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Evidence for the Presence of Two Different Types of Protein Bodies in Wheat Endosperm

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ABSTRACT

Storage proteins of wheat grains (*Triticum* L. em Thell) are deposited in protein bodies inside vacuoles. However, the subcellular sites and mechanisms of their aggregation into protein bodies are not clear. In the present report, we provide evidence for two different types of protein bodies, low- and high-density types that accumulate concurrently and independently in developing wheat endosperm cells. Gliadins were present in both types of protein bodies, whereas the high molecular weight glutenins were localized mainly in the dense ones. Pulse-chase experiments verified that the dense protein bodies were not formed by a gradual increase in density but, presumably, by a distinct, quick process of storage protein aggregation. Subcellular fractionation and electron microscopy studies revealed that the wheat homolog of immunoglobulin heavy-chain-binding protein, an endoplasmic reticulum-resident protein, was present within the dense protein bodies, implying that these were formed by aggregation of storage proteins within the endoplasmic reticulum. The present results suggest that a large part of wheat storage proteins aggregate into protein bodies within the rough endoplasmic reticulum. Because these protein bodies are too large to enter the Golgi, they are likely to be transported directly to vacuoles. This route may operate in concert with the known Golgi-mediated transport to vacuoles in which the storage proteins apparently condense into protein bodies at a postendoplasmic reticulum location. Our results further suggest that although gliadins are transported by either one of these routes, the high molecular weight glutenins use only the Golgi bypass route.

Wheat storage proteins are synthesized in the endosperm and accumulate in dense PB[1] inside vacuoles (24). The major wheat storage proteins are the alcohol-soluble gliadins and the glutenins. The gliadins are monomeric proteins that migrate between M_r 30,000 and 60,000 on SDS-PAGE and are subdivided into α, β, γ, and ω gliadins on the basis of fractionation on acidic PAGE (29). A new classification of gliadins into sulfur-rich (α, β, γ) and sulfur-poor (ω) fractions was recently suggested (12), based on their amino acid sequence and sulfur content. Glutenins aggregate by disulfide and noncovalent bonds to form large complexes, but reduction and subsequent electrophoresis indicate that these proteins are comprised of two groups, LMW-GS and HMW-GS (10). The LMW-GS are similar in M_r and amino acid sequence to the sulfur-rich gliadins and were recently classified as aggregated gliadins (12).

Although the molecular and biochemical properties of wheat storage proteins have been extensively characterized, very little is known about their subcellular transport to vacuoles and the mechanism of their aggregation into PB. Wheat storage proteins are synthesized on membrane-bound polysomes and are then sequestered into the RER (24). EM studies of developing wheat endosperm demonstrated the presence of storage proteins inside vesicles associated with the Golgi, suggesting that storage proteins may utilize this apparatus in their transport from the RER to vacuoles (1, 2, 11, 19, 20). However, in some of these studies, storage proteins were also detected in RER-surrounded PB in the cytoplasm, indicating that the aggregation of storage into PB may also occur within the RER (2, 19, 20; H. Levanony, R. Rubin, Y. Altschuler, and G. Galili, unpublished results). Furthermore, Parker (19) speculated that the PB formed within the RER may be transported directly to vacuoles, bypassing the Golgi apparatus.

In the present report, metrizamide density gradients were utilized to identify the nature of PB formed in wheat endosperm cells and the subcellular site of their formation. Two major types of PB were resolved, light and dense ones, each containing a different composition of storage proteins. The dense PB also contained the ER marker protein, BiP, suggesting that these were formed within the RER. In contrast, the absence of BiP in some of the light PB indicated that these were formed at a post-RER location.

MATERIALS AND METHODS

Plant Material

Hexaploid wheat (*Triticum aestivum*, cv Yamhill) was grown in the greenhouse under 13 h light and 11 h dark at 23°C. Developing grains were collected 9 to 26 DAF.

Production of Antibodies

Serum was raised against subunit 1Dy12 of HMW-GS (23) or against a γ-gliadin (Y. Altschuler and G. Galili, unpublished), overproduced in *Escherichia coli*, and ethanol purified as previously described (5). New Zealand White rabbits were injected subcutaneously at 3-week intervals three times. For each injection, 300 mg of HMW-GS 1Dy12 or γ-gliadin was emulsified in 3 M urea and 50% (v/v) Freund's complete adjuvant. Serum was obtained 10 d after the second and third injections. Anti-yeast-BiP serum was kindly provided by J.P.

