Immunogold Localization of the L3 Protein of Maize Lipid Bodies during Germination and Seedling Growth

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ABSTRACT

We have used antibodies directed against the 16.5 kilodalton protein L3, the most abundant integral protein of maize (Zea mays L. cv Mo 17) lipid bodies, to follow the fate of this protein in scutellar parenchyma cells of maize during germination and subsequent seedling growth. Using gel electrophoresis and immunoblotting as well as immunocytochemical electron microscopy, we found that the amount of L3 decreases gradually during the first 3 to 4 days of seedling growth and more rapidly over the course of the next several days. Immunogold localization of the protein on thin sections indicated that L3 is found exclusively in the surface phospholipid monolayer of lipid bodies. The density of L3 in the surface layer of individual lipid bodies does not change during seedling growth; therefore, the decrease in the amount of L3 can be attributed to a decrease in the number of lipid bodies rather than to selective removal of protein components from the surface of all lipid bodies. Thus, L3 is apparently degraded at the same time as the matrix lipid of each lipid body. Unlike lipase, L3 does not appear to be transferred to other cellular compartments such as vacuoles during late stages of seedling growth.

The triacylglycerols stored in the lipid bodies of seeds are degraded during germination to provide energy and precursor molecules for embryo growth (2, 6). Despite the importance of this process, little is known on a cellular level about the fate of the lipid bodies, and, after the matrix lipid has been depleted, the fate of the components of the phospholipid monolayers that surround the hydrophobic core (10, 16). The lipid bodies in scutella of maize kernels have been studied the most extensively. These lipid bodies contain a limited number of distinct proteins which are believed to be integrated in the surrounding monolayer (11). The protein termed L3 (M, 16,500) and lipase (M, 65,000) are the best studied (8, 9, 11, 13). L3 is the most abundant protein, representing 30% of the total lipid body protein, and is present in mature dry kernels. Analyses of its amino acid sequence, deduced from a cDNA clone, indicate that it has a small hydrophilic segment at the amino terminus, another segment which can form an amphipathic a-helix at the carboxy terminus, and a large central core of hydrophobic amino acids (12). Lipase is a relatively minor protein component. Lipase activity does not appear in the cells until 3 to 4 d after the start of germination. The level of activity gradually increases, peaks at d 5 and then remains high for at least 5 more d (13). The distribution of the enzyme in cells has been studied using cellular fractionation techniques. Lipase activity is found initially (3–4 d) in the lipid body fraction but appears to shift into other cellular compartments, possibly vacuoles, at later stages (6–10 d) (13). In contrast to the lipase data, no information on the distribution of L3 in maize scutellar cells during germination and subsequent seedling growth is available. In this paper, we report on the immunocytochemical localization of the L3 protein and an investigation of its fate following germination. Our data indicate that the amount of L3 decreases gradually over the first 4 d and then decreases dramatically over the course of the next 3 d. Thus, unlike lipase, L3 is apparently degraded at the same time as the matrix lipid of the lipid bodies. In addition, L3 is confined exclusively to the surface of lipid bodies, and, unlike lipase, does not appear to be transferred to other cytoplasmic compartments during later stages of seedling growth.

MATERIALS AND METHODS

Plant Materials. Kernels of inbred maize (Zea mays L. cv Mo 17) were obtained from the Illinois Foundation Seed Corp., (Champaign, IL). Ungerminated kernels were either soaked in water for 3 h (d 0) or were soaked in running tap water overnight and allowed to germinate between moist paper towels at 27° C for 0.5 to 7 d.

SDS-PAGE and Immunoblotting. Scutella from kernels germinated for various lengths of time were chopped with a razor blade and ground with a mortar and pestle in grinding medium containing 0.15 M Tricine-KOH, 10 mM KCl, 1 mM MgCl, 1 mM EDTA, 2 mM DTT, and 0.6 M sucrose (pH 7.5). The volume of each homogenate was adjusted to a fixed value of 1 ml per 5 scutella and insoluble debris was removed by centrifuging the homogenates for 2 s in an Eppendorf centrifuge. The supernatant fraction, including the lipid layer, was treated with SDS and 2-mercaptoethanol and used for gel electrophoresis (7, 15). Samples were run on 12.5% SDS-polyacrylamide gels and the gels were stained with Coomassie blue R. Immunoblotting was carried out as described previously (11).

