Expression of the wild-type and mutated vacuolar storage protein phaseolin in Xenopus oocytes reveals relationships between assembly and intracellular transport

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The role played by subunit assembly in the intracellular transport of the bean storage protein phaseolin, a soluble trimeric glycoprotein, was investigated using Xenopus oocytes injected with RNA. We show that phaseolin assembly is dependent upon the level of synthesis of the protein and is required for intracellular transport out of the endoplasmic reticulum. We also show that a fraction of the assembled phaseolin is permanently retained in a post-endoplasmic reticulum compartment. Deletion of the C-terminal z-helical domain fully prevents in vivo assembly but not endoplasmic reticulum retention. This indicates that this domain is necessary for trimerization but not for interactions of unassembled subunits with endoplasmic reticulum components. The truncated phaseolin has high in vivo stability. The potential implications of these findings on the possibility to improve the nutritional value of phaseolin through genetic engineering are discussed.

Proteins having a signal peptide which allows import into the endoplasmic reticulum enter the pathway of biosynthetic protein transport (reviewed by Pfeffer et al., 1987). There is now evidence that transport from the endoplasmic reticulum to the Golgi complex and from here to the cell surface occurs by default and that additional signals are necessary to be sorted out from this bulk flow. However, it is also clear that in the case of most oligomeric proteins, the newly synthesized unassembled subunits need to fold properly and to assemble themselves into the correct oligomeric structure in order to be competent for transport along the pathway. Unassembled or unfolded subunits, as well as improperly folded oligomers, are normally retained in the endoplasmic reticulum through a process which has been termed quality control (reviewed by Hurtley and Helenius, 1989). The mechanism of retention has not yet been fully clarified and in fact the existence of independent steps of control has been hypothesized (Hurtley and Helenius, 1989; Singh et al., 1990). Some unassembled proteins can however be secreted and most of these are soluble proteins. Examples include the uncombined x and y subunits of the heterodimeric human choricin gonadotropin (Peters et al., 1984), the immunoglobulin light chains (Köhler et al., 1976) and the soluble subunit (but not the membrane-integrated subunit) of class I transplantation antigens (Severisson and Peterson, 1984). Also, truncation of the membrane-anchoring and cytosolic domain of influenza hemagglutinin relieves this protein from the need of trimerization for exit from the endoplasmic reticulum (Singh et al., 1990). Therefore, the requirement for oligomerization appears to be less important for soluble proteins than for membrane proteins. Here, we have investigated the relationships between assembly and intracellular transport of the common bean (Phaseolus vulgaris) storage protein phaseolin, a soluble trimeric plant glycoprotein.

In bean seeds, phaseolin accumulates in specialized vacuoles, termed protein storage vacuoles, and is then hydrolyzed during germination to provide a source of reduced nitrogen to the young seedling. Phaseolin is the product of a small family of highly homologous genes (Slightom et al., 1985). On SDS/PAGE, phaseolin synthesized in vitro by bean cotyledonary mRNA is separated into two size-classes of polypeptides with $M_\text{s}$ 50,000 ($\beta$ class) and 46,000 (y class). The higher $M_\text{s}$ of the $\beta$ class is due to the presence of two direct repeats absent in the $\beta$ class (Slightom et al., 1985). The polypeptides have a co-translationally removed signal peptide and two N-glycosylation sites. The first site is completely glycosylated, while the second site is only partially glycosylated (Bollini et al., 1983; Sturm et al., 1987). Therefore, newly synthesized phaseolin, present in the endoplasmic reticulum, has a four-banded pattern on SDS/PAGE, representing $x$ and $y$ polypeptides with one ($x$ and $y$) or two ($x'$ and $y'$) oligosaccharide chains. Phaseolin is then transported through the Golgi complex, where part of its oligosaccharide chains are modified, and finally deposited into the protein storage vacuoles (Bollini et al., 1982; Sturm et al., 1987). The compartment in which phaseolin trimers assemble has not been determined. A related protein that accumulates in pea cotyledons is present as a mixture of monomers and trimers in an endoplasmic reticulum-enriched subcellular preparation (Chrispeels et al., 1982). This indicates that the endoplasmic reticulum is a site of assembly of this class of proteins but does not rule out the possibility that trimerization can in part occur also later on during transport. The X-ray structure of phaseolin has been determined, contributing to make this trimeric glycoprotein a good model system for the study of the assembly of soluble oligomeric proteins (Lawrence et al., 1990).

Heterologous expression in mRNA-injected Xenopus oocytes has been used to study several aspects of the synthesis of vacuolar storage proteins, including signal sequence cleav-

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Abbreviations. Endo H, endo-$\beta$-N-acetylglucosaminidase H; wtPHSL, wild-type $\beta$ phaseolin; A363, a truncated $\beta$ phaseolin. Enzyme. Endo-$\beta$-N-acetylglucosaminidase H (EC 3.2.1.96).
age (Ereken-Tumer et al., 1982). N-glycosylation (Bassùner et al., 1983; Matthews et al., 1981) and Golgi-mediated oligosaccharide chain processing (Vitale et al., 1986). Phaseolin and other legume storage proteins are secreted by *Xenopus* oocytes (Bassùner et al., 1983). This indicates that these proteins enter the biosynthetic protein transport pathway also in the heterologous system and that they have some sorting signal which is not recognized in animal cells, where they follow the bulk flow transport leading to secretion (in animal cells the analogue of the vacuoles are considered to be the lysosomes). The molecular characteristics of phaseolin synthesized in *Xenopus* oocytes have been in part elucidated. Matthews et al. (1981) first showed that the protein is glycosylated in these cells. We showed that the typical α”, α’, β” and β’ subunits are synthesized (Vitale and Bollini, 1986).