[1] Abbreviations: PB, protein body(ies); LMW-GS, low M_r glutenin subunits; HMW-GS, high M_r glutenin subunits; BiP, immunoglobulin heavy-chain-binding protein.

Vogel (22) and anti-α-gliadin serum was kindly provided by A. Blechl and F.C. Greene.

Subcellular Fractionation of Membrane Homogenates from Developing Wheat Grains, Determination of NADH Cyt c Reductase Activity, and Extraction of Storage Proteins

Developing wheat grains (0.5 g) were homogenized in 0.9 mL of buffer B (20 mM Tris [pH 7.6], 50 mM KCl, 10 mM $MgCl_2$, 0.3 M NaCl, 2 mM EDTA, 10% [w/v] sucrose). The homogenates were filtered through four layers of cheesecloth and then layered on 4.5 mL of a continuous 10 to 50% (w/v) gradient of metrizamide in buffer B containing 15% (w/v) sucrose (28). The tubes were centrifuged for 18 h at 275,000*g* at 4°C, and fractions of 0.4 mL were collected from the top of the gradient. The activity of the ER marker enzyme, NADH Cyt *c* reductase, in each fraction was measured as previously described (26). For storage protein extraction, the fractions were brought to 70% ethanol and 1% β-mercaptoethanol, incubated for 30 min at 60°C, and then centrifuged for 15 min at 14,000 rpm in an Eppendorf centrifuge. The supernatant volume was reduced 50% by Speedvac centrifugation. Following addition of 1.0 mL of 0.3 M NaCl to the supernatant, storage proteins were collected by overnight incubation at 4°C and subsequent precipitation.

SDS-PAGE and Western Blot Analyses

Pellets of total proteins, or ethanol plus β-mercaptoethanol-soluble storage proteins, were dissolved in SDS sample buffer and separated on 12.5% SDS-PAGE (14). Staining of the proteins with Coomassie blue was as previously described (6). For western blot analysis, proteins were transferred from the SDS gel to a nitrocellulose filter (0.2 μm) and immunoblotted with the various antisera as described previously (27). Sera at the following dilutions were used: 1:10,000 for anti-γ-gliadin, 1:1000 for anti-α-gliadin, 1:1000 for anti-HMW-GS 1Dy12, and 1:5000 for anti-yeast-BiP. Goat anti-rabbit-alkaline phosphatase conjugate was used as a secondary antibody.

Immunogold Labeling and EM Analysis of Metrizamide Gradient Fractions

Samples from the representative fractions e and m of the metrizamide gradients were mixed with 2 volumes of cold fixative to give the final concentrations of 1% (v/v) glutaraldehyde, 4% (v/v) formaldehyde, and 50 mM KH_2PO_4/Na_2HPO_4 (pH 7.5). Samples were gently mixed, pelleted at 4°C for 30 min at 48,000*g*, and prepared for EM analysis and immunogold labeling as described previously (16). Anti-γ-gliadin or anti-yeast-BiP sera were diluted 1:500 and 1:50, respectively.

Pulse-Chase Labeling of Developing Grains

Developing grains at approximately 17 DAF were cut into thin slices and incubated for 1 h in [^{35}S]methionine (10 mCi/mL, 800 Ci/mmol; New England Nuclear) at room temperature. The slices were then briefly washed, transferred to buffer B for 4.5 h of chase, and then analyzed by gradients as described above, except that detection of the radioactive bands in the SDS gels was performed by fluorography.

RESULTS

Separation of PB on Metrizamide Density Gradients

PB from developing wheat grains at approximately 17 DAF, a stage representing maximal synthesis of storage proteins, were fractionated on a metrizamide gradient and separated into 14 fractions. Analysis of NADH Cyt C reductase activity showed that the ER membranes sedimented mostly in fractions c to e in the gradient, with a density of approximately 1.08 to 1.13 $g \cdot cm^{-3}$ (Fig. 1). Chloroplast membranes sedimented at fraction f with a density of approximately 1.135 $g \cdot cm^{-3}$ as visualized by their green color (Fig. 1). Storage proteins were extracted from each fraction in alcohol plus β-mercaptoethanol and analyzed by SDS-PAGE (Fig. 2). Storage proteins were enriched at two ranges along the density gradient: in the relatively light fractions, d to h (termed light PB), and in the relatively dense fractions, l to n (termed dense PB). The various storage proteins were unequally distributed along the gradient. The HMW-GS and some proteins of lower M_r were highly enriched in the fractions of the dense PB (Fig. 2, bands marked by arrows). Most storage protein bands, in the size range of gliadins (including the aggregated gliadins known also as LMW-GS), were distributed both in the light and dense PB fractions (Fig. 2, dashed lines), although some of these were enriched in the light PB fractions (Fig. 2, bands marked by bars). This differential distribution of the storage proteins was confirmed by western blot analyses using anti-HMW-GS, anti-γ-, and anti-α-gliadin sera (data not shown). The morphology of the PB was studied by EM, using thin sections from the representative fractions e and m, which were immunogold labeled with anti-γ-gliadin serum. PB from the light fraction (e) were relatively small (approximately 0.5 μm