Tissue Preparation for Electron Microscopy. The scutella of germinated kernels were carefully separated from the embryo and endosperm. The central portion of each scutellum was cut into 1 mm3 pieces and fixed in 4% glutaraldehyde in 0.06 M K-phosphate (pH 7.4) for 24 h at 4° C. The fixed pieces were dehydrated in an ethanol series at 4° C and infiltrated with Lowicryl K4M (Polysciences, Inc.) at −20° C for 10 d. Samples were polymerized in a N2 atmosphere with ultraviolet light for 2 d at −20° C and for 2 d at room temperature.

Immunocytochemistry. L3 protein was localized on thin sec-

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tions using immunogold techniques. Antibodies directed against the L3 protein of the lipid body membrane were prepared as described (11), and an IgG\(^1\) fraction (5 times more concentrated than the IgG in the original serum) was isolated from the immune sera by DEAE Affi-Gel Blue column chromatography (Bio-Rad Corp.). Silver-gold thin sections were mounted on carbon-formvar coated nickel grids. The grids were floated section side down on a drop containing 0.02 M lysine, 0.02 M glycine, and 0.02 M NELCI for 15 min (1). The sections were then blocked by floating the grids on a drop of 5% instant nonfat milk in TBST (20 mM Tris, 0.5 M NaCl, 1% Tween-20, pH 7.5) for 15 min. Sections of d 4 or d 7 samples were blocked for 15 additional min with 5% normal goat serum (Cooper Biomedical, Inc., Malvern, PA) in TBST. Following the blocking step, the grids were incubated for 2 h in a humid chamber on drops of the anti-L3 IgG solution diluted 1:100 in TBST. The sections were rinsed extensively with TBST, reblocked with 5% goat serum in TBST (d 4 and d 7 samples only) and incubated on a drop of protein A-gold (12 nm) in PBST (10 mM sodium phosphate, 0.5 M NaCl, 0.1% Tween-20, pH 7.0) for 30 min.

The grids were rinsed, transferred between several drops of TBST over a period of 10 min, rinsed again with distilled water, and air-dried. Sections were poststained with 2% aqueous uranyl acetate for 3 min and examined in a JEM (Japan Electron Optics, Tokyo) 100 C electron microscope operated at 80 kV.

To check the specificity of labeling, the following series of controls were performed: (a) L3 antibodies were replaced with normal (nonimmune) rabbit serum, (b) L3 antibodies were replaced with antibodies directed against the unrelated Chl-protein CPII of thylakoid membranes (3), (c) L3 antibodies were preabsorbed with lipid body membrane proteins isolated from ungerminated maize kernels (0.5 mg/ml) (11), (d) sections were incubated with the protein A-gold conjugate alone.

**RESULTS AND DISCUSSION**

**Pattern of Degradation of L3 Protein During Seedling Growth.** Homogenates of scutella from ungerminated maize kernels can be resolved into a large number of protein bands using SDS-PAGE (Fig. 1). The most abundant protein of the lipid bodies, L3, is clearly visible as a band of approximately 16,500 M\(_{r}\). As the kernels germinate and the embryos start to grow, reserve materials in the scutella are mobilized and the total amount of protein in scutellar homogenates decreases rapidly. Since different proteins are degraded at different rates, the pattern of protein bands also changes over time (Fig. 1). The 16,500 M\(_{r}\) band gradually disappears as the protein content of the scutella drops. After 4 to 5 d, the L3 band is no longer visible.

An immunoblot of a similar gel using anti-L3 antibodies (Fig. 1) confirms that L3 is degraded during this period. Since other bands do not subsequently appear on the immunoblots, immunoreactive degradation products or modified forms of L3 that persist in the cells are not likely to exist. Substantial amounts of L3 are present for at least the first 3 to 4 d after the start of germination. The protein appears to be gradually degraded from d 1 to 5 and then rapidly disappears. Interestingly, the disappearance of L3 coincides with the appearance of lipase activity and the onset of extensive triacylglycerol hydrolysis in the scutellar cells. Thus, our data suggest that there may be a correlation between the amount of L3 present and the amount of storage lipid in the cells.

