Planta (1994) 192:512–518

Planta

Role of structural domains for maize γ-zein retention in *Xenopus* oocytes

Margarita Torrent [1], Maria Isabel Geli [1], Luis Ruiz-Avila [2], Josep Maria Canals [2], Pere Puigdomènech [1], Dolors Ludevid [1]

[1] Departament de Genètica Molecular, Centre de Investigació i Desenvolupament (CSIC), Jordi Girona 18–26, E-08034 Barcelona, Spain
[2] Departament de Biologia Cellular i Anatomia Patològica. Facultat de Medicina, Universitat de Barcelona, Casanova 143, E-08036 Barcelona, Spain

Received: 5 May 1993 / Accepted: 13 September 1993

Abstract. In order to examine the role of cysteine (Cys)-rich domains in the accumulation of maize (*Zea mays* L.) γ-zein within the endoplasmic-reticulum-derived protein bodies, we studied the localization of γ-zein and of two truncated forms of γ-zein in *Xenopus laevis* oocytes. The two derivatives were constructed from a DNA encoding the γ-zein: one by deletion of the Pro-X linker region (21 amino acids) and the other by deletion of the Cys-rich domain (94 amino acids). In-vitro-synthesized transcripts were injected into oocytes and the distribution of the translation products was then analyzed. The entire γ-zein and both truncated forms of the γ-zein had accumulated efficiently in microsomes and no traces of secretion were observed. We suggest that neither C-terminal Cys-rich nor Pro-X domains are essential for γ-zein retention in oocyte vesicles. Therefore, structural features derived from disulphide bonds are not necessary for γ-zein targeting on the endoplasmic reticulum.

Key words: Protein domain – Protein targeting – Storage protein – *Xenopus* oocytes (protein secretion) – Zein – *Zea*

Introduction

In plant seeds, proteins supposed to have a storage function are deposited in protein bodies (PB). In cereals such as maize (Larkins and Hurkman 1978) and rice (Krishnan et al. 1986), these proteins remain in the endoplasmic reticulum (ER) and they accumulate as aggregates in PBs in the lumen of the ER. In wheat, storage proteins accumulate as PBs inside vacuoles (Kim et al. 1988) but the subcellular location and the mechanisms of their aggregation in PBs are not clear. Recently, Rubin et al. (1992) proposed two different origins for the PBs of wheat endosperm. There is evidence that vacuoles are the site where storage proteins are deposited in legume seeds (Chrispeels 1991). These proteins are oligomers and partial or complete oligomer formation occurs in the ER (Chrispeels et al. 1982).

Xenopus oocytes have been extensively used to study transport of plant storage proteins (Bassuner et al. 1983; Vitale et al. 1986). Recent experiments by Ceriotti et al. (1992) show that trimerization of the phaseolin subunits (a storage protein in bean seeds) is required for the transport of the protein out of the ER in *Xenopus* oocytes. By expressing α- and β-gliadins in *Xenopus* oocytes, Simon et al. (1990) indicated the presence of two different routes for the transport of these wheat storage proteins from the ER to the vacuoles.

In maize, four distinct types of zein polypeptides (α-, β-, γ- and δ-zein) account for 60% of the total endosperm proteins at seed maturity (Shewry and Tatham 1990). Although different experiments have shown that targeting and retention to the ER are similar in both animal and plant cells (Denecke et al. 1990, 1992), evidence to date does not support a model for retention of ER-resident maize zeins. The mechanism of PB assembly in maize is as yet unclear. Argos et al. (1982) suggested that α-zeins form highly structured insoluble aggregates. In-vivo retention of α-zein polypeptides has been observed in *Xenopus* oocytes, where they aggregated within PB-like vesicles (Hurkman et al. 1981; Wallace et al. 1988).

