In Vivo and In Vitro Processing of Seed Reserve Protein in the Endoplasmic Reticulum: Evidence for Two Glycosylation Steps

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ABSTRACT Cotyledons of the common bean (*Phaseolus vulgaris* L.) synthesize large amounts of the reserve protein phaseolin. The polypeptides are synthesized on membrane-bound polysomes, pass through the endoplasmic reticulum (ER) and accumulate in protein bodies. For a study of the biosynthesis and processing of phaseolin, developing cotyledons were labeled with radioactive amino acids, glucosamine and mannose, and isolated fractions (polysomal RNA, polysomes, and rough ER) were used for in vitro protein synthesis. Newly synthesized phaseolin present in the ER of developing cotyledons can be fractionated into four glycopolypeptides by SDS PAGE. In vitro synthesis with polysomal RNA results in the formation of two polypeptides (or size-classes) while run-off synthesis with polysomes isolated from rough ER produces two unglycosylated and two glycosylated polypeptides. The formation of two glycosylated polypeptides by polysome run-off shows that glycosylation is a co-translational event. The two unglycosylated polypeptides formed by polysome run-off are slightly smaller than the two polypeptides formed by in vitro translation of isolated RNA, indicating that a signal peptide may be present on these polypeptides.

Run-off synthesis with rough ER produces a pattern of four polypeptides similar to the one obtained by in vivo labeling. The two abundant glycopolypeptides formed in vivo or in vitro by rough ER are larger than the two glycosylated polypeptides formed by polysome run-off. This result indicates the existence of a second glycosylation event for the abundant polypeptides. Inhibition of glycosylation by Triton X-100 during chain-completion with rough ER was used to show that these two glycosylation steps normally occur sequentially. Both glycosylation steps are inhibited by tunicamycin. Analysis of carbohydrate to protein ratios of the different polypeptides and of trypsin digests of polypeptides labeled with [^{3}H]glucosamine confirmed the conclusion that some glycosylated polypeptides contain two oligosaccharide chains, while others contain only one. An analysis of tryptic peptide maps shows that each of the unglycosylated polypeptides is the precursor for one glycosylated polypeptide with one oligosaccharide chain and one with two oligosaccharide chains.

Legume seeds synthesize and accumulate large amounts of reserve protein in the course of their development. In the mature seed, these proteins are located in membrane-bounded organelles known as protein bodies (17, 35). Protein bodies are small (1–10 μm) protein-filled vacuoles (14, 26) which contain not only reserve proteins but also lectins (3, 6, 20, 34) as well as the acid hydrolases normally found in the lysosomes of animal cells (33). During seedling growth the reserve proteins are hydrolyzed, and the protein bodies participate in the autophagic digestion of cellular macromolecules and structures, indicating that they function in the same way as lysosomes (19). In the common bean *Phaseolus vulgaris* the two most abundant proteins in the protein bodies are the reserve protein phaseolin and the lectin phytohemagglutinin. Both are glycoproteins containing 4–6% carbohydrate mostly as mannose and glucosamine (1, 27). Phaseolin is synthesized on membrane-bound polysomes and sequestered in the lumen of the ER (4) before its transport to the protein bodies (8). Developing

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cotyledons therefore constitute an excellent system to study the biosynthesis, modification, and transport of glycoproteins which accumulate in lysosomelike compartments. So far, most of the work on the processing of glycoproteins has been done with secretory proteins such as immunoglobulins (5) or ovalbumin (21) or with membrane proteins such as the G protein of vesicular stomatitis virus (28).

In vitro biosynthesis of phaseolin with mRNA extracted from developing cotyledons showed that polypeptides of two different size classes (M_r 48,000 and 45,000) were obtained (18, 31). However, fractionation of phaseolin by one-dimensional SDS PAGE showed that at least three (30) or four (7) different polypeptides were present. This larger number of polypeptides in mature phaseolin may be due to co- or posttranslational modification of the initial translation products. By analyzing the polypeptides of phaseolin synthesized in vivo, and in vitro in different translation systems, we obtained evidence for the co-translational addition of oligosaccharide moieties. Some phaseolin polypeptides are glycosylated once, but most are glycosylated twice; the size heterogeneity of mature phaseolin is due in part to the differential glycosylation of polypeptides of the same size-class.

MATERIALS AND METHODS

Plant Material: Seeds of *Phaseolus vulgaris* L. cv. Greensleeves were purchased from Burpee Seed Co. (Warminster, PA) and the plants grown as described (7). Most experiments were carried out with cotyledons weighing 125–175 mg, when accumulation of phaseolin is quite rapid (7, 31).

Isolation of Rough ER, Polysomes, and Polysomal RNA: Seeds were harvested 20–25 d after flowering, immediately frozen in liquid nitrogen, and stored at −80°C. Cotyledons were homogenized in a cold mortar in extraction buffer (7) using 5 ml/g of tissue, and the homogenate was centrifuged for 10 min at 1,000 *g*. The supernatant was centrifuged again at 30,000 *g* to sediment the membranous organelles. The pellet was either suspended directly in a small volume of 40 mM Tris-HCl, pH 8.6, containing 20 mM KCl and 1 mM $MgCl_2$ (for in vitro run-off of rough ER) or stored at −80°C and used for the isolation of membrane-bound polysomes (7).

RNA was isolated from polysomes by the following procedure. Polysomal material equivalent to A_{260} = 40 was dissolved in 1.5 ml of buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 0.5% SDS, 1 mM 2′ and 3′ AMP), and 1.5 ml of a mixture of phenol, chloroform, and isoamylalcohol (50:50:1) were added. The mixture was stirred at room temperature for 10 min and centrifuged at 10,000 *g* for 10 min. The aqueous layer was recovered and the phenol layer re-extracted with 1.5 ml of buffer A. The aqueous layers were combined and made 0.3 M with respect to NaCl and 0.2 M with respect to Na-acetate. Addition of 2.5 vol of ethanol resulted in the precipitation of the polysomal RNA when the solution was kept overnight at −20°C. The RNA was recovered by centrifugation at 10,000 *g* for 10 min, dried under a stream of N_2, dissolved in sterile water, divided in small aliquots, and stored at −80°C.

In vitro translation was performed as described (7) using [^{3}H]leucine (sp act 4.07 TBq·mmol^{-1}) or [^{35}S]methionine (sp act 46.7 TBq·mmol^{-1}) from New England Nuclear Corp. (Boston, MA). Spermidine (120 μM) was included in the translation mixtures.

In Vivo Labeling of Cotyledons with Radioactive Precursors: In vivo labeling was done by the method of Spencer et al. (32) in which cotyledons are placed on a 20-μl drop of precursor solution on a piece of parafilm in a closed petri dish. If the precursor was present in a solution containing ethanol, the solution was lyophilized and the precursor dissolved in sterile water. The following precursors were used: ^{3}H-amino acid (Amersham Corp., Arlington Heights, IL) with specific activities of the amino acids ranging from 5.11 to 2.78 TBq·mmol^{-1}, D-[2-^{3}H]mannose (Amersham Corp.) specific activity 592 GBq·mmol^{-1}, D-[1,6-^{3}H(N)]glucosamine hydrochloride (New England Nuclear Corp.) specific activity 1.15 TBq·mmol^{-1}, ^{14}C (U)-amino acid mixture (New England Nuclear Corp.) with specific activity of the individual amino acids ranging from 20 to 4.3 GBq·mmol^{-1}.