We provide evidence here that, in the oocytes, trimerization of phaseolin is dependent upon the level of synthesis of the protein and is necessary for intracellular transport out of the endoplasmic reticulum. We also identify the C-terminal a-helical domain of phaseolin as necessary for assembly but not for endoplasmic reticulum retention of unassembled subunits and we show that deletion of this domain does not result in increased degradation in the endoplasmic reticulum. These findings may have implications in the planning of experiments aimed at improving the nutritional value of phaseolin through genetic engineering. Finally, we also show that part of the trimeric phaseolin is not secreted by *Xenopus* oocytes. However, unlike unassembled subunits, these trimers are not located in the endoplasmic reticulum but permanently retained in a later compartment, indicating a double destiny for assembled phaseolin in these cells.

### MATERIALS AND METHODS

#### Extraction of bean RNA and injection into oocytes

Cotyledons were isolated from developing seeds of *P. vulgaris* cultivar Greensleaves. RNA was extracted from membrane-bound polysomes, as described by Bollini et al. (1983) and resuspended in water. *Xenopus laevis* oocytes were isolated manually and incubated overnight at 19°C in modified Barth medium [7.5 mM Tris/HCl, pH 7.6, 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.82 mM MgSO₄, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 10 μg/ml penicillin (sodium salt) and streptomycin sulphate, 100 μg/ml gentamicin and 20 U/ml nystatin]. Oocytes that looked healthy after the overnight incubation were injected with 30 nl of different RNA dilutions. Injected oocytes that looked healthy after further overnight incubation at 19°C were used for the pulse-chase experiments, which were also performed at 19°C.

#### Recombinant DNA procedures

Clone β-31, a full-length cDNA encoding β phaseolin (Slightom et al., 1985), was first subcloned into the PSTI site of the plasmid bluescript (Stratagene, San Diego) under the T7 promoter. In preliminary experiments, however, we found that the synthetic mRNA obtained from this plasmid was not translatable in *Xenopus* oocytes. The 5’ untranslated region of the cDNA was therefore removed and the cDNA inserted in the pSP64T vector, which generates synthetic mRNA more suitable for *Xenopus* oocyte expression (Krieg and Melton, 1984). For this purpose, the bluescript insert was amplified with the polymerase chain reaction using two specific oligonucleotides: the sense oligonucleotide beginning at a 5’ and the antisense-orientation oligonucleotide complementing the sequence, starting from the end of the cDNA, at the unique restriction sites, *Hind*III, *EcoRV* and *EcoRI* of the bluescript plasmid. After being sequenced, the insert was digested with *Sma*I and *EcoRV* and subcloned into the end-filled *Bgl*II site of pSP64T. This clone was termed wild-type β phaseolin (wtPHSL). The truncated phaseolin clone, βPHSL 4363, was constructed by deleting the Aspl-Spl site fragment from the coding sequence of wtPHSL (by partial digestion, because there is an *Sspl* site in pSP64T). The Aspl sticky end was then end-filled and blunt ligated to the *Sspl* end: in this way the C terminus of β phaseolin loses the last 59 amino acids and acquires six new amino acids before a stop codon.

**In vitro** transcription, preparation of synthetic mRNA for oocyte injection and **in vitro** translation in rabbit reticulocyte lysate, were essentially as described by Fabbrini et al. (1988), except that SP6 RNA polymerase (Promega Biotec, Madison) was used, at 0.6 U/μl final concentration.

#### Culturing and homogenation of oocytes

Batches of five injected oocytes were pulse-labelled for 2 h with 5 μl/oocyte in modified Barth medium supplemented with 1 μCi/μl of [4,5-3H]leucine (140 Ci/mmol, Amersham, UK). Oocytes were washed extensively with modified Barth medium and then either homogenized or chased for the first 2 h in 5 μl/oocyte of modified Barth medium and for the remaining time in the same amount of modified Barth medium supplemented with 10 mM unlabelled leucine. Synthesis in the presence of tunicamycin (Serva) was performed by injecting tunicamycin (100 μg/ml in 200 μM NaOH) together with the RNA and culturing the oocytes in the presence of 2.5 μg/ml tunicamycin. Synthesis in the presence of 1-deoxymannojirimycin (Miles) was performed by culturing the oocytes in the presence of 5 mM 1-deoxymannojirimycin as described by Fabbrini et al. (1988). At the desired time points the incubation medium was collected, oocytes were first washed with 5 μl/oocyte of modified Barth medium (which were added to the original incubation medium) and then extensively washed with modified Barth medium before homogenation with 40 μl/oocyte of homogenation buffer (20 mM Tris/HCl, pH 7.4, 100 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride). The sample composed by the incubation medium and the first wash was also brought to 40 μl/oocyte with homogenation buffer.

