Biosynthesis and Intracellular Transport of 11S Globulin in Developing Pumpkin Cotyledons

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ABSTRACT

In vitro studies to explore the biosynthesis of 11S globulin developing cotyledons of pumpkin (Cucurbita sp.) demonstrated that 11S globulin is synthesized on membrane-bound polysomes. M, of the translation products (preproglobulin) synthesized by the poly(A)±RNA isolated from developing cotyledons were determined to be 64,000 and 59,000, which are larger than those of the mature globulin subunit (62,000 and 57,000). Preproglobulin is then cotranslationally processed by cleavage of the signal peptide to produce proglobulin. In vitro pulse-chase experiments showed the sequential transformation of the single-chain proglobulin to mature globulin subunit (disulfide-linked doublet polypeptides) indicating posttranslational modification of the proglobulin.

Subcellular fractionation of the pulse-chased intact cotyledons showed that the 35S methionine label is detectable in proglobulin in rough endoplasmic reticulum shortly after the pulse label. With time, the labeled proteins move into other cellular fractions: proglobulin in the density = 1.24 grams per cubic centimeter fraction after 30 minutes and mature globulin subunit associated with protein bodies after 1 to 2 hours. The distribution of proglobulin in sucrose density gradients did not correspond with those of catalase (microbody marker) or fumarase (mitochondria marker). An accumulation of proglobulin occurred in the density = 1.24 grams per cubic centimeter fractions, whereas the mature globulin was scarcely detectable in this fraction. In contrast, proglobulin was not detected by immunochemical blotting analysis in the protein bodies prepared under the mild conditions from cotyledon protoplasts. The results suggest that the d = 1.24 grams per cubic centimeter fractions are engaged in the translocation of proglobulin into the protein bodies.

11S globulin proteins are present in seeds of both mono- and dicotyledonous plants as a major reserve protein. The only reserve protein of pumpkin (Cucurbita sp.) seed is 11S globulin which comprises more than 85% of the total protein of dry seed. Most of the insoluble 11S globulin molecules are limited-hydrolyzed during the early stage of germination by the globulin-specific proteinase to produce the soluble molecular species (11).

Thus, the pumpkin cotyledon can be used to explore the mechanism of biosynthesis as well as mobilization of seed protein. Similar studies with seeds of leguminous species are complicated by the presence of multiple reserve proteins.

The pumpkin 11S globulin subunit consists of two polypeptide chains linked together by a disulfide bond (9, 10). The N-terminal amino acid sequences and C-terminal residues have been previously reported (22). There is a high degree of structural resemblance to other 11S globulins such as glycmin (20) and legumin (5).

The globulin molecules synthesized during the step of seed development, about 30 to 40 d after anthesis, are stored as crystalloids in the spherical protein bodies (5–7 μm) localized in cotyledons of dry seeds (8). The mechanisms involved in the biosynthesis and intracellular transport of seed proteins have received a great deal of attention in recent years (12). In vitro translation systems have shown that a single polypeptide precursor form is synthesized on the RER (12). Higgins et al. (13) have conducted similar studies of the biosynthesis and processing of pea lectin, which is also localized in the protein bodies. However, the mechanism involved in processing the single chain precursor polypeptide to the mature form of 11S globulin (consisting of disulfide-linked doublet polypeptide chains) is unknown. In attempting to answer this unsolved question, we have conducted experiments using the pumpkin 11S globulin as a model system. The specific objective of this study was to identify the intracellular transport of the precursor form of the protein molecules synthesized on the RER.

MATERIALS AND METHODS

Plant Materials. Seeds of pumpkin (Cucurbita sp. cv Kurokawa Amakuri Nankin) were grown during the summer season of 1983 on the university farm and experiments were carried out using cotyledons (120–160 mg/cotyledon pair) of developing plants 35 to 40 d after fertilization.