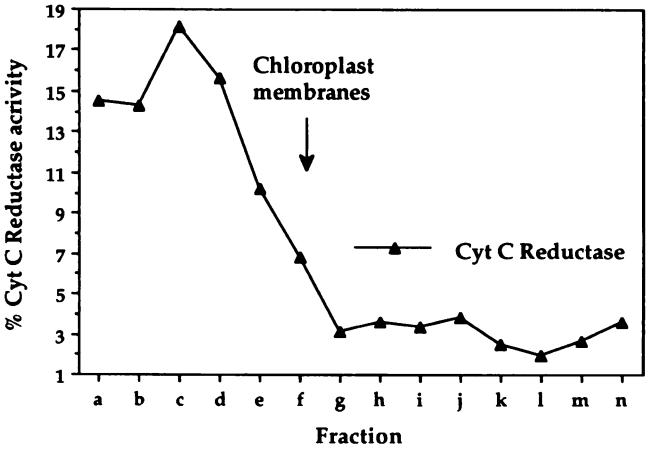

Figure 1. Distribution of NADH Cyt C reductase activity and chloroplast membranes along the metrizamide gradient. PB were separated on a metrizamide gradient, and individual fractions from the top (a) to the bottom (n) were assayed for the activity of NADH Cyt C reductase. The activity of this enzyme in each fraction is given as a percentage of its total activity in the gradient. Chloroplast membranes were visualized by their green color.

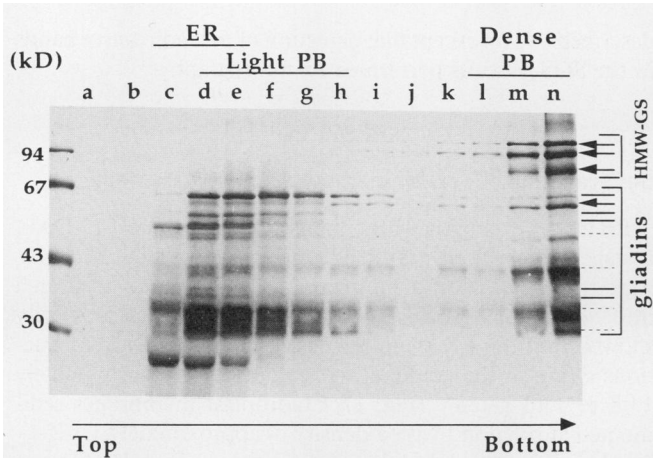

Figure 2. Distribution of storage proteins from endosperms approximately 17 DAF along a metrizamide gradient. PB were separated on a metrizamide gradient, and storage proteins were then extracted from the various fractions. An equivalent amount of protein from one grain was run on SDS-PAGE, and the gels were stained with Coomassie blue. The M_r (in thousands) of protein markers are indicated on the left, and the migration position of HMW-GS and gliadins are indicated on the right. Proteins preferentially located in the light and dense PB are indicated by bars and arrows, respectively; those appearing in both types of PB are indicated by dashed lines.

in diameter) and more darkly stained (Fig. 3a). Thin sectioning of the dense fraction (m) was laborious, apparently due to the presence of a large quantity of starch; the PB in this fraction appeared mostly as large, amorphic, and brightly stained aggregates (Fig. 3b). No gold particles were detected in the PB when the thin sections were treated with preimmune serum as a control (data not shown).