In animal cells it is widely accepted that aggregation in the lumen of the ER of misfolded or incompletely folded proteins can occur in vivo (Marquart and Helenius 1992). Aggregates often contain aberrant disulphide cross-links and they may either be retained or degraded in the ER (Hurtley and Helenius 1989). Disulphide bonds are crucial for the function and stability of the proteins as well as for their maturation and intracellular transport (Braakman et al. 1992). These cross-links are generated in the ER because this compartment provides an oxidizing environment for disulphide forma-

Abbreviations: Cys = cysteine; Dc = truncated form of γ-zein constructed by deletion of the Cys-rich domain; HbP = truncated form of γ-zein constructed by deletion of the Pro-X linker region; MBS = modified Barth's solution; Pro = proline

Correspondence to: D. Ludevid; FAX: 34(3)2045904

tion and for an enzyme, protein disulphide isomerase (PDI), which probably catalyses the process (Freedman 1989). Since the ER is not the terminal compartment for most of the proteins targeted on it, a quality-control system is thus established limiting the exit from the ER to the Golgi complex to those proteins that are fully folded and correctly oligomerized.

The amino-acid sequence deduced from the nucleotide sequence of cDNA (Prat et al. 1985) and genomic clones (Boronat et al. 1986) shows that the γ-zein (also called glutelin-2) gene encodes a protein of 204 residues with no homology with α-zein polypeptides. The γ-zein is a sulphur-rich protein with a clear domain structure (Prat et al. 1985). An N-terminal signal peptide precedes a proline(Pro)-rich repetitive domain composed of eight units of the Pro-Pro-Pro-Val-His-Leu repeat. A C-terminal Cys-rich domain is linked to the repetitive domain by a short spacer formed by the sequence Pro-X. In maize endosperm cells, γ-zein was immunolocalized within ER-derived protein bodies and seen to surround α-zein aggregates (Ludevid et al. 1984). Since γ-zein is a partially soluble protein lacking the KDEL ER-retention signal (Munro and Pelham 1987) like other storage proteins, it offers an appropriate system to study whether oligomerization through disulphide bonds has a role in storage-protein aggregation and retention in the ER.

In this paper we attempt to determine if Cys-rich domains are necessary for retaining the γ-zein in the ER by using *Xenopus* oocytes as a heterologous system. The DNAs encoding a γ-zein and two deletion derivatives were engineered, the corresponding transcripts injected into *Xenopus* oocytes and the distribution of the translation products analyzed. We suggest that the Pro-X and Cys-rich domains are not involved in the intracellular retention of γ-zein. It would thus appear that either the N-terminal (Pro)-rich domain or the C-terminal tail contain the information necessary to target this protein on the ER.

Materials and methods

Plasmid constructions. Three constructions were cloned under the *T3* promoter of the plasmid Bluescript (pBSKS, Stratagene, La Jolla, Cal. USA) used as a transcription vector. Plasmid pKSG2 was obtained by subcloning the PvuI (blunt-ended)-XbaI restriction fragment of p22.3 (Boronat et al. 1986) containing the complete γ-zein coding sequence and polyadenylation signals, in EcoRVXbaI restriction sites of pBSKS. This clone contains the wild-type sequence of γ-zein (γ-zein, Fig. 1). Plasmid pHbP2 was obtained by two cloning steps: (i) the 300-bp SalI–Hae3 restriction fragment of pKSG2 was cloned in pBSKS restricted with SalI–EcoRV (pKSC9) and (ii) the 600-bp PvuII–XbaI fragment of pKSG2 was cloned in SmaI–XbaI restriction sites of pKSC9. The new construction pHbP2, contains the coding sequence of a γ-zein mutant, in which the Pro-X domain of the protein has been deleted (HbP, Fig. 1). As a consequence of the cloning strategy a useful EcoRI restriction site was introduced between two characteristic domains (repeat and Cys domains) of the γ-zein coding sequence. Plasmid pKSDC was obtained by ligation of the plasmid pKSG2 after digestion with PstI. This construct contains the coding sequence of a new γ-zein mutant, in which the Cys-domain of the protein has been deleted (DC, Fig. 1).