#### Analysis of phaseolin

Cell homogenates or media were centrifuged for 3 min on an Heraeus Biofuge A at 15000 × g and total phaseolin was immunoprecipitated from the supernatants using the method described by Gething et al. (1986) using rabbit anti-(bean phaseolin) antiserum. To separate unassembled and assembled phaseolin, supernatants of the 3-min centrifugation were loaded on top of 5–25% (mass/vol.) sucrose gradients made in 20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 0.1% Triton X-100 and centrifuged for 16 h at 40000 rpm, 20°C on a Beckman SW 40Ti rotor. 0.6-ml fractions were collected and the phaseolin immunoprecipitated as described above. Immunoprecipitated phaseolin was analyzed by SDS/PAGE and fluorography as described (Bollini et al., 1983). Quantification of phaseolin was performed through microdensitometry of fluorographs, using a Camag TLC Scanner II.
Digestion with trypsin

Supernatants of the 3-min centrifugation of oocyte homogenates were fractionated on linear sucrose gradients as described above. Phaseolin was immunoprecipitated from an aliquot of each fraction and analyzed by SDS/PAGE to locate fractions containing monomers or trimers, which were then pooled separately. An aliquot (1.2 ml) from each pool of fractions or from the supernatant of the 3-min centrifugation of oocyte incubation media (brought to 1.2 ml with the buffer used for sucrose gradients) was brought to 200 pg/ml bovine serum albumin and divided into two halves which were warmed to 37°C. Trypsin (from a 100 pg/ml stock in 1 mM HCl) was added to one half to a final concentration of 10 pg/ml and an equivalent volume of 1 mM HCl was added to the other half of each sample. After incubation for 10 min at 37°C, the reaction was stopped by adding soybean trypsin inhibitor (from a 200-pg/ml stock solution) to a final concentration of 20 pg/ml and the phaseolin was immunoprecipitated as described above but the Sepharose beads were further washed twice using distilled water. The sample was then centrifuged for 5 min at 13000 rpm in a Heraeus Biofuge A. The supernatant was recovered, diluted tenfold with 0.1 M sodium citrate, pH 5.5 and 500 µg bovine serum albumin (fraction V, Baker), 1 µl antiprotease mix (containing, in 50% DMSO: 2 mg/ml antipain, chymostatin, leupeptin, pepstatin and bestatin; 10 T. I. U/ml aprotinin) and 5 µl 100 mM phenylmethylsulfonyl fluoride were added. The sample was divided in two aliquots and 20 mU endoglycosidase H (Boehringer) or 20 pl 1-deoxymannojirimycin and phaseolin was immunoprecipitated from cell homogenates (o) or incubation media (m) equivalent to one (RNA: 1) or two (RNA: 2/25) oocytes and analyzed by SDS/PAGE and fluorography. The positions of the four phaseolin subunits are indicated. (B) As in panel (A) but oocytes were treated with 1-deoxymannojirimycin and phaseolin was immunoprecipitated from cell homogenates (o) and incubation media (m) equivalent to two (RNA: 1) or five (RNA: 2/25) oocytes.

RESULTS

Unassembled phaseolin subunits are not secreted from Xenopus oocytes

Since phaseolin extracted from bean seeds is trimeric, we determined whether proper assembly occurs in Xenopus oocytes and investigated the oligomeric state of the secreted polypeptides. Oocytes injected with 30 ng (this amount is arbitrarily termed in the figures) of a bean cotyledonary RNA preparation was pulse-labelled with [3H]leucine for 2 h and chased for 2 h or 24 h. Phaseolin was immunoprecipitated from cell homogenates (o) or incubation media (m) equivalent to one (RNA: 1) or two (RNA: 2/25) oocytes and analyzed by SDS/PAGE and fluorography. The positions of the four phaseolin subunits are indicated. (B) As in panel (A) but oocytes were treated with 1-deoxymannojirimycin and phaseolin was measured. The four-banded pattern (Fig. 1A, lane 1). No phaseolin was detectable in the oocyte medium at this time (not shown in this experiment, but see Fig. 4, lanes 1 and 2). After a 24-h chase the pattern of phaseolin was less clear, probably due to processing of the oligosaccharide chains (see below) and about 40% of the protein had been secreted into the medium (Fig. 1A, lanes 2 and 3). Moreover, at 24 h of chase, some of the intracellular phaseolin polypeptides migrated slightly faster than their secreted counterparts.

Velocity-gradient centrifugation was used to assess the assembly state of phaseolin. Immunoprecipitation of phaseolin from the gradient fractions showed that at 2 h of chase, phaseolin could be fractionated into two peaks (Fig. 2, panel A). A small amount peaked in the same position of bean-synthesized phaseolin trimers, which were used as markers (markers not shown in the figure). Most of the protein however peaked slightly before the marker bovine serum albumin (M, 69000), indicating that it was still monomeric. After the 24-h chase, again intracellular phaseolin was a mixture of trimers and monomers while all the phaseolin recovered from the medium was assembled into trimers (Fig. 2B and C). Extracellular proteolytic degradation is not likely to be responsible for the observed lack of monomers in the medium since immunoprecipitated monomeric phaseolin, bound to anti-phaseolin IgG—Sepharose beads, was fully stable upon incubation for 24 h in injected oocyte-conditioned medium (not shown). In addition, inclusion of bovine serum albumin at 1 mg/ml in the medium during the pulse/chase did not result in the recovery of secreted phaseolin monomers (not shown).
Our results indicate that phaseolin is assembled into trimers in the oocytes and that secretion temporally follows protein assembly.