Accumulation of PB in the Light and Dense Fractions at Early and Late Stages of Grain Development

To study the course of storage protein accumulation in the two types of PB at different stages of grain development, PB from relatively young (approximately 9 DAF) and more mature (approximately 26 DAF) grains were separated on metrizamide gradients. The younger grains contained relatively low amounts of storage proteins that were hardly detected in gels stained with Coomassie blue (data not shown) but were clearly seen in western blots (Fig. 4). The majority of storage proteins that reacted with the anti-α- and anti-γ-gliadin antibodies appeared in the light PB (Fig. 4, B and C, lanes c–e), but some gliadins were also detected in the dense PB (Fig. 4, B and C, lanes m and n). However, already at this early developmental stage, the HMW-GS were highly enriched in the dense PB (Fig. 4A). Interestingly, smaller amounts of HMW-GS from these young grains were also detected in fraction d, which contained the RER and light PB, and to a lesser extent in the intermediate-density fractions e to l (Fig. 4A).

The distribution of storage proteins among the light and dense PB from older grains at approximately 26 DAF was very similar to that of grains at approximately 17 DAF (data not shown).

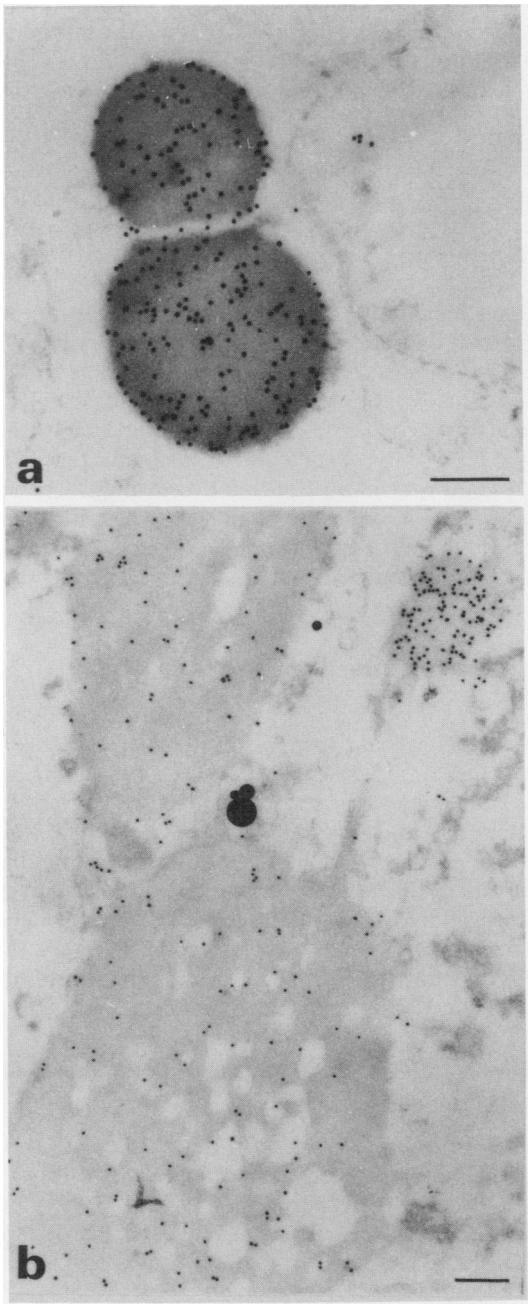

Figure 3. EM analysis of PB from different fractions of the metrizamide gradient. PB were separated on a metrizamide gradient, and the representative fractions e and m were prepared for EM analysis and immunogold labeling with anti-γ-gliadin serum. a, PB from the light fraction e. b, PB from the dense fraction m. Bars, 0.3 μm.