**Organization of Scutellar Parenchyma Cells.** Like the cells of most seed storage tissues, the cytoplasm of maize scutellar parenchyma cells if packed with large numbers of protein bodies and lipid bodies (Fig. 2). Protein bodies contain dense protein depositions which, in this tissue, gradually disappear over the course of 4 to 7 d after the start of germination (data not shown). As their contents are digested, protein bodies fuse into large centrally located vacuoles. Lipid bodies in maize scutellar tissue are quite variable in size (0.2–2.5 \(\mu\)m). They appear electron translucent in thin sections since the matrix triacylglycerols are extracted during tissue preparation. Large numbers of lipid bodies remain in the scutellar cells for at least 4 d after the start of germination (Fig. 4); by d 7, very few lipid bodies are left (Fig. 5).

Lipid bodies in scutellar cells are often found closely appressed to protein bodies (Figs. 2 [arrows], 3, 4) or vacuole (Fig. 5) membranes. Although the surface monolayers of lipid bodies have shown to be directly continuous with the outer leaflets of the bilayers of protein bodies in barley aleurone cells (4), the nature of the association in maize scutellar cells is unclear and will require further investigation to determine whether the membrane is actually continuous.

**Distribution of L3 Protein at Early Stages of Seedling Growth.** As shown in Figure 3, the L3 protein can be readily localized by immunocytochemical methods on thin sections of scutellar cells from maize kernels germinated for 0.5 d. Lipid bodies of all sizes contain substantial quantities of anti-L3 label (Fig. 3). As has been shown previously for lipid body membrane proteins in developing soybeans (5), label is found exclusively over the surface layer of the lipid bodies; it is not present over the triacylglycerol core region. The protein is not localized in specific regions of the membrane but appears to be distributed evenly over the surface of the lipid bodies (7.0 ± 1.7 [1 \(\sigma\)] gold particles/\(\mu\)m cross-sectioned surface).

Label is never found over other membranes in scutellar cells. Therefore, if the membranes of lipid bodies and protein bodies are directly continuous as in barley aleurone cells (4), L3 either does not diffuse from the lipid body monolayer to the protein body bilayer, or is not stably maintained there, possibly because of the very bulky, apparently nonmembrane-spanning, hydrophobic domain of the protein (12).

**Distribution of L3 Protein at Later Stages of Seedling Growth.** Since previous investigations indicated that the subcellular distribution of lipase changes over the course of germination and seedling growth (13), we also investigated the distribution of L3 in cells of kernels germinated for 4 or 7 d. Our immunogold localizations of L3 confirm the results obtained with immunoblotting techniques. Significant amounts of both L3 and lipid bodies are still present at d 4; however, by d 7, the number of lipid bodies and the total amount of L3 in the cells are dramatically reduced. In both d 4 (Fig. 4) and d 7 cells (Fig. 5), L3 is still exclusively localized in the lipid body monolayer; i.e. its distribution, unlike the distribution of lipase, does not change during seedling growth. Interestingly, the density of label in the lipid body monolayer also does not change much during the course of germination and seedling growth. Lipid bodies in d 4 and d 7 cells contain roughly the same amount of L3 per unit surface area (d 4: 6.1 ± 1.5 [1 \(\sigma\)] gold particles/\(\mu\)m cross-sectioned surface; d 7: 5.8 ± 1.7 [1 \(\sigma\)] gold particles/\(\mu\)m cross-sectioned surface) as lipid bodies in 0.5 d cells. Therefore, degradation of matrix lipid and of the protein components in the surrounding boundary layer appears to be coordinated. Lipid bodies with reduced levels of L3 are not seen and small membrane ghosts containing L3 proteins are only rarely found in the cytoplasm (Fig. 5, inset).

L3 also does not accumulate in the interior of vacuoles or in their membranes even when the two organelles are closely appressed. If the membranes of the two organelles are directly connected (13) and L3 is transferred to the vacuolar membrane, then L3 is either degraded very rapidly or is antigenically altered such that our antibodies no longer recognize the protein.

**Immunocytochemical Controls.** Immunolabeling of the L3 pro-

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1 Abbreviations: IgG, immunoglobulin; TBST, Tris buffered saline with Tween-20; PBST, phosphate buffered saline with Tween-20.