The lack of secretion of monomers could either be merely due to the fact that assembly is faster than secretion or indicate that assembly is required for phaseolin secretion. It has recently been shown that the expression level influences the rate of assembly of influenza hemagglutinin (a trimeric membrane glycoprotein) in *Xenopus* oocytes (Ceriotti and Colman, 1990). We investigated whether a decrease in the phaseolin rate of synthesis, achieved by reducing the amount of RNA injected, could prevent efficient oligomerization. Decrease in the amount of RNA injected into oocytes from the same animal resulted in the progressive reduction of phaseolin synthesis and of the percentage of phaseolin recovered in the medium. In the experiment shown in Figs 1 and 2, a 25-fold reduction of injected RNA caused an equal decrease in the synthesis of the protein. At this level of synthesis there was no detectable secreted phaseolin after a 24-h chase (Fig. 1 A and B, lane 6) and all the phaseolin recovered from the cell homogenate was in a monomeric form, at 2 h as well as at 24 h of chase (Fig. 2 D and E). Therefore, when no assembly occurs, there is no secretion. This result rules out the possibility that monomers are not secreted solely because assembly is faster than secretion. We conclude that phaseolin assembly is a post-translational event which depends upon the level of synthesis of the protein and that unassembled phaseolin cannot be secreted.

At 24 h of chase, monomers appeared to be represented only by fully glycosylated subunits (Fig. 2 B and E). However, post-translational processing of the oligosaccharide chains could have altered the $M_r$ of the phaseolin polypeptides. As a consequence, the identification of the individual subunits may be uncertain. To overcome this problem, the experiment shown in Fig. 1 B was performed in the presence of an inhibitor of carbohydrate processing. The mannose analogue 1-deoxymannojirimycin inhibits Golgi-mediated processing of the oligosaccharide chains in mammalian cells as well as in *Xenopus* oocytes (Fuhrmann et al., 1984; Bishoff and Kornfeld, 1984; Fabbri et al., 1988). At a high expression level of phaseolin, synthesis in the presence of 1-deoxymannojirimycin resulted in the recovery of a small amount of $\beta'$ subunits in the incubation medium (Fig. 1 B, lane 3). This indicates that in normal conditions the $\beta'$ subunit increases its $M_r$ because of carbohydrate processing and this in turn results in comigration with $\beta''$ [see also the results of the endo-$\beta$-$N$-acetylgalactosaminidase (endo H) digestion shown below]. However, most of the $\beta'$ polypeptides present at 2 h of chase could not be recovered at 24 h of chase even in the presence of 1-deoxymannojirimycin, indicating that they were either degraded or post-translationally glycosylated to yield the fully glycosylated form (Fig. 1 B, lanes 1, 2 and 3). Sucrose-gradient analysis (not shown) indicated that also in this experiment the vast majority of single-glycosylated subunits were still monomeric at 2 h of chase. We therefore suggest that the process leading to $\alpha'$ and $\beta'$ disappearance was active only on the unassembled subunits. As predicted by this hypothesis, no single-glycosylated polypeptides could be recovered after 24 h of chase in oocytes that had been injected with a reduced amount of RNA, where oligomerization and secretion were fully inhibited (Fig. 1 B, lanes 4–6; sucrose gradients not shown). Quantitative analysis by densitometry of the fluorograph shown in Fig. 1 B and of the results of other experiments (see below) indicated that post-translational glycosylation (rather than selective degradation) is mainly responsible for the observed decrease in the recovery of single-glycosylated subunits. Although $N$-glycosylation is usually a co-translational event, post-translational addition of oligosaccharide chains has been described in the case of both membrane (Ronnett and Lane, 1981) and soluble (Valle et al., 1983) secretory proteins. Most important, post-translational glycosylation indicated that unassembled phaseolin subunits are not secreted because they are unable to leave the endoplasmic reticulum.

**Phaseolin subunits assemble into trimers before acquiring endo H resistance**

To obtain information on the subcellular location of phaseolin assembly, we digested the protein with endo-$\beta$-$N$-acetylgalactosaminidase H (endo H). This enzyme hydrolyses high mannose oligosaccharide chains between the two inner $N$-acetylglucosamine residues but does not hydrolyse complex chains originating from the Golgi-mediated processing of the high mannose structures. Therefore, resistance to endo H is considered as an indication that a protein has reached at least the Golgi complex along the transport pathway. In bean cotyledonary cells, single-glycosylated phaseolin subunits acquire endo H resistance while fully glycosylated subunits have only high mannose chains, probably because the presence of the second chain changes the conformation of phaseolin in a way that does not allow access by processing enzymes (Sturm et al., 1987). Trimeric or monomeric phaseolin, isolated from the sucrose gradient fractions of oocyte homogenates, were digested with endo H (Fig. 3). The positions of unglycosylated phaseolin polypeptides (termed $\alpha'$ and $\beta'$) synthesized in the presence of tunicamycin are indicated on the right of Fig. 3. The pattern resulting from digestion of secreted phaseolin (composed exclusively of trimers) revealed that some of these subunits changed their mobility while others did not (lanes 9 and 10). In particular, $\alpha''$ (the slower migrating band in lane 9) disappeared and a new band of similar intensity appeared,
Fig. 3. Endoglycosidase H digestion of monomeric and trimeric phaseolin. Oocytes were injected with 30 ng/oocyte bean RNA and labelled for 2 h with [3H]leucine. Either at 2 h or 24 h of chase, oocytes were collected, homogenized and the homogenates were fractionated on 5–25% sucrose gradients. At 24 h of chase, the oocyte medium was also collected and fractionated as above. Phaseolin contained in monomer (M) and trimer (T) peaks was immunoprecipitated and incubated with (+) or without (−) endo H as described in Materials and Methods. Analysis was by SDS/PAGE and fluorography. The positions of unglycosylated α′ and β′ phaseolin subunits are indicated on the right-hand-margin.