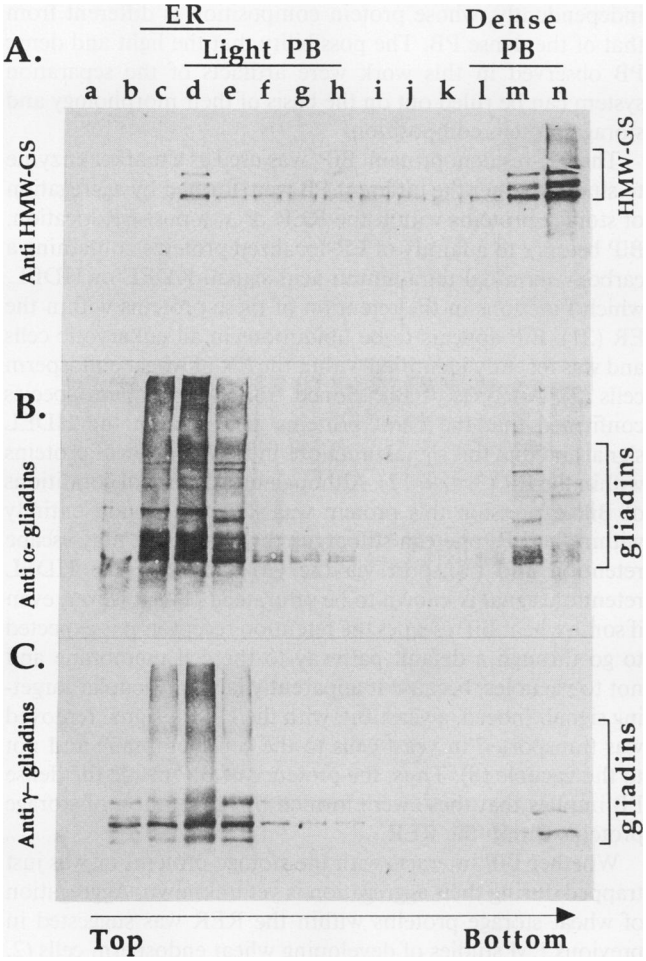

Figure 4. Western blot analysis of storage proteins from developing grains at approximately 9 DAF separated on the metrizamide gradient. PB from young grains at approximately 9 DAF were fractionated on a metrizamide gradient. Storage proteins from the various fractions were reacted in western blots with sera raised against HMW-GS (A), anti-α-gliadins (B), and anti-γ-gliadins (C). The migration positions of HMW-GS and gliadins are indicated on the right.

Formation of the Light and Dense PB

Pulse-chase analysis was performed to learn about formation of the two types of PB detected in the metrizamide gradient. Slices from developing wheat grains at approximately 17 DAF were labeled with [^{35}S]methionine for 1 h and then either harvested or chased in a cold medium for 4.5 h. PB were then fractionated on metrizamide gradients, storage proteins were extracted and separated by SDS-PAGE, and the gels were fluorographed. Most of the labeling appeared to be associated with gliadins (and LMW-GS) in the range of M_r 30,000 to 50,000 and none with the HMW-GS (Fig. 5, A and B). The reason for this is unclear. After 1 h pulse labeling, the storage proteins were located mostly in the ER-containing fractions c to e, although some was also detected in the heavier fraction f (Fig. 5A). When the pulse labeling was followed by 4.5 h of chase (Fig. 5B), labeled storage proteins accumulated in the light (fractions d–h) and dense PB (fraction m). However, no labeled protein was detected in the intermediate-density fractions i to l even following the 4.5-h chase (Fig. 5B), strongly suggesting that the dense PB were not formed by a gradual increase in density but by a distinctive, quick process of storage protein aggregation. Interestingly, following the chase period, one labeled band was detected exclusively in fractions d and m (Fig. 5B, band marked by an arrow). Because the distribution of this band in the gradient was similar to that of the HMW-GS (Fig. 4A), it might be an LMW-GS that interacted with the HMW-GS by noncovalent and disulfide bond formations.

Association of BiP with the PB

We previously showed that BiP is present in the ER of wheat endosperm cells (7). Therefore, we utilized BiP as an ER marker protein to test the origin of the two PB types observed in the metrizamide gradient. For this study, we utilized serum raised against the last 216 amino acids of yeast BiP, which is quite specific for this protein (22). This serum was previously shown in our laboratory to cross-react very strongly with wheat BiP, very weakly with the cytoplasmic HSC70, and not at all with the mitochondrial HSP70 cognate (7). Western blot analysis was carried out using aliquots from the fractions of the metrizamide density gradient used in