Isolation of ER: To isolate ER (after in vivo incorporation of precursors and not for in vitro translation of rough ER), cotyledons were homogenized in 12% (wt/wt) sucrose dissolved in buffer B (200 mM Tris-HCl, pH 8.6, 20 mM KCl, 1 mM EDTA). The homogenate was centrifuged at 1,000 *g* for 10 min and the supernatant loaded on a discontinuous sucrose gradient consisting of 7 ml of 20% (wt/wt) sucrose in buffer B on top of 3 ml of 35% (wt/wt) sucrose in buffer B. After centrifugation at 150,000 *g* for 90 min the ER-rich organelle fraction was recovered from the top of the 35% sucrose layer. The load-portion of the gradient contained the cytosolic components as well as the contents of the protein bodies since most protein bodies are ruptured with this procedure (8, 13).

Immunological Techniques and Affinity Chromatography: The preparation of antiserum against phaseolin has been described (7). IgG specific for phaseolin was prepared by affinity chromatography using phaseolin-Sepharose as an affinity gel. Coupling of Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) to phaseolin and phaseolin-specific IgG was carried out according to the manufacturer's specifications. Isolation of radioactive phaseolin polypeptides with Sepharose-IgG (Phaseolin) was carried out as described by Badenoch-Jones et al. (2). Separation of glycosylated from unglycosylated phaseolin polypeptides was carried out with concanavalin A-Sepharose 4B (Sigma Chemical Co., St. Louis, MO). The mixture of polypeptides was dissolved in buffer C (50 mM Na-phosphate, pH 7.0, 1% NaCl, 0.05% SDS) and mixed with the affinity resin equilibrated with the same buffer. After incubation overnight at 4°C the proteins which did not bind to the concanavalin A were eluted with buffer C, and the glycosylated proteins were eluted with 1 M α-methyl-D-mannoside (Sigma Chemical Co., grade III) in buffer C. The unglycosylated polypeptides of phaseolin were recovered from the mixture of unbound proteins with immunoaffinity gel.

Electrophoresis on Polyacrylamide Gels or Thin Layer Plates: SDS PAGE of polypeptides was done as described (7) using the system of Laemmli and Favre (22). Electrophoresis of oligopeptides on thin-layer plates (20 × 20 cm, Merck & Co., Inc., Rahway, NJ) was done at pH 4.7 in *n*-butanol/pyridine/acetic acid/H_2O (2:1:1:36) for 1.5 h at 400 V. Fluorography of polyacrylamide gels was done as described (10). Fluorography of thin-layer plates was done by spraying the plates with Enhance (New England Nuclear Corp.) followed by exposure to Kodak safety films XAR-5.

Tryptic Digestion of Phaseolin Polypeptides: Phaseolin was digested with trypsin as described by Bollini and Vitale (9). Recovery of individual polypeptides and subsequent digestion was carried out as follows. For the analysis of glycopeptides, ER from [^{3}H]glucosamine-labeled cotyledons was fractionated by SDS PAGE and the gel stained to locate the four phaseolin polypeptides. The gel was destained, rinsed with water, and the bands were excised. The gel slices containing the protein were lyophilized, and a small portion was set aside for re-electrophoresis to assess purity. For the analysis of [^{35}S]methionine-labeled peptides, unglycosylated and glycosylated polypeptides synthesized in vitro by chain completion in the presence of [^{35}S]methionine by polysomes and rough ER, respectively, were fractionated by SDS PAGE. The polypetpides were localized by autoradiography and the corresponding gel-portions excised. The slices were treated twice for 6 h at 30°C with 1 ml of 1% NH_4HCO_3 containing trypsin (the ratio of trypsin to protein was approximately 1:10). The two extracts were combined and the slices re-extracted for 18 h at room temperature with 0.5% NH_4HCO_3 to recover more digestion products. The pooled extracts were lyophilized several times to eliminate the ammoniumbicarbonate, then redissolved in 2% NH_4OH. Tryptic digests were fractionated on thin-layer plates either by electrophoresis (see above) or by ascending chromatography for 5 h at room temperature with *n*-butanol:acetic acid:water (200:30:75) as solvent.

RESULTS

Previous results from our laboratory show that phaseolin of *P. vulgaris* cv. Greensleeves consists of at least four polypeptides (or size-classes of polypeptides) which can be separated by one-dimensional SDS PAGE (7, 8). Two abundant polypeptides alternate with two less abundant ones under our standard conditions of electrophoresis. These four polypeptides will be referred to as A, B, C, and D, in order of increasing mobility on the gels. Newly synthesized polypeptides are transiently associated with the endoplasmic reticulum before they are transported to the protein bodies (8). The electrophoretic pattern of newly synthesised polypeptides of phaseolin present in the ER is shown in Fig. 1. Cotyledons were labeled with ^{3}H-amino acids, [^{3}H]glucosamine, or [^{3}H]mannose, and the ER was isolated on discontinuous sucrose gradients. The polypeptides of the ER were fractionated by SDS PAGE and a fluorogram was prepared. The results show that the ER contains a number of polypeptides which can be readily labeled with ^{3}H-amino acids (Fig. 1, lane *1*), [^{3}H]glucosamine (Fig. 1, lane *2*), or [^{3}H]mannose (Fig. 1, lane *3*). Phaseolin polypeptides

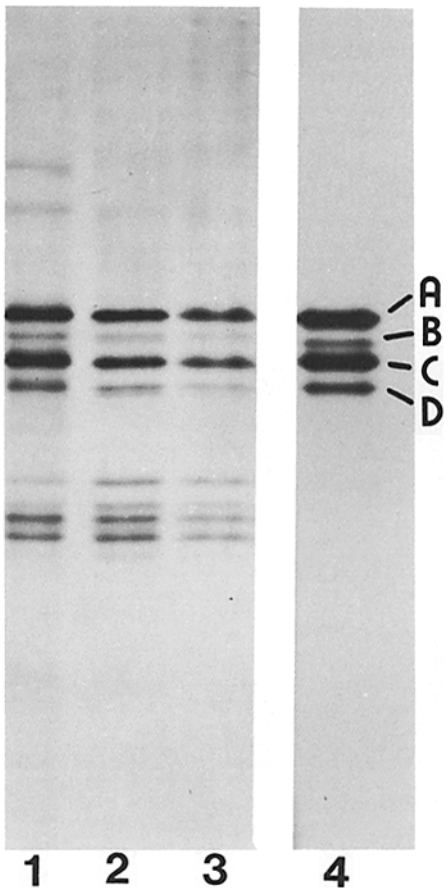

FIGURE 1 Fluorograph of polypeptides in the ER labeled with radioactive amino acids, glucosamine, and mannose. Cotyledons (2) were labeled for 5 h with 7.5×10^5 Bq each of [^{3}H]amino acids (lane *1*), [^{3}H]glucosamine (lane *2*) or [^{3}H]mannose (lane *3*). The ER was isolated from the homogenate on discontinuous sucrose gradient, and the polypeptides fractionated by SDS PAGE. Lane *4* shows phaseolin isolated with immunoaffinity gel from the sample shown in lane *1*.