In-vitro transcription and translation. Plasmids containing γ-zein coding sequence (pKSG2), Pro-X domain deletion (pHbP2) and Cys-rich domain deletion (pKSDC) were linearized by digestion with XbaI and capped transcripts were transcribed in vitro following standard protocols (Sambrook et al. 1989) using T3 polymerase and the cap analogue G(5′)ppp(5′)G. In-vitro translation and translocation of the synthetic transcripts were carried out using a wheat-germ cell-free system as described by Torrent et al. (1986) and [^{35}S]cysteine as a labelled amino-acid precursor. Nuclease-treated canine pancreatic microsomal membranes (CM; Amersham, Buckinghamshire, UK) were added at the beginning of the translation period. Incubation was carried out for 60 min at 25° C. When indicated, aliquots of translation mixtures were treated with 100 μg · ml^{-1} proteinase K (PK; Merck, Darmstadt, Germany) at 4° C for 30 min in the presence or in the absence of 0.5% Nonidet P40 (NP40; Fluka, Buchs, Switzerland). Protease reaction was stopped by adding phenylmethylsulfonyl fluoride (PMSF) to 10 mM. After precipitation in 15% trichloroacetic acid (TCA), the different samples were analyzed by SDS–PAGE and fluorography as described below.

Microinjection and culturing of oocytes. Female *Xenopus laevis* were obtained from commercial suppliers (Blades Biological, Kent, UK; Centre d'elevage de Xénopes du CRBM, Montpellier, France) and anaesthetized with 3-aminobenzoic acid ethyl ester (1 mg · ml^{-1}, Sigma, St. Louis, Mo., USA). Individual oocytes at Dumont stages V and VI (Colman 1984) were manually stripped from the ovary and maintained in culture overnight at 18° C in modified Barth's solution (MBS; Colman 1984). Healthy oocytes were defolliculated by incubation with 0.5 mg · ml^{-1} collagenase (type II; Sigma). The vegetative poles of defolliculated oocytes were injected with 50 nl of the synthetic transcripts at concentrations of 0.1–0.4 μg · μl^{-1}. Oocytes were then incubated at 18° C in excess MBS medium supplemented with gentamicin (100 μg · ml^{-1}; Sigma) and nystatin (20 U · ml^{-1}; Sigma). For pulse-chase experiments, injected oocytes were incubated overnight to permit cRNA recruitment. Oocytes that looked healthy after the overnight incubation were cultivated in small wells containing 5 μl per oocyte of 37.10^6 Bq · ml^{-1} [^{35}S]methionine or 37.10^6 Bq · ml^{-1} [^{35}S]cysteine in MBS suplemented with gentamicin, nystatin and different protease inhibitors (0.1 mM PMSF, 0.5 μg · ml^{-1} leupeptin, 10 μg · ml^{-1} aprotinin, 0.7 μg · ml^{-1} pepstatin). Protein pulse-labelling was performed for 2 h and afterwards labelled oocytes were extensively washed with MBS and incubated in MBS supplemented as indicated above plus 10 mM unlabelled methionine or cysteine for the desired time. All incubations were performed at 18° C. At the end of the chase period, oocytes and incubation media were collected separately and immunoprecipitated as described below. The incubation medium was only analyzed in those wells in which all the oocytes appeared morphologically healthy.

Analysis of synthesized proteins. Oocytes were homogenized with 40 μl per oocyte of buffer A (50 mM Tris-HCl, pH 7.5; 100 mM NaCl; 1 mM EDTA; 1% Triton X–100; 1 mM PMSF) by passing them ten times through the tip of a 200-μl micropipette and then incubated on ice for 10 min before precipitation in 15% TCA. The TCA-precipitated pellets were processed for 15% SDS–PAGE (Laemmli 1970). Immunoblotting of proteins subjected to SDS–PAGE was performed as described by Ludevid (1985). The nitrocellulose sheets were incubated with anti-γ-zein polyclonal antibody (dilution 1:2000; Ludevid et al. 1985) and alkaline phosphatase-conjugated swine anti-rabbit immunoglobulins were used for antibody detection. Immunoprecipitation was carried out essentially according to Borgese and Gaetani (1980) using rabbit anti-γ-zein serum. In order to suppress background, homogenized oocytes and incubation media were preincubated with non-immune serum (dilution 1:25) in buffer B (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1 mM EDTA; 1% Triton X–100; 0.1% SDS; 1 mM PMSF) for 30 min at room temperature. Afterwards, protein A–Sepharose was added and the mixtures were incubated for 30 min at