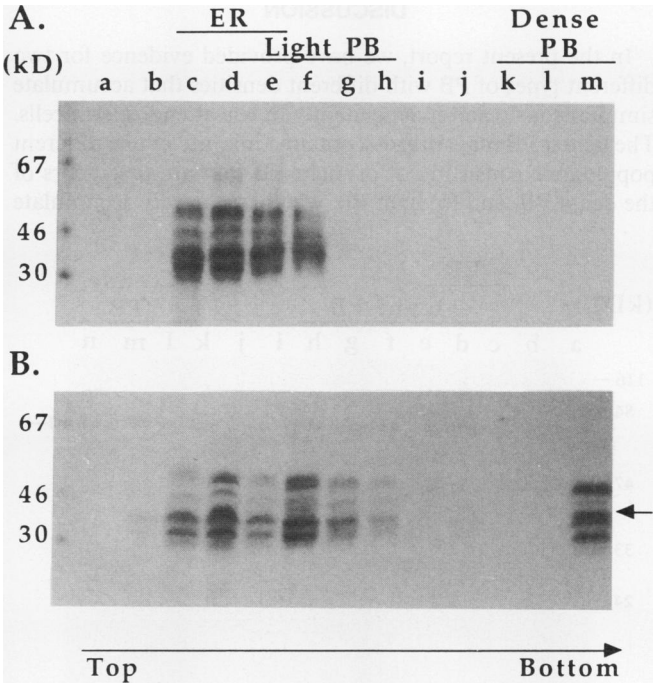

Figure 5. Pulse-chase labeling of storage proteins in developing grains. Three thin slices from a developing grain at approximately 17 DAF were pulse labeled with [^{35}S]methionine for 1 h (A) or pulse labeled for 1 h and chased for an additional 4.5 h in the absence of the radioactive amino acid (B). PB were then fractionated on the metrizamide gradients. Storage proteins from the various fractions were alcohol extracted and separated on SDS-PAGE, and the gels were fluorographed. The M_r (in thousands) of protein markers are indicated on the left. A protein band that, following the chase, was present only in fractions d and m is marked by an arrow on the right.

Figure 1 reacting with anti-yeast-BiP serum. Clearly, the strongest reaction was found in fractions c to e containing the ER, as expected, due to the specificity of the serum to wheat BiP (Fig. 6). Yet, clearly, BiP also cosedimented with the dense PB (Fig. 6, lanes l–n). Furthermore, the cosedimentation of BiP with the dense PB was not due to artifactual adhesion of ER membranes to these PB because EM analysis revealed that BiP-specific gold particles were present inside the PB (Fig. 7A). Although a considerable amount of BiP also appeared in fractions containing the light PB (Fig. 6 lanes d–h), it was difficult to assess its association with the light PB because some of these fractions also contained the ER membranes. Association of BiP with the light PB was also difficult to assess in the EM analysis, because BiP-specific gold labeling was found inside some (Fig. 7B), but clearly not in all, of them (data not shown). Thus, it is possible that the BiP-containing light PB were formed within the RER, whereas PB lacking BiP were formed by condensation of storage proteins at a post-ER location. Gold labeling of the PB with the anti-yeast-BiP serum was specific because no such labeling was detected when the EM sections were treated with preimmune serum (Fig. 7, C and D). Moreover, the BiP-specific gold labeling in the PB was not eliminated when the EM sections were pretreated with periodate to remove glycans (data not shown).

DISCUSSION

In the present report, we have provided evidence for two different types of PB with different densities that accumulate simultaneously and independently in wheat endosperm cells. The lighter PB may, in fact, contain a mixture of two different populations consisting of (a) light PB that are precursors of the dense PB, and (b) light PB, which apparently accumulate

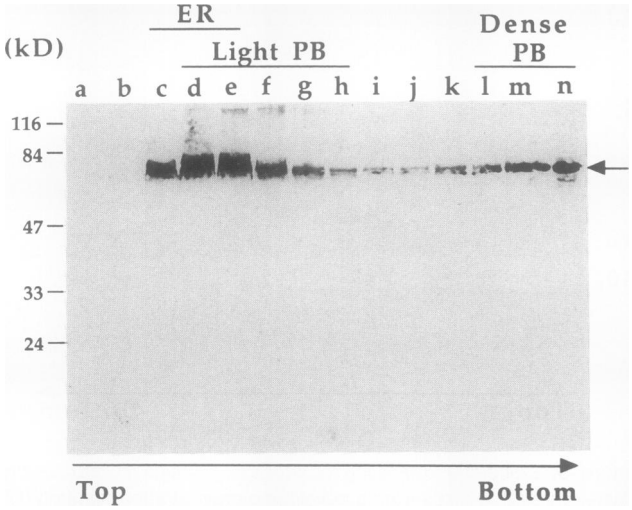

Figure 6. Distribution of BiP in the metrizamide gradient. Grains at approximately 17 DAF were homogenized in buffer B and separated on a metrizamide gradient. Total proteins from each fraction were then separated on SDS-PAGE and reacted in a western blot with anti-yeast-BiP serum. The M_r (in thousands) of protein markers are indicated on the left, and the position of BiP is marked by an arrow on the right.

independently, whose protein composition is different from that of the dense PB. The possibility that the light and dense PB observed in this work were artifacts of the separation system can be ruled out on the basis of their morphology and storage protein composition.