Trimerization does not obligate phaseolin to secretion

The results reported in Fig. 2 indicate that a substantial portion of trimeric phaseolin is still inside the oocyte even after a 24-h chase. We investigated whether this assembled phaseolin is en route to secretion or, for some reason, is unavailable for secretion. We injected oocytes with 30 ng RNA/oocyte and also extended the chase to 48 h (Fig. 4A). At this time point phaseolin was immunoprecipitated from the homogenate of some oocytes and from their medium (Fig. 4A, lanes 5 and 6), whilst other oocytes were separated from their medium (from which phaseolin was also immunoprecipitated to serve as a control; Fig. 4A, lane 7), washed and incubated for additional 24 h (72 h chase) in fresh medium. There was very little secretion between 48 h and 72 h (Fig. 4A, lanes 8 and 9), indicating that the vast majority of phaseolin, still present in the oocytes at 48 h of chase, was blocked (or sorted out) at some step along the secretory pathway. At 48 h of chase the great majority of intracellular phaseolin was trimeric (Fig. 4B), only part of the β′ subunit and a very low amount of the α′ subunit (visible only after very long film exposure) being still unassembled. Therefore, the block in secretion was not a result of a lack of assembly. Block was neither due to a decrease in the activity of the oocyte secretory pathway, which could have resulted from the long incubation, since oocytes labelled 48 h after RNA injection secreted large amounts of radioactive phaseolin in the following 24 h (not shown). By overexposing the fluorograph shown in Fig. 4A, we could observe that the small amount of labelled phaseolin secreted between 48 h and 72 h of chase (lane 11) was constituted almost exclusively by polypeptides having some mobility of the monomeric form of intracellular β′, the only subunit which was still unassembled in the oocytes at 48 h of chase. Phaseolin secreted between 48 h and 72 h of chase was fully assembled into trimers (Fig. 4B). These results indicate that monomers, even after 48 h inside the cell, are still available for assembly and secretion. Conversely, the vast majority of trimers present in the oocyte, at 48 h of chase are blocked in what could be a dead end of the secretory pathway.
Methods. Control and trypsin-treated samples were immunoprecipitated and analysed by SDS-PAGE and fluorography. dMM, deoxymannojirimycin or tunicamycin were also resistant to trypsin digestion (Fig. 5, lanes 1–4), although the recovery of tryptic fragments from unglycosylated phaseolin was slightly lower. Conversely, monomers present in the cell after both a 2-h pulse and a 24-h chase did not yield any immunoprecipitable fragment after trypsin digestion, either because phaseolin had been extensively degraded or because fragments derived from monomers had a conformation no longer recognizable by our antibodies (Fig. 5, lanes 9, 10, 13, 14). These results indicate that oligomerization either conceals some critical tryptic digestion sites or causes major changes in the structure of phaseolin subunits. Acquisition of trypsin resistance is an early event (at the end of the pulse, trimers are resistant to trypsin but not to endo H) and is not markedly affected by glycosylation or processing of the side chains.

The C-terminal \( \alpha \)-helical domain is necessary for trimer formation but not for retention of monomers in the endoplasmic reticulum

Our results establish the expression in Xenopus oocytes as an \emph{in vivo} system to define the role played by the different domains of the phaseolin polypeptide with respect to trimerization and endoplasmic reticulum retention of unassembled subunits. The determination of the crystal structure of phaseolin helps in the planning of such experiments (Lawrence et al., 1990). At the three-dimensional level, each phaseolin subunit is composed by two structurally similar units. Each unit is made up by a \( \beta \)-barrel and an \( \alpha \)-helical domain. In the trimer, the \( \alpha \)-helical domain of the N-terminal unit is in contact with the \( \alpha \)-helical domain of the C-terminal unit of an adjacent polypeptide. The domains are about 60 amino acids long and are in the terminal part of each unit, along the primary sequence. Taking advantage of the presence of two unique restriction sites in a \( \beta \) phaseolin cDNA clone (see Materials and Methods), we deleted the last 59 amino acids of the polypeptide, which constitute the C-terminal \( \alpha \)-helical domain. In the resulting construct, the open reading frame extends for six new amino acids starting from position 363 before the occurrence of a stop codon (Fig. 6A). Synthetic mRNA obtained from wtPHSL cDNA clone or from the truncated clone (A363) were first tested by \emph{in vitro} translation: A363 had the expected decrease in molecular mass with respect to wtPHSL (Fig. 6B, lanes 1 and 2). When expressed in oocytes, at the end of pulse labelling, wtPHSL was represented as a single, poorly defined band (Fig. 6B, lanes 7 and 9). Indicating that phaseolin was in part singly glycosylated and in part fully glycosylated. After a 24-h chase, wtPHSL was largely secreted and this and the intracellular form migrated as a single, poorly defined band (Fig. 6B, lanes 7 and 9). Sucrose-gradient analysis showed that wtPHSL was largely present as a monomer at 0 h of chase while at 24 h of chase most of the protein was assembled and only trimers were secreted (Fig. 7A, B and C). Endo H digestion fully removed the side-chains of both monomeric and trimeric wtPHSL at 0 h of chase (Fig. 8, lanes 3 and 4, and 7 and 8). At 24 h of chase, the enzyme removed the two side-chains of monomeric

Monomeric and trimeric phaseolin differ in their susceptibility to proteolytic digestion