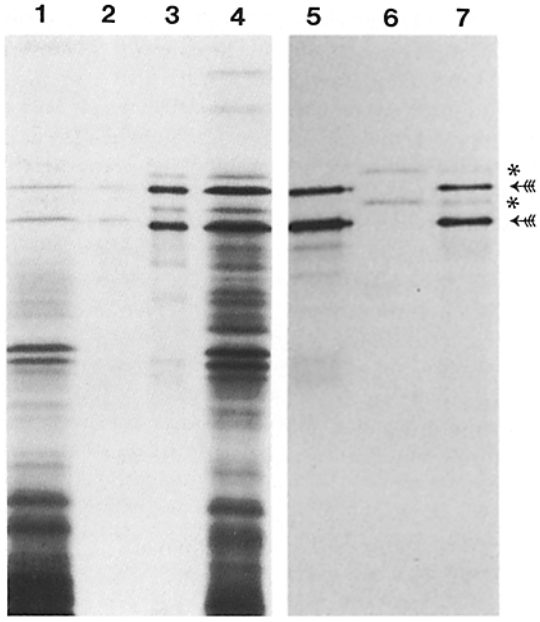

FIGURE 2 Fluorograph of polypeptides synthesized in vitro either with polysomal RNA or by run-off of isolated polysomes. In vitro translation systems contained 10 μg of polysomal RNA (in 100-μl assay system) or polysomes equivalent to $A_{260} = 0.8$ (in 100-μl assay system). Incubation for 45 min at 30°C with 4.8×10^5 Bq of [^{3}H]-leucine; isolation of phaseolin by affinity chromatography with IgG-Sepharose or concanavalin A-Sepharose. (Lane 1) Total *in vitro* translation products obtained with polysomal RNA. (Lane 2) Phaseolin isolated with IgG-Sepharose from products shown in lane *1*. (Lane 3) Phaseolin isolated with IgG-Sepharose from products shown in lane *4*. Lane 4: Total in vitro translation products obtained by chain completion of polysomes isolated from rough ER. Lane 5: Unglycosylated phaseolin polypeptides isolated from the products in lane *7* by treatment with concanavalin A-Sepharose. Lane 6: Glycosylated phaseolin polypeptides isolated as in lane *5*. Lane 7: As lane 3. Asterisks indicate glycosylated polypeptides; arrows indicate unglycosylated polypeptides.

were isolated with antibodies against phaseolin from the ^{3}H-amino acid-labeled ER and are shown in Fig. 1, lane *4*. The results indicate that the four most heavily labeled polypeptides in the ER are those of phaseolin, and that all four bands are glycopolypeptides containing mannose and glucosamine. All four peptides bind to a concanavalin A–Sepharose column and can be eluted with α-methylmannoside (data not shown, but see later).

When RNA isolated from developing cotyledons is translated in vitro, two different phaseolin polypeptides (or size-classes of polypeptides) could be resolved by SDS PAGE (18, 31). A more complex pattern of phaseolin polypeptides was obtained when polysomes detached from ER membranes were allowed to complete in vitro polypeptides already initiated in vivo. This more complex pattern containing three or four bands was not identical with, but resembled more or less, the pattern found after in vivo labeling of excised cotyledons (7, 31). To find out whether the differences resulted from co-translational processing events we analyzed the translation products of isolated polysomal RNA and polysomal run-off side-by-side on a polyacrylamide gel (Fig. 2). The total translation products obtained with polysomes detached from ER membranes are shown in Fig. 2, lane *4*. The polypeptides of phaseolin isolated from the total polysomal run-off with antibodies against phaseolin are shown in Fig. 2, lanes *3* and *7*. There are two abundant polypeptides (arrows) and two less abundant polypeptides (asterisks) in the M_r 50,000 range, as well as some contaminating smaller polypeptides (Fig. 2). Fractionation by SDS PAGE of the products of in vitro synthesis with polysomal RNA as template indicates the synthesis of numerous polypeptides (Fig. 2, lane *1*), two of which bind to antibodies against phaseolin (Fig. 2, lane *2*). The two polypeptides in Fig. 2, lane *2*, have a slightly lower mobility than the two abundant polypeptides in Fig. 2, lane *3*. This slight difference in mobility is similar to that found for the polypeptides of conglycinin and may represent the presence of a signal sequence on the products made with mRNA as template (29).

The products of polysomal run-off synthesis were fractionated on concanavalin A-Sepharose to determine whether some chains were already glycosylated. Polysomes were detached from ER-membranes with detergent and allowed to complete already initiated polypeptides (Fig. 2, lane *4*). Phaseolin polypeptides were isolated from this mixture with antibodies against phaseolin (Fig. 2, lane *7*) and fractionated with concanavalin A–Sepharose into a fraction which did not bind (Fig. 2, lane *5*) and a fraction which bound to the affinity gel and could be eluted with α-methyl mannoside (Fig. 2, lane *6*). The results show that the isolated polysomes completed the synthesis of two unglycosylated polypeptides (most abundant) and two

glycosylated polypeptides (least abundant). The results indicate that glycosylation is a co-translational event, because some nascent chains present on the polysomes were already glycosylated.

The relative abundance of glycosylated and unglycosylated chains completed in vitro could be manipulated by changing the amount of polysomes added to the in vitro system. With an amount of polysomal material equivalent to an A_{260} of 0.1 in a total in vitro system with a volume of 25 μl, most of radioactivity was in unglycosylated chains (Fig. 3, lane *1*). However, when 5 and 20 times as much polysomal material was added to the system with the same total incubation volume (25 μl), more and more of the radioactivity was in glycosylated chains (Fig. 3, lanes *2* and *3*). These conditions may favor the completion of the largest (and glycosylated) nascent chains at the expense of the shorter (and unglycosylated) nascent chains. A comparison of the size of the glycosylated polypeptides made by polysome run-off with the polypeptides made in vivo (Fig. 3, lane *4*) shows that the two glycopolypeptides made in vitro have approximately the same size as the two less abundant glycopolypeptides (B and D) but differ considerably from the abundant glycopolypeptides A and C. Glycosylated polypeptides as large as A and C were never produced by polysome run-off.