room temperature. After discarding the protein A-sepharose, the samples were incubated in buffer B with anti-γ-zein serum (dilution 1:200) and protein A-sepharose. Immunoprecipitated proteins were analyzed by SDS–PAGE and fluorography.

Preparation of microsomal fraction and protease treatment. Three days after injection, unfrozen oocytes were homogenized at 0° C in batches of 20 in 0.4 ml of buffer C (20 mM Tris-HCl, pH 7.6; 50 mM KCl; 10 mM MgCl; 100 mM NaCl; 1 mM EDTA) containing 10% (w/v) sucrose, and processed essentially as described by Colman (1984). The homogenates were layered onto a step gradient of 1 ml 20% (w/v) sucrose and 1 ml 55% (w/v) sucrose in buffer C and centrifuged at 45 000 rpm in an SW60 rotor (Beckman, Palo Alto, Cal., USA) for 60 min at 4° C. The microsomal fraction was then removed from the 20%/55% interface. Microsomal fractions were diluted three times with buffer C and divided into three equal parts. Samples with and without 1% N P40 were digested with 100 $\mu g \cdot ml^{-1}$ proteinase K for 90 min at 20° C. Reactions were stopped by adding PMSF to 10 mM. After 10 min on ice samples were precipitated with 15% TCA and processed for SDS–PAGE and immunoblotting.

Results

In-vitro translocation of γ-zein and truncated γ-zein across microsomes. The γ-zein protein sequence is composed of different characteristic domains (Prat et al. 1985) as is shown in the hydropathic profile in Fig. 1. A 19-amino-acid signal peptide is followed by a short N-terminal domain and a highly repetitive region (Repeat) containing eight repeats of the unit Pro-Pro-Pro-Val-His-Leu. This repeat is linked to the C-terminal domain by a Pro-X region where X are amino acids rich in Cys. The non-repetitive C-terminal domain contains most of the Cys residues and conserved regions related to those of other prolamines (Shwery and Tatham 1991). In order to investigate whether structural features derived from disulphide bonds contain information aimed at retaining the γ-zein in the ER, we constructed two derivatives from the entire coding sequence of γ-zein. Deletions of sequences corresponding to the Pro-X domain (21 amino acids) and the Cys-rich domain (94 amino acids) were engineered in order to abolish putative disulphide cross-links of the encoded proteins. As shown in Fig. 1, deleted constructions were cloned including the sequence corresponding to the signal peptide. Both truncated HbP (Pro-X deletion) and DC (Cys-rich deletion) proteins contain the first 10 amino acids and the repeat domain of mature γ-zein. The last 17 amino acids of the C-terminal tail were also conserved in both cases.

To determine whether the truncated proteins encoded by the constructions were membrane-translocation-competent, in-vitro transcription-translation experiments were carried out in the presence of dog pancreas microsomes. Synthetic transcripts were synthesized using bacteriophage T3 RNA polymerase and were translated in vitro in a wheat-germ cell-free system. Cysteine was used as a labelled amino-acid precursor, since the truncated DC γ-zein had only the first codon as methionine. Translation products were immunoprecipitated with a polyclonal antiserum raised against γ-zein (Ludevid et al. 1985). Both HbP-and DC-synthesized proteins were recognized by an anti-γ-zein antibody despite the possible structural changes induced by deletions (results not shown). As can be seen in Fig. 2 (lanes 3, 7, 11), translocated products were protected from proteinase digestion. It should be noted that there was a low yield of translocated protein (about 30%) in all cases, probably due to the low translocation competence of the microsomal membranes used. In previous in-vitro studies, we have observed that canine microsomes translocate maize zeins poorly (Campos et al. 1988). However, proteinase-K