Trypsin treatment of native phaseolin purified from bean cotyledons produces major, stable fragments having an apparent molecular mass approximately corresponding to the two halves of the phaseolin subunits (Johnson et al., 1982). This indicates that assembled phaseolin has large domains rather resistant to proteolysis. Resistance to proteolytic digestion has been used as evidence of differences between transported and retained variants of secretory proteins and to follow conformational changes occurring during transport along the secretory pathway (Gething et al., 1986; Hille et al., 1990; Williams et al., 1988). Therefore, we used a trypsin-resistance assay to compare the folding state of monomeric and trimeric phaseolin. Proteolytic digestion was performed on sucrose gradient fractions containing monomers or trimers and phaseolin was then immunoprecipitated and analyzed (Fig. 5). Proteolysis was performed before immunoprecipitation to avoid possible masking of digestion sites by the bound antibody. Trypsin treatment of intracellular or secreted trimers produced immunoprecipitable fragments (Fig. 5, lanes 5–8, and 11 and 12). Some of these had only a slight increase in mobility, indicating that part of the protein underwent removal of small terminal peptides, while the presence of fragments with \( M_c \) 27000 and 24000 suggested that part of phaseolin was cleaved in the central region of the polypeptide. Upon incubation with a 15-fold excess of trypsin, only the fragments corresponding to the halves of the phaseolin subunits were generated, indicating that the others were intermediates of digestion (not shown). These results indicate that retained and secreted phaseolin trimers are both folded in their typical protease-resistant structure and suggest that retention of assembled phaseolin is not due to major conformational defects. Phaseolin trimers secreted by oocytes treated with tunicamycin or 1-deoxymannojirimycin were also resistant to trypsin digestion (Fig. 5, lanes 1–4), although the recovery of tryptic fragments from unglycosylated phaseolin was slightly lower. Conversely, monomers present in the cell after both a 2-h pulse and a 24-h chase did not yield any immunoprecipitable fragment after trypsin digestion, either because phaseolin had been extensively degraded or because fragments derived from monomers had a conformation no longer recognizable by our antibodies (Fig. 5, lanes 9, 10, 13, 14). These results indicate that oligomerization either conceals some critical tryptic digestion sites or causes major changes in the structure of phaseolin subunits. Acquisition of trypsin resistance is an early event (at the end of the pulse, trimers are resistant to trypsin but not to endo H) and is not markedly affected by glycosylation or processing of the side chains.

![Fig. 5. Trypsin digestion of monomeric and trimeric phaseolin.](image-url) Oocytes were injected with 30 ng/oocyte bean RNA and pulse labelled for 2 h with \( ^{35} \text{S} \) methionine. Some of the oocytes were also treated with 1-deoxymannojirimycin or tunicamycin as described in Materials and Methods. Either at the end of the labelling period or after a 24-h chase, the medium was collected and the oocytes were homogenized. The homogenates were fractionated on a 5–25% sucrose gradient. Fractions containing either monomeric (M) or trimeric (T) phaseolin were pooled and treated with trypsin as described in Materials and Methods. Control and trypsin-treated samples were immunoprecipitated and analysed by SDS/PAGE and fluorography. dMM, deoxymannojirimycin; Tm, oocytes treated with tunicamycin. The positions of molecular mass markers (kDa) are indicated on the right-hand-margin.

Fig. 5. Trypsin digestion of monomeric and trimeric phaseolin. Oocytes were injected with 30 ng/oocyte bean RNA and pulse labelled for 2 h with \( ^{35} \text{S} \) methionine. Some of the oocytes were also treated with 1-deoxymannojirimycin or tunicamycin as described in Materials and Methods. Either at the end of the labelling period or after a 24-h chase, the medium was collected and the oocytes were homogenized. The homogenates were fractionated on a 5–25% sucrose gradient. Fractions containing either monomeric (M) or trimeric (T) phaseolin were pooled and treated with trypsin as described in Materials and Methods. Control and trypsin-treated samples were immunoprecipitated and analysed by SDS/PAGE and fluorography. dMM, deoxymannojirimycin; Tm, oocytes treated with tunicamycin. The positions of molecular mass markers (kDa) are indicated on the right-hand-margin.
intracellular wtPHSL, represented only by the \( \beta'' \) subunit (Fig. 8, lanes 9 and 10), but the lower part of the poorly defined band representing trimeric protein, both intracellular and secreted, was resistant to digestion (Fig. 8, lanes 13–16). This strongly suggests that the assembled protein was composed by \( \beta' \) with one complex chain and \( \beta'' \) with two high mannose chains. The results reported in Figs 6–8 indicate that the single \( \beta \) phaseolin polypeptide synthesized upon injection of synthetic mRNA behaved like the phaseolin polypeptides synthesized upon injection of the total membrane-bound bean RNA. Therefore, the synthesis, assembly, transport and processing of phaseolin in the heterologous system is the result of activity of endogenous oocyte components.

The truncated \( \Delta363 \) was also synthesized in the oocytes as a mixture of single and fully glycosylated polypeptides (Fig. 6B, compare lane 2 with lane 4). At 24 h of chase no secreted protein was detectable and intracellular \( \Delta363 \) was represented by a well-defined band having very similar mobility to the fully glycosylated polypeptide (Fig. 6B, compare lanes 8 and 10 to lane 4). Sucrose-gradient analysis showed that \( \Delta363 \) did not assemble at all, even after 24 h of chase (Fig. 7D and E). Endo H fully deglycosylated \( \Delta363 \) isolated at either 0 h or 24 h of chase (Fig. 8, lanes 5 and 6, and 11 and 12). Therefore, removal of the C-terminal domain fully inhibited assembly, Golgi-mediated processing and secretion of phaseolin. This was not the result of a particularly low expression level of the truncated polypeptide: the levels of synthesis of wtPHSL and \( \Delta363 \) were similar and much higher than the highest levels which we could obtain using bean RNA. We conclude that the C-terminal domain is necessary for both assembly and secretion in vivo. Removal of this domain, however, does not affect phaseolin stability in the oocytes. In fact, quantitation by fluorograph densitometry showed that in the experiment reported in Fig. 6 the recovery of \( \Delta363 \) at 24 h of chase with respect to 0 h of chase was 62% while that of wtPHSL (intracellular plus secreted) was 47%. Also in another, completely independent experiment (not shown) the recovery was higher for \( \Delta363 \) than for wtPHSL.