To confirm that the glycosylated polypeptides made in the chain completion system had been co-translationally glycosylated, we first allowed chain completion to proceed in the presence of nonradioactive amino acids, and radioactive amino acids were added later and later (2–15 min after the start of the incubation). The polypeptides produced in this manner were then fractionated by SDS PAGE and visualized by fluorography (Fig. 4). If chain completion was allowed to proceed for 10 min with cold amino acids (Fig. 4, lane *4*) before the addition of radioactive amino acids, no radioactive glycosylated polypeptides were formed. This result confirmed that glycosylation was a co-translational event and that the glycosylated polypeptides were more nearly completed than the unglycosylated ones

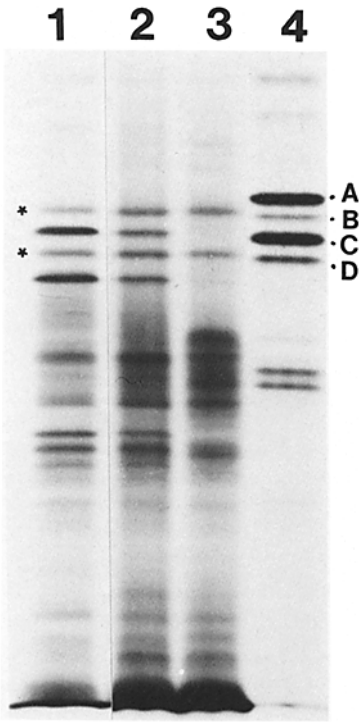

FIGURE 3 Fluorograph of polypeptides made by run-off from isolated polysomes, and polypeptides made in vivo. Polysomal material (A_{260} = 0.1 for lane *1*; A_{260} = 0.5 for lane *2*; A_{260} = 2.0 for lane *3*) incubated in 25 μl for chain completion assay; incubation at 30°C for 45 min with 1.6 Bq of [^{3}H]leucine. Lane *4* contains ER isolated from cotyledons labeled with [^{3}H]-amino acids. Asterisks indicate glycosylated polypeptides.

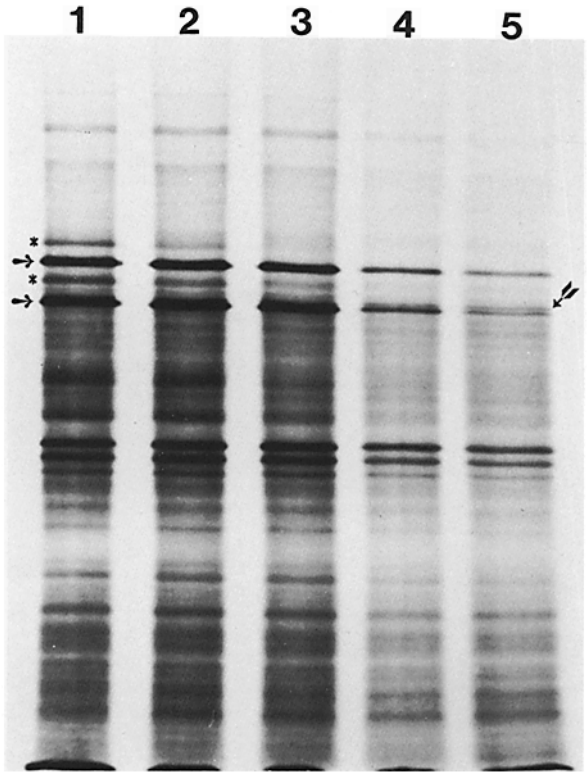

FIGURE 4 Fluorograph of polypeptides made by run-off from isolated polysomes with later and later addition of radioactive amino acids. Polysomal material (A_{260} = 0.3 in 25-μl assay mixture) incubated at 30°C for 45 min with 1.1 × 10^5 Bq of [^{3}H]leucine, [^{3}H]-leucine was added at the beginning of the incubation (lane *1*), or after 2 min (lane *2*), 5 min (lane *3*), 10 min (lane *4*), or 15 min (lane *5*). Asterisks indicate glycosylated polypeptides; arrows indicate unglycosylated polypeptides.

at the time the polysomes were isolated. This experiment also shows that a very late addition of radioactive amino acids (Fig. 4, lane *5*) results in the formation of a doublet of radioactive polypeptides (arrow on the right hand side of Fig. 4). We interpret the presence of this doublet as evidence that the polysomes contained some polypeptides the signal sequence of which had not yet been removed at the time the polysomes were detached from the membranes.

Since run-off by isolated polysomes did not produce glycosylated polypeptides so large as those present in vivo in the ER, we used rough ER instead of isolated polysomes in the chain-completion system. The products of synthesis were again fractionated by SDS PAGE and visualized by fluorography (Fig. 5). Lane *4* shows all the polypeptides which were labeled, while lanes *2* and *3* (Fig. 5) show the polypeptides of phaseolin extracted from the total mixture with immunoaffinity gel. Lanes *2* and *3* (Fig. 5) represent two different in vitro translations. When the polypeptides formed by rough ER chain-completion were compared with those formed by polysome chain-completion (Fig. 5, lane *1*) we found that two new polypeptides (asterisks) were formed. The size of these new polypeptides closely corresponded to that of the two abundant polypeptides (Fig. 5, *A* and *C*) made in vivo and present in the ER (Fig. 5, lanes *5* and *6*). In vitro chain completion with rough ER produced a pattern of polypeptides more complex than that obtained by in vivo labeling (compare in Fig. 5, lanes *3* and *5*). This higher degree of complexity may result from the formation of unglycosylated polypeptides (the fastest running

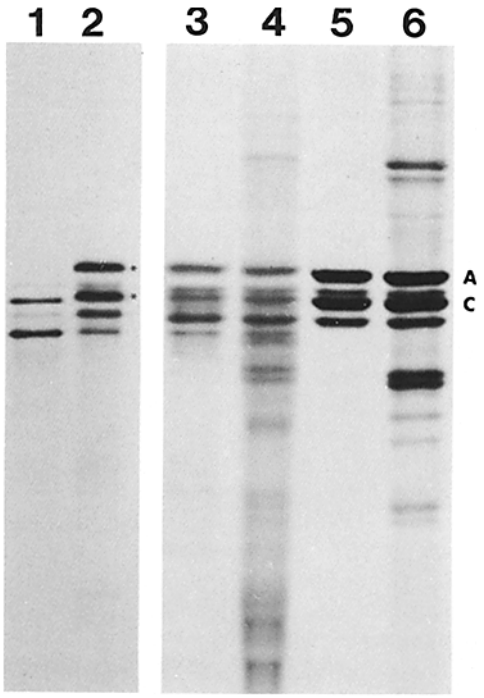

FIGURE 5 Fluorograph of polypeptides synthesized in vitro using chain completion of nascent chains associated with rough ER, and synthesized in vivo. Rough ER equivalent to $A_{260} = 0.5$ was translated in vitro in 50-μl assay system at 30°C for 45 min in the presence of 1.8×10^5 Bq of [^{3}H]leucine. (Lane *1*) Polypeptides of phaseolin synthesized in a chain completion assay with isolated polysomes detached from the membranes; phaseolin isolated with immunoaffinity gel. (Lanes *2* and *3*) Polypeptides of phaseolin synthesized in a chain completion assay with isolated rough ER, phaseolin isolated with immunoaffinity gel, lanes *2* and *3* represent two different incubations. (Lane *4*) Total products synthesized in vitro by rough ER. (Lane *5*) Polypeptides of phaseolin synthesized in vivo and isolated from the ER with immunoaffinity gel from products shown in lane *6*. (Lane *6*) Polypeptides synthesized in vivo and associated with the ER of cotyledons labeled with [^{3}H]amino acids.

polypeptides in Fig. 5, lanes *2* and *3*) or partially glycosylated polypeptides.