Isolation of Poly(A)+-RNA. Frozen cotyledons (10 g) were ground in liquid N2 to a fine powder, to which was added 50 ml of 50 mM Tricine-KOH (pH 9.5) containing 150 mM NaCl, 20 mM EDTA, and 2% SDS. An equal volume of phenol-chloroform (1:1, v/v) was added to the suspension, and RNA was extracted into the aqueous phase by vigorous stirring. After centrifugation, the aqueous phase was treated once again with 30 ml of phenol-chloroform (1:1). The subsequent procedures were basically the same as those described by Miyata et al. (19).

Isolation of Polysomes. Developing cotyledons (27 g) or etiolated cotyledons (3-d) (30 g) were pulverized to a fine powder in liquid N2. To the powder was added 70 ml of 0.2 M Tris-HCl (pH 8.5) containing 60 mM KCl, 50 mM MgCl2, 0.1 mM EDTA, 0.2 M sucrose, 5 mM DTT, and yeast RNA (3 mg/ml). Subsequent procedures were essentially as described by Larkins et al. (15), except that at the final step both free and membrane-bound polysomes were pelleted by centrifugation at 230,000 g for 3 h in a Beckman R45Ti rotor. The pellets were resuspended in a small volume of 20 mM Hepes-KOH (pH 7.5) containing 120 mM KCl, 2.5 mM MgCl2, 0.25 M sucrose, and 1 mM DTT.

In Vitro Protein Synthesis. Poly(A)+-RNA or isolated polysomes were used in an in vitro translation system containing

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wheat germ extracts as described by Roberts and Paterson (27) or Larkins et al. (15), respectively. The reaction mixture (50 μl) for translation of poly(A)⁺-RNA contained [³⁵S]methionine (50 μCi) and 2.5 μg of poly(A)⁺-RNA. The reaction mixture (72 μl) for either free or membrane-bound polysomes contained [³⁵S]methionine (30 μCi) and 1.5 4.3S μts of polysomes. Subsequent procedures were exactly those described by Yamaguchi et al. (30). Characterization of the globulin precursor synthesized in this system was performed using specific anti-11S globulin IgG and the immunoprecipitates were subjected to SDS gel electrophoresis and fluorography.

Preparation of Protein Bodies. Protoplasts were prepared as described by Nishimura and Bevers (21). Sliced tissues from seven developing cotyledon halves were incubated in 5 ml of an enzyme solution (2% Cellulase 'Onozuka' R-10 and 0.1% Pectolyase dissolved in 0.7 M mannitol). After 4 to 5 h, the solution was removed, and 5 ml of 0.7 M mannitol was added to the slices and the flask was briefly swirled by hand. The mannitol solution was collected by filtration through a Nylon bolting cloth (35 mesh) and the filtrate was layered on 2 ml of 40% (w/v) sucrose. After slow speed centrifugation at 200g for 25°C, the supernatant was collected and used as the protein body fraction. The isolated protein bodies were inspected by an Olympus BHS phase-contrast microscope and found to be free from contamination by other cellular components (Fig. 4). The extracts from the protein body fraction and the whole cotyledon tissue were then subjected to immunological (western) blotting analysis.

Sucrose Density Gradient Centrifugation. Developing cotyledons (5 g) were homogenized in a Petri dish placed in an ice bath by chopping with a stainless steel razor blade for 5 min using 3 ml of chilled medium containing 150 mM Tricine-KOH (pH 7.5), 1 mM EDTA, and 13% (w/v) sucrose. The homogenates were passed through four layers of cheesecloth and the filtrate was directly layered onto a sucrose gradient consisting of a 1-m1 cushion of 60% (w/w) sucrose, 11 ml of sucrose solution increasing linearly from 30 to 60% sucrose, and 2 ml of 20% sucrose in a 17-ml tube. The gradient solution was then centrifuged at 21,000 rpm for 3 h in a Beckman Spinco model L8-70 ultracentrifuge using an SW 27-1 rotor at 4°C. After centrifugation, fractions (0.4 ml) were collected by an ISCO Density Gradient Fractorator (model 640) and used for the assays of each respective marker enzyme, the sucrose density, and the protein content, which was analyzed with a Bio-Rad Protein Assay Kit using bovine γ-globulin as a standard. The localization of globulin or proglobulin was determined using SDS gel electrophoresis or immunological (western) blotting analysis, respectively.