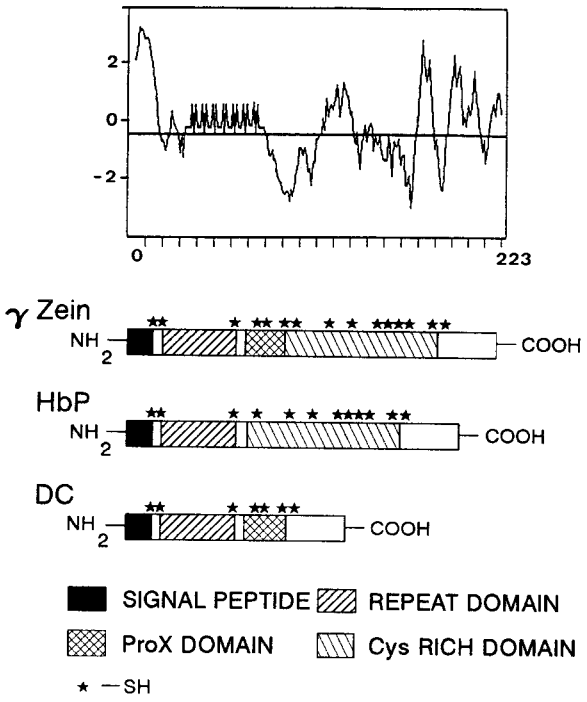

Fig. 1. Hydrophylicity profile of γ-zein and diagrams of γ-zein, HbP and DC proteins showing the distribution of structural domains as well the positions of Cys residues

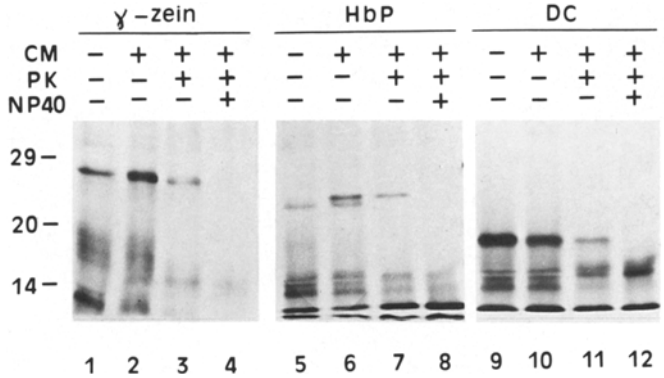

Fig. 2. In-vitro translation and translocation of transcripts corresponding to γ-zein, HbP and DC proteins. *Lanes 1, 5, 9,* total translation products; *lanes 2, 6, 10,* total translation products after translocation into canine microsomal membranes (*CM*); *lanes 3, 7, 11,* translocated products resistant to proteinase K (*PK*) action; *lanes 4, 8, 12,* total translation products after PK treatment in the presence of 0.5% Nonidet P40 (*NP40*). Marker molecular weights are indicated on the *left* (in kilodaltons)

digested translocated proteins when detergent was added (lanes 4, 8, 12), reflecting the fact that the signal peptide remains as a functional target for cross-membranes. However, with the exception of the Pro-X-deleted protein (HbP), cleavage of the signal peptide could not be observed in terms of electrophoretic mobility (Fig. 2, lanes 6, 7). In this case, the translocated protein has a lower electrophoretic mobility than the pre-protein. One explanation could be that HbP was modified post-translationally. However, such a situation is not probable, since HbP and γ-zein have almost identical polypeptide chains and such modification should be present in both polypeptides. Another explanation is the anomalous SDS–PAGE mobility observed in most of the storage proteins and the highly repetitive Pro-rich proteins (Stiefel et al. 1988). In fact, the processing of the γ-zein signal peptide was only detected by using two-dimensional electrophoresis (Torrent et al. 1986) and the molecular weight of γ-zein deduced from SDS–PAGE migration was about 6 kDa higher than the molecular weight predicted by amino-acid sequence (22 kDa). Both HbP and DC also show anomalous electrophoretic mobilities. Although their apparent molecular weights are 26 kDa and 17 kDa, respectively, the predicted molecular weights from amino-acid sequences are 20 kDa and 12 kDa, respectively. These phenomena are probably due to special conformational features of these proteins.