The results presented in Fig. 5 indicate that there may be two glycosylation steps: one which is co-translational, and one which either is co- or may be posttranslational. To obtain direct evidence concerning the temporal relationship of these two glycosylation steps, we added Triton X-100 to the chain-completion system at different times after the reaction had been initiated. Glycosylation of nascent polypeptides by membrane-bound enzymes can be completely inhibited by Triton X-100 without affecting in vitro protein synthesis (28). When Triton X-100 was added at the beginning of the incubation of rough ER with the translational mixture (Fig. 6, lane *2*), the polypeptide pattern resembled the one obtained with polysomes (Fig. 6, lane *1*): two heavily-labeled unglycosylated polypeptides alternated with two lightly labeled glycopolypeptides. Later and later additions of Triton X-100 (Fig. 6, lanes *3–7*) showed a decrease in the intensity of the fastest running unglycosylated polypeptide (double arrow), a transient increase in the two glycosylated polypeptides marked with asterisks, and the appearance of a fully mature polypeptide (Fig. 6, *A*) if the membranes were left intact for at least 10 min. We interpret these changes as evidence for two sequential glycosylation steps. The two unglycosylated polypeptides (marked with arrows) gradually decrease, undergoing a first glycosylation (polypeptides marked with asterisks reach their maximum intensity after 10 min) and then a second glycosylation giving rise to the mature glycopolypeptides A and C (Fig. 6). There was no change in the intensity of the polypeptide(s) in position C (Fig. 6). This is interpreted as being due to the simultaneous disappearance of an unglycosylated polypeptide (single arrow) and the appearance of the fully glycosylated polypeptide C (Fig. 6).

The analysis of Fig. 6 indicates that not all the polypeptides undergo the second glycosylation; polypeptides B and D (Fig. 1) should therefore contain less carbohydrate than A and C (Fig. 1). The idea that the four chains do not contain the same amount of carbohydrate was investigated by labeling the cotyledons with ^{3}H-amino acids, [^{3}H]glucosamine, or [^{3}H]mannose. The ER of such cotyledons was isolated and the polypeptides were fractionated by SDS PAGE. Fluorographs of these gels were made, and the phaseolin region of the films was scanned to determine the intensity of each radioactive band. The results were expressed as a percentage of radioactivity from [^{3}H]mannose or [^{3}H]glucosamine of the radioactivity from ^{3}H-amino acid. This allowed us to measure the amount of carbohydrate in each band and normalize it to the same amount of protein. The results show that on a protein basis bands A and C (the abundant polypeptides) had nearly twice as much glucosamine and mannose as bands B and D (the less abundant polypeptides). The results obtained by scanning the fluorographs were confirmed by double-labeling experiments in which the cotyledons were labeled with ^{14}C-amino acids and [^{3}H]glucosamine or ^{14}C-amino acids and [^{3}H]mannose. The isolated ER was fractionated by SDS PAGE, and the four different polypeptides were recovered from the gel by electroelution. The ratios of ^{3}H/^{14}C with glucosamine and with mannose were measured for each of the four polypeptides. The results (Table I) show that bands A and C contained ~50% more glucosamine than bands B and D (compared to the amount of ^{14}C-amino acid) and ~80–100% more mannose. These results are consistent with the interpretation that the two most abundant polypeptides contain more and/or longer oligosaccharide chains than the less abundant polypeptides.

To find out how many oligosaccharide chains each of the glycopolypeptides contained, we isolated radioactive phaseolin polypeptides from the ER of cotyledons labeled with [^{3}H]-

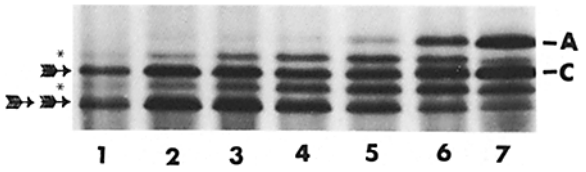

FIGURE 6 Fluorograph of the phaseolin polypeptides made by run-off synthesis with isolated polysomes and rough ER. In vitro translation system with [^{3}H]leucine and polysomal RNA ($A_{260} = 0.25$) or rough ER ($A_{260} = 0.5$), total incubation for 60 min at 30°C. Only the region of the gel containing the phaseolin polypeptides is shown; the slower-running unglycosylated polypeptide (single arrow) is not separated from the glycosylated polypeptide C. (Lane *1*) Polypeptides synthesized by polysome-runoff. (Lane *2–7*) Polypeptides synthesized by rough ER with later and later additions of Triton X-100 (1% vol/vol, final concentration). Detergent was added at the beginning of the incubation (lane *2*), after 2 min (lane *3*), after 5 min (lane *4*), after 10 min (lane *5*), after 20 min (lane *6*), or at the end (lane 7). Arrows denote unglycosylated polypeptides, asterisks denote polypeptides glycosylated once; *A* and *C* represent the mature polypeptides glycosylated twice.

glucosamine and separated them by SDS PAGE. The four polypeptides were recovered from the gel and subjected to digestion by trypsin. The trypsin digests were then spotted on a thin-layer plate and subjected to electrophoresis. The radioactive glyco-oligopeptides were visualized by fluorography and are shown in Fig. 7. Each spot represents a radioactive oligosaccharide (labeled with [^{3}H]glucosamine) attached to a nonradioactive oligopeptide. When total phaseolin (before fractionation by SDS PAGE) was treated in the manner described above, the digestion products contained three different glycosylated oligopeptides (Fig. 7, lane *T*). Very little undigested material remained at the origin (Fig. 7, arrow). When the glycopolypeptides A, B, C, and D were analyzed in this manner the digestion products of A and C contained one additional glycosylated oligopeptide not present in B and D. This result supports our previous conclusion that the abundant polypeptides A and C contain an additional glycosylation site and oligosaccharide side-chain. It is also clear from Fig. 7 that the three oligosaccharides are not present in the same amounts. In particular, the slowest moving one is present in much smaller amounts than the other two. It is not clear whether this reflects partial glycosylation of a particular site or, more likely, heterogeneity at the polypeptide level.

TABLE I

	Glucosamine, ^{3}H/^{14}C		Mannose, ^{3}H/^{14}C	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
A	1.70	1.76	0.51	0.47
B	1.19	1.24	0.29	0.28
C	1.68	1.61	0.56	0.63
D	1.13	1.14	0.26	0.23

Cotyledons were labeled for 3.5 h with [^{3}H]glucosamine and ^{14}C-amino acids, or [^{3}H]mannose and ^{14}C-amino acids. The ER was isolated on discontinuous sucrose gradients and the polypeptides were fractionated by SDS PAGE. The four polypeptides of phaseolin were recovered by electroelution, and radioactivity in ^{3}H and ^{14}C was determined. The ratios indicate that polypeptides A and C have ~50% more glucosamine and 100% more mannose than polypeptides B and D.