Pulse-Chase Labeling. Five μl of [³⁵S]methionine (58 μCi) was administered to the inner surface of the isolated cotyledon on moistened filter paper in a Petri dish and incubation was performed at 25°C. One to six cotyledons were used per treatment. After 15 or 30 min of [³⁵S]methionine uptake, the tissue was briefly rinsed in 10 mM unlabeled methionine solution, followed by further incubation for 0.5, 1, 2, or 3 h of chase period. At the indicated times, cotyledons were homogenized with 2% SDS solution containing 10 mM Tris-HCl (pH 6.8), 5% 2-mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue in a mortar. After centrifugation, 1 to 3 μl of the supernatant solution was subjected to SDS gel electrophoresis and fluorography.

For subcellular separation of the labeled cotyledons, the procedures of pulse-chase labeling and sucrose density gradient centrifugation were exactly the same as those described above.

Immunological (Western) Blotting. Electrophoretic blotting was performed basically as described by Vaessen et al. (29) and Yamaguchi et al. (30).

Marker Enzyme Assays. All enzyme assays were carried out at room temperature (25°C). Assay methods of catalase (17), NADPH-Cyt c reductase (16), fumarase (25), and alcohol dehydrogenase (26) followed those described in the literature.

Estimation of S Values. Sucrose density gradient centrifugation was performed according to the method of Martin and Ames (18). The internal marker enzymes, i.e. bovine liver catalase (0.5 k units) and yeast alcohol dehydrogenase (100 μg), were added to 200 μl of the extract from the developing cotyledons (0.06 g). The sample solution was layered onto 4.5 ml of the linear sucrose gradient (8–24% [w/v]) containing (a) 0.1 M Tris-HCl (pH 7.5) and 2 M NaCl or (b) 0.1 M Tris-HCl (pH 7.5), 1% Tween, and 0.25 M NaCl. Centrifugation was carried out at 40,000 rpm for 48 h (a) or 24 h (b) in a Beckman Spinco model L8-70 ultracentrifuge using an SW 65 Ti rotor at 4°C. After centrifugation, fractions (0.1 ml) were collected by an ISCO Density Gradient Fractorator (model 640), and aliquots were assayed for catalase and alcohol dehydrogenase and subjected to immunological (western) blotting analysis.

RESULTS

Precursor Forms of 11S Globulin. As a first step to study the in vitro synthesis of pumpkin 11S globulin, we prepared both free and membrane-bound polysomes from cotyledons of either developing seeds or etiolated seedlings, and examined the nature of the translation products directed by these preparations. From the data shown in Table 1, given on the basis of the same quantity of RNA, the maximal incorporation of [³⁵S]methionine into the immunoprecipitable polypeptides was observed with membrane-bound polysomes obtained from the developing cotyledons. The results indicated that the 11S globulin is synthesized on the RER, and that the globulin mRNA is not present in the cotyledons of germinating seedlings.

The fluorographic analysis of Figure 1 clearly shows that M₆ of the in vitro translation products directed by poly(A)⁺-RNA are 64,000 and 59,000, which are approximately 2,000 kD larger than those of the constituent subunits (α and β) of the mature globulin molecules. Consequently, the protein appears to be synthesized as a larger precursor form (proglobulin).

The time-sequential formation of in vitro translation products on membrane-bound polysomes has been analyzed employing the 'read-out' system. The initial products detected are M₆, 62,000 and 57,000 polypeptides, followed by the formation of proglobulin molecules having a larger molecular size (data not shown). These results indicate that cotranslational cleavage of the signal peptide of proglobulin occurs on the RER to produce proglobulin molecules.