**FIG. 1.** SDS-PAGE of homogenates of maize scutella obtained at various times (0–9 d) after the start of germination. Lanes were loaded equally on a per seed basis; positions of $M_r$ markers (45,000, 31,000, 21,500, 14,400) are shown on the far left. The gel at left was stained for protein; the immunoblot at right was made using anti-L3 antibodies. L3 (arrows) is the most abundant protein in lipid bodies isolated from ungerminated kernels (lane L). The amount of L3 in scutellar homogenates decreases gradually over the course of germination and subsequent seedling growth. By 6 to 7 d after the start of germination, L3 has largely disappeared from the cells.

**FIG. 2.** Parenchyma cell of scutellum isolated from a maize kernel germinated for 0.5 d. The cytoplasm is packed with protein bodies (PB) and lipid bodies (L), some of which are appressed against protein body membranes (arrows) ($\times$15,000, bar = 1 $\mu$m).

Protein appears to be very specific. If L3 specific serum is replaced with control nonimmune serum (Fig. 6), very few or no gold particles are observed over the lipid body membranes. If L3 antibodies in the immune serum are preabsorbed with an excess of lipid body membrane proteins, labeling of the lipid body membrane completely disappears (Fig. 7). Finally, labeling is not due to nonspecific sticking of protein A-gold or antibodies to lipid body membranes, since no gold particles were observed over these membranes if L3 antibodies were deleted or replaced with antibodies to the Chl-protein CPII*, an unrelated hydro-
FIG. 3-5. Maize scutellar parenchyma cells immunolabeled with antibodies directed against the L3 protein. Anti-L3 labeling is found at a uniform density over the membranes of lipid bodies (L) regardless of their size or stage of seedling growth. Protein body (PB) or vacuole (V) membranes (arrowheads) are not labeled with the anti-L3 antibody.

FIG. 3. Immunolabeling of cell from maize kernel germinated 0.5 d (×40,000, bar = 0.5 μm).

FIG. 4. Immunolabeling of cell from maize kernel germinated 4 d (×40,000, bar = 0.5 μm).

FIG. 5. Immunolabeling of cell from maize kernel germinated 7 d (×30,000, bar = 0.5 μm). (Inset), Labeled cytoplasmic membrane ghosts (arrow) are also occasionally seen at later stages (7 d) (× 50,000, bar = 0.2 μm).
phobic membrane complex isolated from thylakoids (data not shown).

**Functional Implications.** The localization and amount of L3 protein in the surface layer of lipid bodies in maize scutellar cells, as well as the apparent polar architecture of the L3 protein and its pattern of degradation are consistent with its postulated structural role (11). An evenly distributed coat of L3 could help stabilize the phospholipid interface between the cytoplasm and hydrophobic lipid core as well as prevent lipid bodies from fusing indiscriminately with themselves or with other organelles. During the course of germination and seedling growth, as matrix lipid is degraded, the boundary layer of phospholipids and proteins must shrink in size. Since the density of L3 in the lipid body monolayer appears to be independent of both the size of the lipid bodies and the stage of seedling growth, L3 and phospholipids are apparently removed from the monolayer as the lipid is degraded. Since pools of L3 do not appear anywhere within the cells, L3 is also apparently degraded at that time. The pattern of degradation of L3 is in marked contrast to that of lipase, which plays an enzymic role in triacylglycerol hydrolysis and persists until later in seedling growth.

Our previous studies in barley aleurone cells (4) and maize scutellar cells (13) indicate that lipase can be associated with cellular compartments other than lipid bodies, and therefore, appears to exist stably, at times, in bilayer membranes. Unfortunately, we have been unable to directly confirm this observation since the lipase antibodies presently available (8) do not recognize the protein on Lowcryl sections of glutaraldehyde-fixed scutellar cells. However, using immunocytochemistry, we have been able to demonstrate that L3 is not associated with a bilayer membrane during germination and seedling growth. Due to the bulky nature of its hydrophobic domain (12), we might, in fact, expect L3 to be extremely unstable in a bilayer membrane. If this is the case, we can speculate that L3 should also not stably associate with bilayer membranes during lipid body biogenesis. Previous investigators have suggested that lipid bodies form when storage lipids accumulate between the two phospholipid layers of ER membranes and then are released into the cytoplasm (14). We suggest that, during this process, L3 might concentrate in areas where there are pockets of storage triacylglycerol disrupting the bilayer. It is also possible that L3 could serve as a foci for the nucleation of such pockets.

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