To find out how many unglycosylated polypeptides of phaseolin are synthesized in vivo, we treated cotyledons with tunicamycin (an inhibitor of the en bloc transfer of high-mannose type oligosaccharides) and then labeled them with ^{3}H-amino acids. In preliminary experiments we tested the effect of tunicamycin on the incorporation of [^{3}H]glucosamine. In contrast to experiments done with pea cotyledons (2) we found little inhibition of incorporation of [^{3}H]glucosamine when using 50 μg/ml of the inhibitor. Some (30–50%) inhibition was obtained with 500 μg/ml of tunicamycin and this concentration was used in subsequent experiments. Cotyledons were pretreated with a 20-μl drop of 500 μg/ml of tunicamycin for 3 h, then allowed to incorporate ^{3}H-amino acids for 3 h, and the ER was isolated. The radioactive polypeptides were fractionated by SDS PAGE and visualized by fluorography. The results show that the pattern of phaseolin polypeptides synthesized in the presence of tunicamycin (Fig. 8, total ER in lane *3*) was more complex than in its absence (Fig. 8, lane *5*) and appeared to be a mixture of glycosylated and unglycosylated polypeptides resulting from a partial inhibition of glycosylation. The ER obtained from the tunicamycin-treated cotyledons was dissolved with 1% Triton X-100, and the polypeptides were fractionated on concanavalin A-Sepharose. The fraction which bound to the affinity column was eluted with α-methylmannoside and is shown in lane 4 (Fig. 8). The fraction which did not bind to the affinity resin was challenged with antibodies against phaseolin, and the unglycosylated phaseolin polypeptides are shown in lane *2* (Fig. 8). Phaseolin isolated from polysomal run-off is shown in lane *1* (Fig. 8). The results show that in the presence of tunicamycin two unglycosylated polypeptides were synthesized; the size of these two polypep-

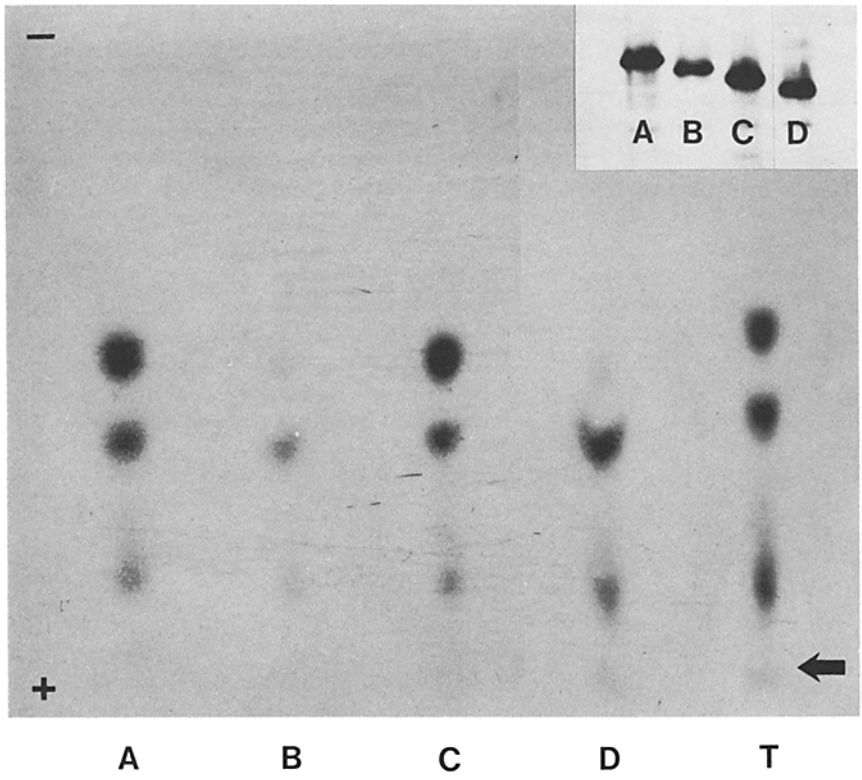

FIGURE 7 Fluorograph of glycosylated oligopeptides separated by thin-layer electrophoresis. Cotyledons (5) were labeled for 6.5 h each with 9 × 10^5 Bq of [^{3}H]glucosamine. The ER was isolated on discontinuous sucrose gradient and fractionated by SDS PAGE. The individual polypeptides of phaseolin were cut out and digested with trypsin. The oligopeptides were recovered and material representing 10,000 cpm (bands *A*, *C* and total phaseolin [*T*]) or 5,000 cpm (bands *C* and *D*) was spotted on a thin-layer plate and subjected to electrophoresis. The plate was sprayed with "Enhance" and a fluorograph prepared. *A*, *B*, *C*, and *D* correspond to the different polypeptides of phaseolin, arrow indicates origin.

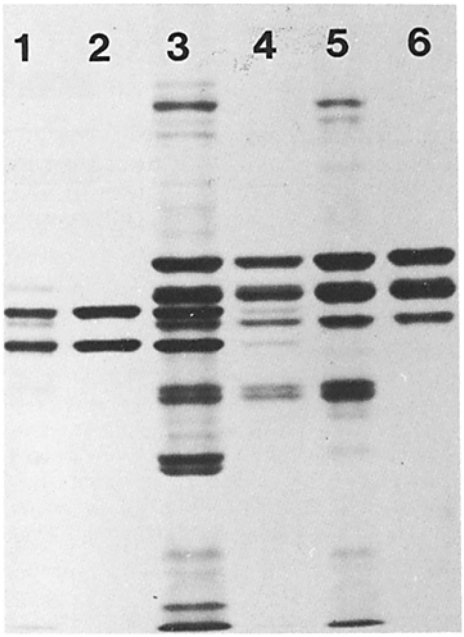

FIGURE 8 Fluorograph of polypeptides associated with the ER and synthesized in vivo in the presence or absence of tunicamycin. Cotyledons (7) were treated with 20 μl each of water or tunicamycin (500 mg/ml) for 3 h. Then 7.2×10^5 Bq of ^{3}H-amino acids was added to each cotyledon and incorporation allowed to proceed for 3 h. The ER was isolated on discontinuous sucrose gradients and dissolved with 1% Triton X-100 before separation of glycosylated from unglycosylated polypeptides with concanavalin A-Sepharose (tunicamycin-treated sample). (Lane *1*) Run-off synthesis from polysomes; phaseolin recovered with immunoaffinity gel (as in Fig. 2, lane *3*). (Lane *2*) Unglycosylated phaseolin polypeptides recovered from products shown in lane *3* by sequential treatment with concanavalin A-Sepharose and IgG-Sepharose. (Lane *3*) Total ER from tunicamycin treated cotyledons. (Lane *4*) Glycosylated polypeptides present in the ER of tunicamycin-treated cotyledons, separated from unglycosylated polypeptides with concanavalin A-Sepharose. (Lane *5*) Total ER of control cotyledons. (Lane *6*) Phaseolin polypeptides extracted from products shown in lane *5* with IgG-Sepharose. Note that in this particular gel which contains only 12% acylamide polypeptides *B* and *C* did not separate.

tides in the ER is similar to the size of the unglycosylated polypeptides made in vitro by polysomal run-off (compare the abundant polypeptides in lane *1* with the polypeptides in lane 2 in Fig. 8). The results confirm the conclusion that the four glycosylated polypeptides arise from two size-classes of polypeptides.