The mature form of pumpkin 11S globulin contains two major subunits (α: M₆, 62,000 and β: M₆, 57,000) (Fig. 1, lane 2), each containing two polypeptide chains linked by a disulfide bond (9, 10). 2-Mercaptoethanol treatment produces smaller cleaved products (γ: M₆, 34,000-36,000 and δ: M₆, 22,000-26,000) (lane 3). However, this treatment does not alter the electrophoretic mobilities of the in vitro translation products (proglobulin) (lane 1), indicating that both γ and δ chains are synthesized as a single polypeptide chain.

Table 1. In Vitro Translation of 11S Globulin Directed by Free and Membrane-Bound Polysomes from Either Developing or Etiolated Cotyledons

<table>
<thead>
<tr>
<th>Polysomes</th>
<th>Incorporation of [³⁵S]Methionine*</th>
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<td>Developing cotyledons</td>
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<td>dpm</td>
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<tr>
<td>Membrane bound</td>
<td>114,400</td>
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<td>Free</td>
<td>6,570</td>
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* Incorporation of [³⁵S]methionine into immunoprecipitable polypeptides was measured on the basis of the same quantity of RNA.
were bodies (d) density fractionation (translational number 22), subjected to microsomes and/or organelles, i.e. microbodies (glyoxysomes). To assure that the fractions were not derived from the degradation of proteins, protoplasts were prepared from the developing cotyledons and used to isolate the intact protein bodies. Immunochemical methods did not identify proglobulin in the protein bodies (Fig. 4), thus showing that d = 1.24 g/cm³ fractions are not derived from these protein bodies. It is evident that at least three organelles, i.e. ER, d = 1.24 g/cm³ fractions and protein bodies, are involved in the biosynthesis and intracellular transport of the globulin molecules.

The pulse-chase [35S]methionine labeled cotyledons were subjected to subcellular fractionation experiments. Results presented in Figure 5 show that immediately after the pulse (0 h), radioactivity was detected in proglobulin localized in the ER and not in prepreglobulin. After 30 min chase, radioactivity had spread into the proglobulin present in two different organelles, ER and d = 1.24 g/cm³ fractions. During the subsequent 1 to 2 h chase periods, the γ and δ chains of globulin molecules in the protein body fraction became prominently labeled. For the purpose of further exploring the mechanism of the molecular assembly of the globulin subunit, the S value of the proglobulin or globulin molecules was determined to be 8S or 12S, respectively.

**DISCUSSION**

On the basis of these data, we propose that prepreglobulin synthesized on the RER is cotranslationally processed to make up trimeric proglobulin, which is then transported to the d = 1.24 g/cm³ fractions. Subsequent posttranslational processing results in the formation of hexameric molecules of the mature 11S globulin which are deposited in the protein bodies. The biosynthetic mechanism of the pumpkin 11S globulin can be diagrammatically summarized as in Figure 6. The Mᵣ of the in vitro synthesized singlet polypeptide (prepreglobulin) was shown to be approximately 2000 D larger than that of the doublet globulin subunit composed of disulfide-linked polypeptide chains.

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**Fig. 1.** In vitro synthesis of 11S globulin directed by poly(A)⁺-RNA. Lane 1. Fluorogram of immunoprecipitate of 11S globulin directed by poly(A)⁺-RNA. Lane 2 and 3. SDS gel electrophoretic pattern of mature globulin with (lane 3) or without (lane 2) 2-mercaptoethanol. α and β are represented to subunits of proglobulin or unreduced mature globulin, and γ and δ are represented to constituent polypeptide chains of reduced mature globulin. Lane 4. Mᵣ marker proteins: phosphorylase b (94,000), BSA (67,000), ovalbumin (43,000), α-carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactoalbumin (14,400).

**In vivo** pulse-chase labeling of the globulin molecules revealed that radioactivity was initially detected in proglobulin (Mᵣ 62,000 and 57,000), and after 30 to 60 min in both γ and δ chains of the mature form (Fig. 2). A radioactive band of Mᵣ 51,000 was detectable after 1 h. Although this component was precipitated by anti-11S globulin IgG, it is unlikely to be derived from the proteolytic hydrolysis of proglobulin, based on peptide mapping analysis (unpublished observation).