The ER-resident protein, BiP, was used as a marker enzyme to study whether the different PB were formed by aggregation of storage proteins within the RER or at a post-ER location. BiP belongs to a family of ER-localized proteins containing a carboxy terminal tetra-amino acid signal, KDEL or HDEL, which functions in the retention of these proteins within the ER (21). BiP appears to be ubiquitous in all eukaryotic cells and was recently identified within the ER of wheat endosperm cells (7). Analyses of BiP cloned from several plant species confirmed that the plant proteins also contain the HDEL signal and that this signal functions in the retention of proteins within the ER (3, 4, 9, 17). Although under normal conditions of BiP expression this protein was shown to retain entirely within the ER, one can still argue that some BiP may escape retention and transport via the Golgi because the HDEL retention signal is known to be saturated (21). However, even if some wheat BiP escapes the retention receptor, it is expected to go through a default pathway to the cell membrane and not to vacuoles, because it apparently lacks a vacuolar targeting signal. Indeed, a yeast BiP with the HDEL signal removed was transported in yeast cells to the cell membrane and not to the vacuole (8). Thus, the presence of BiP inside the dense PB implies that these were formed by aggregation of storage proteins within the RER.

Whether BiP interacts with the storage proteins or was just trapped during their aggregation is yet unknown. Aggregation of wheat storage proteins within the RER was suggested in previous EM studies of developing wheat endosperm cells (2, 18, 19). Such aggregation is also expected based on the water insolubility of wheat prolamines, as was, in fact, previously shown for prolamines from maize and rice (13, 15). Yet, in wheat, as opposed to maize and rice, the PB formed within the RER are not retained within this organelle but continue on a subcellular transport to vacuoles. The mechanism of this transport is not clear.

Analyses of the EM sections also showed that some of the light PB contained BiP, whereas others lacked this protein. This supports our suggestion that the light fractions contained a mixture of two populations of PB: (a) the BiP-containing, light PB population, which were probably precursors of the RER-derived dense PB, and (b) the PB population lacking BiP, which were formed independently. Because the Golgi apparatus is known to be involved in the transport of at least part of the wheat storage proteins to vacuoles, it is possible that the light PB, lacking BiP, were formed by proteins that were routed via this apparatus and condensed into PB at a post-ER location. It cannot be determined from the present report whether the two types of PB are present within the same or in different endosperm cells. However, we recently observed in EM sections of developing wheat grains that small and large PB are present in the same endosperm cells, suggesting that the two types of PB are formed in the same cell (H. Levanony, R. Rubin, Y. Altschuler, and G. Galili, unpublished observations).