The results presented so far are interpreted to indicate that the two unglycosylated polypeptides give rise to four glycosylated polypeptides, two of which have one oligosaccharide side-chain and two of which have two side-chains. The SDS PAGE gel patterns indicate but do not provide proof that A and B are derived from the slower migrating unglycosylated polypeptide, and C and D from the faster one. This interpretation was confirmed by analyzing the tryptic peptides of the polypeptides of [^{35}S]methionine-labeled phaseolin. Polysomes and rough ER were used in a chain completion system to generate [^{35}S]methionine-labeled polypeptides. These polypeptides were fractionated with concanavalin A-Sepharose 4B, and glycosylated polypeptides were separated from unglycosylated ones. The unglycosylated polypeptides synthesized by the isolated polysomes and the glycosylated polypeptides made by the rough ER were used for further analysis. The polypeptides were fractionated by SDS PAGE, and the polypeptides were located by autoradiography and then excised from the dried gel. These polypeptides were digested with trypsin, and the tryptic peptides were separated by thin-layer chromatography. The methionine-labeled peptides were located by autoradiography of the dried plate (Fig. 9). The notation for the polypeptides used here is the same one used in Fig. 10. The two unglycosylated polypeptides have a different number of [^{35}S]methionine peptides: the slower one (A_oB_o) has four, while the faster one (C_oD_o) has two. The pattern of the glycosylated polypeptides resembles the pattern of the unglycosylated polypeptides from which they were derived. Thus, A and B made by the rough ER resemble A_oB_o made by the polysomes, and C and D made by the rough ER resemble C_oD_o made by the polysomes. The map of C contains, in addition to two abundant spots, a contamination of the spots characteristic of A_oB_o. This is probably due to the incomplete separation of glycosylated and unglycosylated polypeptides by concanavalin A-Sepharose (see

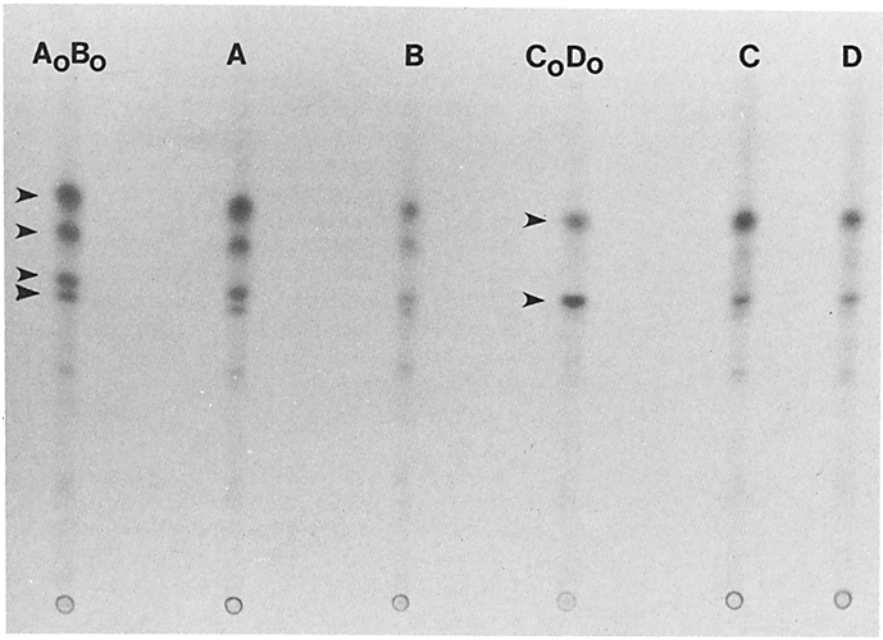

FIGURE 9 Autoradiograph of tryptic digest of [^{35}S]methionine-labeled phaseolin polypeptides separated by thin-layer chromatography. Polysomes and rough ER were used in a chain completion system with 11 GBq of [^{35}S]methionine. The polypeptides were fractionated with concanavalin A-Sepharose 4B, and the unglycosylated polypeptides made by the polysomes and the glycosylated polypeptides made by the rough ER were subjected to SDS PAGE. The polypeptides of phaseolin were located by autoradiography, excised from the gel, and digested with trypsin. The tryptic digests were fractionated by thin-layer chromatography and an autoradiograph was prepared. A_oB_o and C_oD_o are, respectively, the slower and faster moving unglycosylated polypeptides made by polysomes. A, B, C, and D are the glycosylated polypeptides made by rough ER. The arrows indicate the major ^{35}S-labeled peptides.

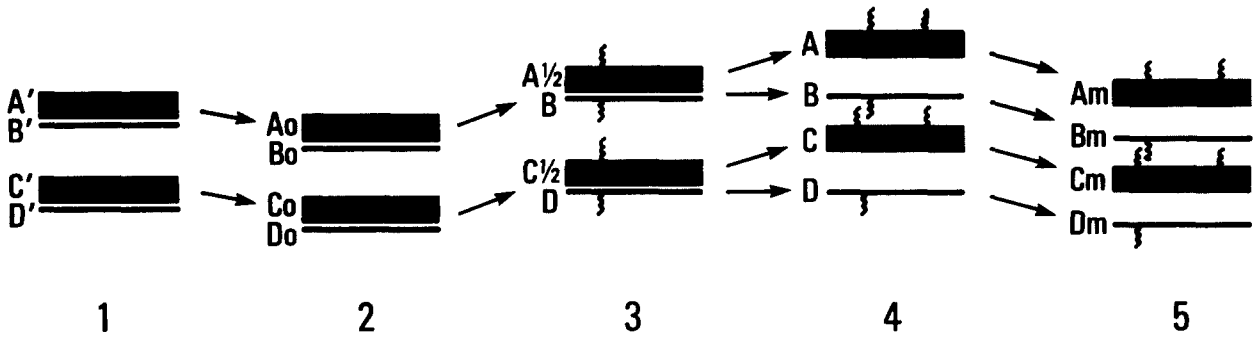

FIGURE 10 Schematic drawings of the different processing steps of the phaseolin polypeptides. Note that polypeptides which are drawn closely together such as *A'* and *B'* or A_o and B_o are not visible as distinct bands on the gels. *A* and *B* are only resolved on the gels after one of the polypeptides (*A*) has received an additional oligosaccharide side-chain. The processing steps are as follows: (*1*) Polypeptides *A'*, *B'*, *C'*, and *D'* synthesized in vitro with polysomal RNA as template; signal peptides present. (*2*) Unglycosylated polypeptides A_o, B_o, C_o, and D_o synthesized in vitro by polysome run-off; signal peptides absent. (*3*) Glycosylated polypeptides *A½*, *B*, *C½*, and *D* synthesized in vitro by polysome runoff; signal peptides absent; one oligosaccharide side-chain present. (*4*) Glycosylated polypeptides *A*, *B*, *C*, and *D* synthesized in vitro by run-off of rough ER, or synthesized in vivo and present in the ER; a second oligosaccharide present on *A* and *C*. (5) Mature glycosylated polypeptides A_m, B_m, C_m, and D_m present in protein bodies after in vivo labeling and chase; nature of the processing step not entirely clear.

for example lane *4* in Fig. 8). Since the position of C overlaps with the unglycosylated A_oB_o polypeptides (see discussion of Fig. 6), the C polypeptide which was recovered in this experiment was contaminated with a small amount of A_oB_o. The peptide maps presented in Fig. 9 confirm that each of the unglycosylated precursors gives rise to two different glycosylated products.