Overall, these results show that the single proglobulin polypeptide is posttranslationally processed to the doublet globulin subunit containing both γ and δ chains.

**Intracellular Transport of Globulin.** The crucial questions remaining to be answered relate to identifying the sites of post-translational processing of proglobulin molecules and assembly to form 11S globulin molecule. To answer the first question, we have subjected homogenates of cotyledons to subcellular fractionation on linear sucrose density gradients. As shown in Figure 3, proglobulin was detected in both ER (fraction number 7) and density (d) = 1.24 g/cm³ (51.7% [w/w] sucrose) fraction (fraction number 22), whereas most of the mature globulin molecules were contained in the bottom fraction, which is likely to be protein bodies and/or suborganelar inclusions, i.e. crystalloids (Fig. 3C).

**Fig. 2.** Labeling patterns of polypeptides during pulse-chase periods. Cotyledons were exposed to [35S]methionine for 15 min (left) or 30 min (right) and subsequently transferred to medium containing unlabeled methionine. Cotyledons were homogenized at the selected chase periods as indicated, and an aliquot of the supernatant fraction obtained after centrifugation was subjected to SDS gel electrophoresis and fluorography as described in the text.

What is the structural entity of the d = 1.24 g/cm³ fractions? From the measurements of several marker enzyme activities, they are clearly denser than ER, mitochondria, or microbodies (glyoxysomes). To assure that the fractions were not derived from the degradation of protein bodies, protoplasts were prepared from the developing cotyledons and used to isolate the intact protein bodies. Immunochemical methods did not identify proglobulin in the protein bodies (Fig. 4), thus showing that d = 1.24 g/cm³ fractions are not derived from these protein bodies. It is evident that at least three organelles, i.e. ER, d = 1.24 g/cm³ fractions and protein bodies, are involved in the biosynthesis and intracellular transport of the globulin molecules.

The pulse-chase [35S]methionine labeled cotyledons were subjected to subcellular fractionation experiments. Results presented in Figure 5 show that immediately after the pulse (0 h), radioactivity was detected in proglobulin localized in the ER and not in preglobulin. After 30 min chase, radioactivity had spread into the proglobulin present in two different organelles, ER and d = 1.24 g/cm³ fractions. During the subsequent 1 to 2 h chase periods, the γ and δ chains of globulin molecules in the protein body fraction became prominently labeled. For the purpose of further exploring the mechanism of the molecular assembly of the globulin subunit, the S value of the proglobulin or globulin molecules was determined to be 8S or 12S, respectively.
were (C). Locations for analysis (27-1).

Bassuner (1), chased cotyledons, the size fractionation process of proglobulin and mature globulin (C). Locations of the marker enzyme activities, NADPH-Cyt c reductase (ER), catalase (microbodies), and fumarase (mitochondria) are indicated by arrows. Protein content was determined by a Bio-Rad Protein Assay Kit using γ-globulin as a standard. (- - -), Protein content; ( . . . ), concentration of sucrose: P, precipitate; G, mature 11S globulin. (Fig. 1), and the signal peptide was cotranslationally cleaved. In contrast, Bassuner et al. (1) have recently reported that the M, of the in vitro translation product of the Vicia faba legumin (11S globulin) has a size similar to that of the mature molecule.

Employing subcellular fractionation experiments of the pulse-chased cotyledons, we have found that the proglobulin molecules, produced after cotranslational processing on the RER, are transported to the d = 1.24 g/cm³ organelles (Fig. 5). Indeed, a marked accumulation of proglobulin in the d = 1.24 g/cm³ organelles is clearly demonstrable (Fig. 3). Similar results have been reported for the intracellular transport of phytohemagglutinin in French bean (Phaseolus vulgaris L.) (2). It was found that phytohemagglutinin is transiently bound to the dense vesicles (d = 1.22 g/cm³) derived from the Golgi membranes. Electron micrographs have demonstrated the structure of vesicles as electron dense small particles (0.1–0.4 μm). The presence of the electron dense small vesicles has also been reported in developing rice endosperm tissues (23). There is thus a good possibility that the d = 1.24 g/cm³ fractions presently observed are structurally related to the dense vesicles identified by other workers.