We also observed that in two completely independent experiments, the absolute amount of fully glycosylated \( \Delta363 \) recovered after the chase was higher than that recovered after the pulse (Table 1, and Fig. 6, lanes 4 and 8). This indicates that \( \Delta363 \) is post translationally glycosylated and therefore that it is unable to leave the endoplasmic reticulum. We con-
Table 1. Glycosilation of A363 phaseolin. Absolute amounts of β' and β'' are given, assuming the sum of the two at 0 h of chase is equal to 100. n.d., not detectable.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Subunit</th>
<th>Chase duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>24 h</td>
<td>62</td>
</tr>
<tr>
<td>1</td>
<td>609</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>620</td>
<td>87</td>
</tr>
<tr>
<td>n.d.</td>
<td>40</td>
<td>13</td>
</tr>
<tr>
<td>n.d.</td>
<td>60</td>
<td>71</td>
</tr>
</tbody>
</table>

It is reasonable to hypothesize that the mechanisms that prevent exit of misfolded or unassembled proteins operates in similar ways in all eukaryotic cells. In fact, endoplasmic-reticulum-resident proteins that may participate in these processes, like the glucose-regulated proteins, are present in animal as well as yeast and plant cells (reviewed by Pelham, 1989). It is, however, possible that trimerization is a prerequisite for phaseolin transport in the oocytes but not in bean cells. It will be very difficult to inhibit the assembly of wild-type phaseolin in plant cells, as we did in the oocytes. Here we produced a truncated phaseolin polypeptide which cannot assemble even at a high expression level (see below): the cell biology of this polypeptide can be studied in transformed plant cells. If the requirement for oligomerization also applies to the transport of phaseolin to protein storage vacuoles in bean cells, trials aimed at improvement of the nutritional characteristics of phaseolin, through genetic engineering, will have to consider any effect that the introduced mutations could have on phaseolin tertiary and quaternary structure.

**DISCUSSION**

Oligomerization and intracellular transport of phaseolin in *Xenopus* oocytes

During the last few years studies on animal proteins have provided evidence that folding and oligomerization play a key role in the intracellular transport of proteins synthesized on the endoplasmic reticulum. Acquisition of the correct quaternary structure is in most cases an absolute requirement for exit from this compartment (reviewed by Hurtley and Helenius, 1989). Binding to specific endoplasmic-reticulum-resident proteins which may facilitate folding or assembly (Bole et al., 1986; Hendershot, 1990; Saitia et al., 1990) can be in some, but not all cases responsible for retention of unassembled or partially assembled subunits of multimeric proteins. As it has been recently pointed out (Hurtley and Helenius, 1989; Singh et al., 1990), this requirement seems to be very strict in the case of membrane proteins, while a few examples of soluble proteins are known in which transport does not absolutely require assembly (see Introduction).

Bean cotyledonary cells accumulate in a highly packed form large amounts of the trimeric soluble storage protein phaseolin. As in the case of the other storage proteins of legume seeds, the structural characteristics of this protein that allow for its efficient transport and stable deposition are still not well understood.

Here we have shown that when phaseolin polypeptides are expressed in *Xenopus* oocytes they assemble post-translationally into trimers which are then largely secreted, while monomers are only found intracellularly and are not secreted. Oligomerization is not simply kinetically faster than secretion. In fact, when oligomerization is completely inhibited by lowering the expression level, no phaseolin secretion occurs. We think it is unlikely that expression level as such influences phaseolin transport since secretion of monomeric soluble proteins has been shown not to be inhibited by lowering the expression level (Cutler et al., 1981) and the truncated, assembly defective phaseolin mutant A363 is not available for intracellular transport even at a high expression level (see below). We therefore conclude that assembly into trimers is required for the intracellular transport of phaseolin in *Xenopus* oocytes. Trimerization of phaseolin precedes acquisition of endo H resistance, strongly suggesting that phaseolin assembles before its arrival in the Golgi complex. Moreover, we have been unable to detect endo H resistant α' and β' monomers. Unassembled phaseolin remains available for trimerization and secretion even at more than 48 h after synthesis and is subjected to post-translational glycosylation. We conclude from these observations that the endoplasmic reticulum is the site of phaseolin assembly and of retention of monomers. From our data we cannot distinguish whether retention operates through a prompt recycling of monomers from a pre-Golgi compartment back to the endoplasmic reticulum, or through the actual inability of monomers to enter the vesicles that leave the endoplasmic reticulum.