DISCUSSION

The results presented in this paper and elsewhere (8) detail the sequence of events in the processing of the glycosylated polypeptides of phaseolin. They confirm and extend the work of Sengupta et al. (29) who studied the processing of the soybean storage protein conglycinin. Together, these studies provide a detailed analysis of the processing steps for a glycoprotein which accumulates in the lysosomelike compartments of plant cells (vacuoles and protein bodies). The co- and posttranslational modification steps for which we obtained evidence are depicted in Fig. 10 and described in the legend. It should be noted that A' and B' as well as C' and D' are shown as separate polypeptides, but that they are not resolved on the gels. Rather, they are present as two polypeptide bands of A' + B' and C' + D'. Our results confirm the findings of Hall et al. (18) that phaseolin is synthesized as two size-classes of unglycosylated polypeptides. This was demonstrated by using polysomal RNA as template, with polysome run-off, and by labeling in vivo in the presence of tunicamycin. Fractionation of the unglycosylated polypeptides obtained by polysome run-off on two-dimensional gels showed that at least five different polypeptides were present (data not shown), indicating that the two polypeptide bands (A' + B' and C' + D') represent size-classes rather than individual polypeptides. This is in agreement with recent findings that fractionation of mature phaseolin by isoelectric focusing (9) or on two-dimensional gels (11) showed that the storage protein has numerous glycopolypeptides. Storage proteins of seeds have been shown to be coded for by small families of closely related genes (16).

Signal Sequence

When polysomal RNA was used as a template for in vitro protein synthesis the polypeptides which are formed were slightly larger than the unglycosylated polypeptides formed by chain-completion of nascent chains on isolated polysomes. Removal of the signal sequences results in the formation of A_o, B_o, C_o and D_o. At this stage of processing, A and B or C and D are still not resolved by SDS PAGE. Signal sequences have been described for many proteins which are secreted, become integrated into membranes or are transported to specific organelles such as chloroplasts and mitochondria. As far as vacuolar and protein body proteins are concerned, signal sequences have been observed for chymotrypsin inhibitor, a protein which accumulates in the vacuoles of tomato leaves (25), for the corn storage protein zein (12, 23), and for the soybean storage proteins glycinin (15) and conglycinin (29). Attempts to demonstrate signal sequences for phaseolin have so far been unsuccessful (18, 24). We interpret the size-difference between the in vitro products of polysome run-off and polysomal RNA as evidence that most of the nascent chains had already lost their signal sequence when the polysomes were isolated. To visualize the nascent polypeptides which had not yet lost their signal sequence, it was necessary to allow chain-completion to proceed for 15 min with nonradioactive amino acids before adding [^{3}H]leucine. This allowed many nearly completed chains to be completed and only the ones which had been intiated recently (prior to isolation of polysomes) to become radioactive. A significant proportion of these chains are larger in size (Fig. 4, lane *5*) presumably because they still have their signal sequences.

Two Sequential Glycosylation Steps

Chain-completion with isolated polysomes resulted in the formation of two glycosylated polypeptides showing that glycosylation of storage proteins is a cotranslational event. The two glycosylated polypeptides had the same size as the less abundant glycopolypeptides B and D. Altering the conditions for chain-completion by greatly increasing the amount of nascent chains in the translation system favored the completion of glycosylated chains, possibly because they were nearest to completion. However, we never obtained a pattern of four glycopolypeptides similar to the one found by labeling in vivo. Such a pattern could be obtained when chain completion was carried out with polysomes still attached to the membranes. Some of the glycopolypeptides formed in this system were larger than those made by isolated polysomes, indicating that

a second glycosylation had occurred in vitro. The sequential nature of these two glycosylation steps was confirmed by the use of Triton X-100, which was added to the rough ER chain-completion system at later and later times during the incubation. We confirmed the finding that the detergent did not inhibit in vitro protein synthesis (28), and were able to show that later and later additions of detergent caused a gradual decrease in unglycosylated polypeptides, a transient increase in polypeptides glycosylated once, and the late appearance of polypeptides glycosylated twice. The interpretation that the abundant glycopolypeptides A and C contain an additional carbohydrate group was confirmed by an analysis of the four glycopolypeptides synthesized in vivo.

Analysis of the Glycopolypeptides

Using radioactive precursors we determined the carbohydrate to protein ratios in newly synthesized phaseolin. This analysis showed that the abundant polypeptides had one and a half times more glucosamine and two times more mannose than the less abundant ones. An analysis of the glycosylated oligopeptides present in a trypsin digest of the individual glycopolypeptides also showed that the abundant polypeptides A and C have an additional glycosylation site, when compared to B and D. Together, these data lead us to suggest that B and D are glycosylated once, while A and C are glycosylated twice. The presence of the additional (and least abundant) glycosylated oligopeptide among the trypsin digestion products is attributed to heterogeneity at the polypeptide level.

Precursor-product Relationship

Three different approaches confirm that two (size-classes of) unglycosylated polypeptides are synthesized: in vitro synthesis with polyA RNA, in vitro runoff with polysomes, and in vivo synthesis in the presence of tunicamycin. The maps of the tryptic peptides (Fig. 9) confirm that the polypeptide with the lower mobility on gels (A_oB_o) gives rise to two glycopolypeptides, one of which has two oligosaccharide side-chains (A) while the other ones has only one (B). The polypeptide with the higher mobility (C_oD_o) also gives rise to two glycopolypeptides, one of which has two oligosaccharide side-chains (C) while the other one has only one (D).

Transport to the Protein Bodies

Transport of glycosylated polypeptides to the protein bodies is accompanied by a final processing step resulting in a slightly greater mobility of the glycopolypeptides on SDS polyacrylamide gels (8, 29). This last processing step is depicted in Fig. 9 and was also observed by Sengupta et al. (29) for soybean conglycinin. The exact nature of this processing step remains to be elucidated. Protein bodies contain glycosidases such as α-mannosidase as well as exopeptidases such as carboxypeptidase (32). The increase in mobility may therefore result from a small change in the size of the oligosaccharide chain, the polypeptide moiety, or both.

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