In young grains, most of the gliadins were found in the

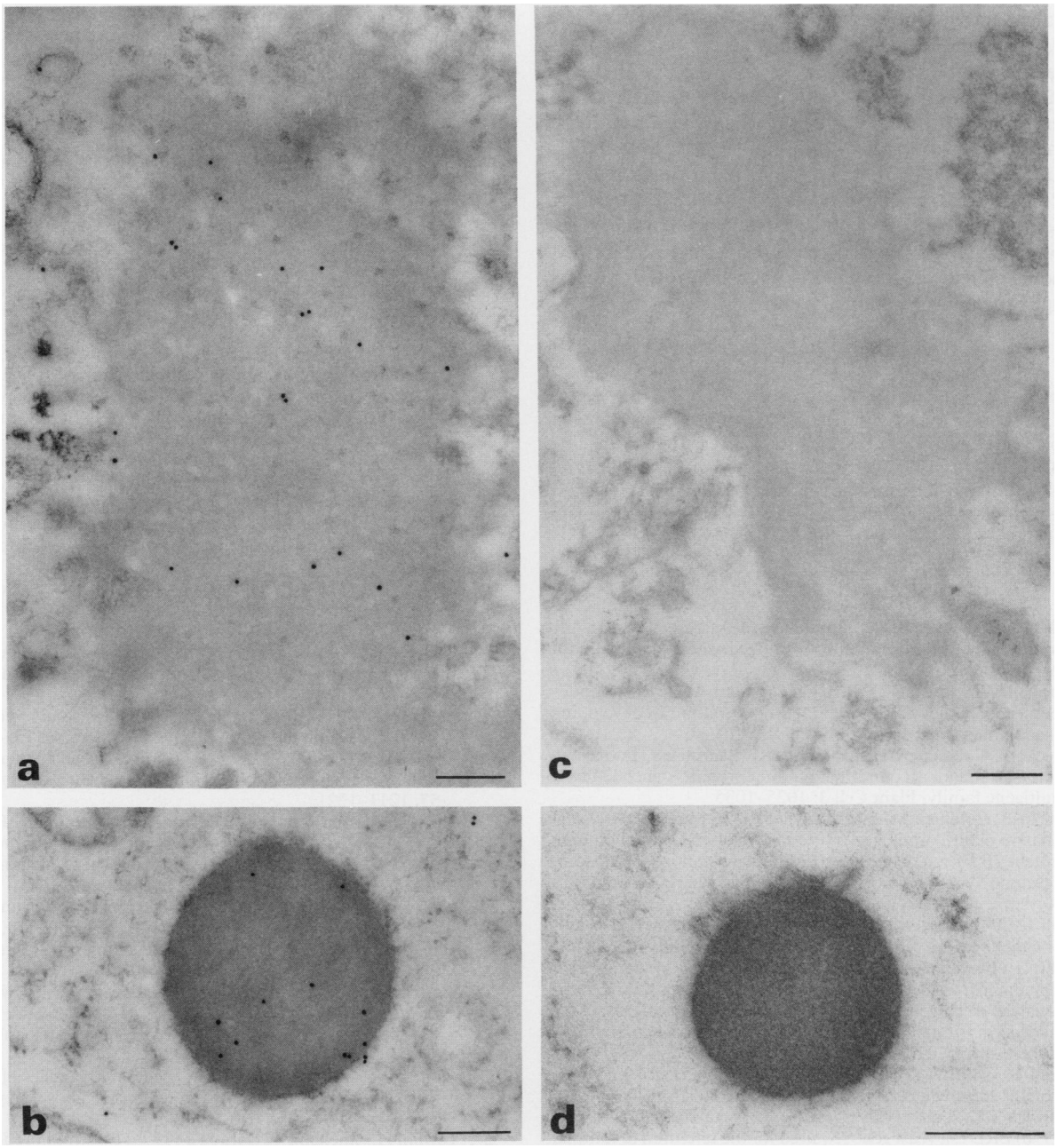

Figure 7. Immunogold localization of BiP within the light and dense PB in the metrizamide gradient. PB were separated on a metrizamide gradient, and the representative fractions m (a and c) and e (b and d) were prepared for EM analysis and immunogold labeling with anti-yeast-BiP serum (a and b) or with preimmune serum as controls (c and d). Bars, 0.25 μm.

light PB, whereas at more mature stages they were found in both PB types. This indicates that, during early grain development, a considerable amount of the gliadins were transported to vacuoles via the Golgi forming the light PB, and the remainder aggregated within the RER forming the dense PB. As the grains matured, relatively more gliadins aggregated within the RER. A similar conclusion was previously obtained by Parker (19) based on EM studies. This hypothesis is also supported by our recent expression of a wheat γ-gliadin in a surrogate system. When the γ-gliadin was synthesized in *Xenopus laevis* oocytes, a large part of the protein was secreted via the Golgi into the medium (oocytes lack vacuoles and, therefore, the prolamines were secreted by the default pathway), whereas the remainder was aggregated within the oocytes to form dense PB (25; Y. Altschuler, R. Harel, and G. Galili, unpublished).

In contrast to the gliadins, the HMW-GS were highly enriched in the dense PB during the entire period of grain development. This suggests that the HMW-GS and presumably also the LMW-GS, which interact with the HMW-GS by

noncovalent and disulfide bonds (10), aggregate mostly, if not entirely, within the RER. The significance of the aggregation of glutenins within the RER is not known, but they are apparently not essential for formation of the dense PB because we recently showed that a single γ-gliadin could form dense PB within *Xenopus* oocytes (Y. Altschuler, R. Harel, and G. Galili, unpublished). Thus, it is possible that formation of the dense PB is an RER-specific process that is mediated by other ER-resident proteins. This may also be supported by the pulse-chase experiments showing that the dense PB were not formed by a gradual increase in density but by a distinct, quick process of storage protein aggregation.

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