslational integration into microsomes, which would account for the presence of labeled product in the supernatant layers (lanes 2 and 3). The results presented in Figure 4 show that cotranslational insertion of oleosin is specific for microsomal membranes and that the monolayer oil body membrane cannot act as a substitute. These results imply that newly synthesized oleosin cannot be directly inserted into the oil body membrane and must first cotranslationally integrate into the ER membrane bilayer prior to transfer to the oil body monolayer membrane.

Two distinctly different, detailed secondary structure models have been proposed for the oleosins (Huang, 1992; Li et al., 1992). The two models have features in common, including an aminoterminal hydrophilic a-helix, which would form a domain that could interact with the cytoplasm and a carboxy-terminal amphipathic a-helix, which would form a domain that could interact with the lipid/water interface. However, the two models differ in the proposed structure of the central hydrophobic region. Huang has proposed that this domain consists of antiparallel a-strands that would form a long, rigid structure penetrating into the TAG core. This proposed structure has a distinctive Pro knot at the tip composed of three highly conserved Pro residues. Murphy and coworkers (Li et al., 1992) have modeled the central region as a flat a-strand sheet lying under the surface of the monolayer, which is a structure found in mammalian apolipoproteins. Both models are based on secondary structure predictions from computer algorithms originally designed to predict structures of soluble, globular proteins. These algorithms are generally considered to be unreliable for the strongly hydrophobic regions found in the transmembrane domains of membrane proteins. Recent results obtained from analysis of CD spectra showed that the secondary structure of oleosin is composed of 10 to 15% a-helix, 60 to 65% a-structure (40-48% a-strand), and 20 to 30% unordered structure (Li et al., 1992); therefore, the experimental data available are consistent with either model.

It is important to consider these models in light of the results presented in this study. The physical dimensions of the predicted rigid a-strand structure (Huang, 1992) exceed the 7-nm cross-sectional dimension of a membrane bilayer and, therefore, would not be stable in the ER membrane. The flat a-strand structure predicted by Li et al. (1992) could possibly fit in a bilayer, between the two phospholipid layers, but there are no other proteins known to be stably integrated into the ER with this conformation (Pugsley, 1989). Most integral membrane proteins have a similar structural motif of one or more hydrophobic transmembrane stretches. However, these stretches are usually about 15 to 20 amino acids long and the central domain of oleosin is 3 times that length. Therefore, neither of the proposed models for the detailed secondary structure of oleosin adequately accounts for its stability in a bilayer membrane. One interpretation of our results is that oleosins must be integrated into the ER bilayer membrane prior to its transfer and integration into the oil body monolayer membrane. If one of these models does indeed predict the correct secondary structure of oleosins in the oil body membrane, then oleosin must be able to adopt a precursor secondary structure that permits stable integration into the ER membrane bilayer.

We propose that TAG synthesized on the ER is sequestered between the bilayers, as most models suggest (Fig. 5). Simultaneously, oleosin is synthesized on the ER and is cotranslationally inserted into the ER membrane bilayer. The oleosin protein undergoes a spontaneous or catalyzed conformational change upon association with accumulating TAG to allow stable insertion into an oil body monolayer. The oil body then buds off from the distal ends of the ER. According to our hypothesis, oleosin does not contain targeting information in its sequence or structure that directs specific intercellular transport from the ER to the oil body, but rather, oleosins insert into the oil body membrane by general affinity and conformational change.

Figure 5. Model of oil body assembly and hypothesis of oleosin targeting. Oleosin is synthesized on the ER and is cotranslationally inserted into the ER membrane bilayer. The oleosin protein undergoes a spontaneous or catalyzed conformational change upon association with accumulating TAG to allow stable insertion into an oil body monolayer. The oil body then buds off from the distal ends of the ER. According to our hypothesis, oleosin does not contain targeting information in its sequence or structure that directs specific intercellular transport from the ER to the oil body, but rather, oleosins insert into the oil body membrane by general affinity and conformational change.

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In our model, oleosin inserts itself into the oil body by affinity for a hydrophobic environment and conformational changes and not by a specific targeting sequence. This model should be testable in vitro assay systems by simultaneously mediating oleosin synthesis and TAG synthesis with seed microsomes. These experiments are currently in progress.

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