The role of the C-terminal α-helical domain

Our results indicate that there must be structural features of phaseolin that determine interaction with early components of the transport pathway when the subunits are in a monomeric form but not when they are assembled into trimers. This interaction avoids exit of monomers from the endoplasmic reticulum. Such structural features can be either determined by the same domains involved in assembly or by other parts of the molecule. The first hypothesis represents a simpler model: masking of the domain by assembly would directly abolish endoplasmic reticulum retention. The three-dimensional structure of phaseolin indicates that in trimers the N-proximal α-helical domain of one subunit is in contact with the C-terminal α-helical domain of the adjacent subunit (Lawrence et al., 1990). We have shown that the truncated β
phaseolin A363, from which the C-terminal domain had been deleted, does not assemble and does not leave the endoplasmic reticulum. Therefore, this domain contains information necessary for assembly but not for endoplasmic reticulum retention of monomers. Sucrose-gradient analysis showed that A363 is a monomer (rather than being an aggregate) and SDS/PAGE analysis showed that it is synthesized as a mixture of single and fully glycosylated polypeptides, like wtPHSL. From these observations, we suggest that the deleted domain is also not necessary for the correct folding of phaseolin and that retention in the endoplasmic reticulum is not the result of misfolding and subsequent formation of stable aggregates, as is instead the case of retention of other mutated proteins (Hurtley and Helenius, 1989).

Parts of phaseolin other than that deleted in A363 interact with early components of the transport pathway. Alternatively, the overall physicochemical characteristics of unassembled subunits determine this interaction. In the latter case it would follow that deletion of the last 59 amino acids does not change such characteristics markedly. In the former case, the N-proximal a-helical domain would be another possible candidate for mediating endoplasmic reticulum retention. We are trying to test this hypothesis by mutating this region. Before data on the three-dimensional structure of phaseolin were available, Hoffman et al. (1988) had studied the expression in transgenic tobacco seeds of a modified phaseolin gene in which a methionine-rich fragment was introduced, in an effort to increase the nutritional value of the protein. The 15 amino acid insert was placed inside the N-proximal a-helical domain. The accumulation of mutated phaseolin was greatly reduced with respect to the wild-type protein. The authors clearly showed that this was due to protein instability and sucrose-gradient analysis revealed that the small amount of accumulated phaseolin was a mixture of unassembled and assembled subunits. Also, the protein could be detected only in the endoplasmic reticulum and in what have been identified as transport vesicles, while it was absent from the protein bodies. As has been pointed out (Lawrence et al., 1990) it is conceivable that the introduced peptide has severely altered the folding and assembly of the protein. At the light of the results presented here, this may have in turn altered the transport competency of the protein or stability in the protein storage vacuoles.

Stability and intracellular transport

The half-life of A363 is longer than 24 h (see Table 1) and even higher than that of wtPHSL. Since we have shown that unassembled phaseolin is more susceptible to in vitro proteolysis than the assembled protein, we suggest that increased stability may be a consequence of the fact that A363 is retained in the endoplasmic reticulum. From the results of the long-term secretion experiment it appears that most of the loss of wild-type phaseolin occurs because of degradation in the late compartment where part of trimers are retained (compare lanes 5 and 8 in Fig. 4), rather than because of extracellular degradation.

Since folding and assembly of newly synthesized proteins occur in the endoplasmic reticulum, this is obviously not a highly hydrolytic compartment. However, retained proteins unable to fold properly or to assemble, will be eventually degraded, with kinetics that are highly polypeptide specific (for a review see Klausner and Sittia, 1990). A determinant for rapid endoplasmic reticulum degradation has been mapped in the transmembrane region of the T-cell receptor a chain (Bonifacino et al., 1990). The same region is also the site of assembly of the a chain with another subunit of the T-cell receptor but is not necessary for endoplasmic reticulum retention. Phaseolin apparently does not contain determinants for rapid degradation of unassembled subunits, as indicated by the fates of both wild-type polypeptides (at low expression, when assembly cannot occur) and of A363. This observation could have some practical implications. A subunit of the soybean storage protein b conglycinin (a phaseolin-like protein) appears to have 20–30-times decreased stability when expressed in leaves than when expressed in seeds of transgenic petunia (Lawton et al., 1987). Probably this is due to the higher proteolytic activity of leaf vacuoles with respect to seed protein storage vacuoles (Chrispeels and Tague, 1991). If, for any reason, expression of seed storage proteins will be desirable in non-seed tissues, the possibility that the synthesis of mutated polypeptides which cannot assemble could result in higher stability due to retention in the endoplasmic reticulum should be tested.

Assembled phaseolin can have two different destinies

We determined that, in Xenopus oocytes, once phaseolin trimers are assembled, they are transported through the Golgi complex and then in part secreted and in part retained within the cell. This oocyte-retained phaseolin is not present as a stable aggregate (a common feature of retained unfolded proteins) and cannot be distinguished conformationally from secreted phaseolin using a protease-sensitivity assay. Although the final location of retained trimers remains to be determined, we have shown that these acquire endo H resistance, indicating that they have at least reached the Golgi complex.

Recently, Roitsch and Lehle (1991) have reported that the yeast vacuolar enzyme carboxypeptidase Y, when expressed in Xenopus oocytes, is targeted to the lysosomes. Targeting is not dependent upon the mannose-6-phosphate signal, but is dependent upon the presence of a discrete amino acid sequence that contains the tetrapeptide QRPL, a positive targeting signal for the yeast vacuole (Valls et al., 1990). A similar signal is present on some vacuolar proteins, included the bean storage proteins phytohemagglutinin and phaseolin, and directs phytohemagglutinin to the yeast vacuole, although it is not known whether this is the actual targeting signal for the delivery to the bean protein storage vacuoles (Tague et al., 1990). It is possible that indeed the vacuolar targeting signal of phaseolin is being recognized, although not efficiently, in the oocytes and promotes the delivery of part of phaseolin to the lysosomes. This would suggest that the not yet clarified mannose-6-phosphate-independent mechanisms operating for targeting proteins to the inner hydrolytic compartments are shared, with not great differences, by plant, yeast and animal cells. This hypothesis, however, awaits more data to be confirmed.

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