It is generally accepted that in animal cells both secretory and lysosomal proteins are transported via the Golgi complex (7), where the posttranslational modifications occur. However, at present no conclusive experimental evidence is available supporting this mechanism operating in the biosynthetic pathway of nonglycosylated type 11S globulin molecules (12). If the d = 1.24 g/cm³ fractions are analogous to the Chrispeels’ dense vesicles (d = 1.22 g/cm³), we must take into account the possible role of the Golgi complex during the intracellular transport of the pumpkin 11S globulin molecules.

Other crucial questions remaining to be answered are the mechanisms of assemblage to form the mature form of 11S globulin, as well as its posttranslational processing. The quaternary structure of proglobulin and the mature globulin appears to correspond with the precursor and mature molecule of legumin in developing pea cotyledons (3). However, in contrast to our observations concerning the transient accumulation of the trimeric proglobulin in the d = 1.24 g/cm³ fractions (Figs. 3 and 5) and its scarce presence in the protein bodies (Fig. 4), Chrispeels et al. (3) proposed that the legumin precursor (trimer) is directly transported to the protein bodies from ER. They also reported that the legumin precursor is processed in the protein bodies to produce the mature form of legumin. However, they only examined ER and protein bodies, and it is conceivable from such experimental protocols that the protein bodies obtained are nothomogeneous but contain the denser membranous components, the latter being probably engaged in the accumulation and/or the posttranslational processing of the legumin precursor.

Subcellular fractionation of the pulse-chased cotyledons clearly demonstrated an accumulation of proglobulin in the d = 1.24 g/cm³ vesicles (Fig. 5). On the contrary, protein bodies are only associated with the mature globulin species, with little proglobulin molecules being accumulated (Figs. 3–5). All these findings substantiate the view that the two organelles are distinctly different in their protein compositions. In corroboration with the results of other investigators, it is reasonable to postulate that the posttranslational processing proceeds after the d = 1.24 g/cm³
Fig. 5. Subcellular fractionation of pulse-chase labeled cotyledons by sucrose density gradient centrifugation. After a prior pulse with $[^3]$H-methionine for 15 min, cotyledons were subsequently transferred to a chase medium containing unlabeled methionine for the selected chase periods as indicated (0, 0.5, 1, and 2 h), and then immediately homogenized by chopping with a razor blade for 5 min. After filtration of the homogenate, an aliquot was subjected to sucrose density gradient centrifugation at 21,000 rpm for 3 h using an SW 27-1 rotor of a Beckman Spinco ultracentrifuge at 4°C. After fractionation, an aliquot of each fraction was subjected to SDS gel electrophoresis and fluorography. D, d = 1.24 g/cm$^3$ fractions; PB, protein bodies; P, precipitate.

Fig. 6. Hypothetical mechanism of biosynthesis and intracellular transport of 11S globulin molecule in developing pumpkin cotyledons. Black arrows indicate the cleavage of signal peptide in pro(11S)globulin and posttranslational processing of globulin, respectively. Site of the introduction of disulfide bridge is not shown. Quaternary structure is based on the date of x-ray analysis of sunflower and rape seed 11S globulins (24) and Brazil nut 11S globulin (14); RER, RER.

Activities in the secretory granules (28) will help us in the full characterization of d = 1.24 g/cm$^3$ fractions in future experiments. Docherty et al. (6) have recently reported that a unique acid thiol protease (M, 31,500) may play a role in the processing of pro(11S)globulin. Further studies on the processing enzyme(s) as well as structural comparison between pro(11S)globulin and globulin will give us a clue to understanding the general mechanism of the posttranslational processing of seed reserve proteins.

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