Expression of γ-zein, HbP and DC in Xenopus *oocytes.* *Xenopus* oocytes have demonstrated great reliability in the targeting of plant proteins introduced by direct microinjection of mRNAs (Ceriotti et al. 1991). To establish that such reliability extends to γ-zein and truncated γ-zeins, oocytes were microinjected with synthetic transcripts from pKSG2, pHbP2 and pKSDC linearized templates as described in *Materials and methods*. After 3 d, injected oocytes and non-injected oocytes (as control samples) were homogenized and proteins from the homogenates as well as from the incubation media were analyzed by SDS–PAGE and immunoblot. The results shown in Fig. 3 indicate that γ-zein and truncated γ-zein products, HbP and DC (lanes 1–3) were highly expressed and accumulated in the *Xenopus* oocytes. The antiserum against γ-zein recognized truncated γ-zeins and did not cross-react with any polypeptides in control oocytes (Fig. 3C). The total protein loaded in each lane corresponds to two oocytes. From previous titration experiments (results not shown) with the antiserum and the purified γ-zein, we can assume that in 72 h, one oocyte could accumulate about 20 ng each of γ-zein and its truncated forms. The bands migrate according to apparent molecular weights of 28,26 and 17 kDa which coincide with the apparent molecular weights observed in the case of translocated proteins in in-vitro experiments. After 3 d of oocyte incubation, neither γ-zein nor HbP and DC traces were detected in the incubation media (data not shown). Although low-rate secretions cannot be detected by immunoblotting, these results suggest that γ-zein and its deletion forms were retained in the oocytes.

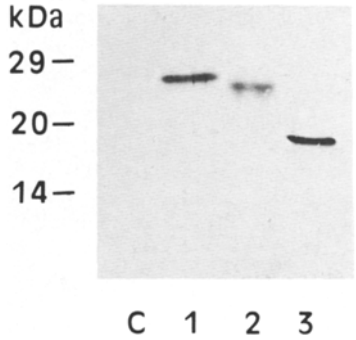

Fig. 3. Accumulation of γ-zein, HbP and DC proteins in *X. laevis* oocytes. Oocytes were injected with either γ-zein, HbP or DC cRNAs and incubated for 3 d in MBS medium (see *Materials and methods*). Protein extracts from two oocytes were electrophoresed and immunoblotted using anti-γ-zein serum. *Lane C*, non-injected oocytes used as a control; *lane 1*, oocytes injected with γ-zein RNA; *lane 2*, oocytes injected with HbP RNA; *lane 3*, oocytes injected with DC RNA. Marker molecular weights are indicated on the *left*

Deletion of the Cys-rich and Pro-X domains of γ-zein does not influence protein stability and routing. Deletions and/or small amino-acid alterations engineered in a polypeptide chain could alter their original stability and routing in vivo. Here, we investigate whether deletion of Cys-rich and Pro-X domains of γ-zein could alter protein stability and routing in *Xenopus* oocytes. The data shown in Fig. 3 indicate that a long period of incubation allowed high levels of synthesis and intracellular deposition of γ-zein, HbP and DC. To determine the time course of the accumulation of these polypeptides, injected oocytes were pulse-labelled for 2 h and then chased for 24 h and 48 h in an unlabelled medium. Antiserum against γ-zein was used to immunoprecipitate both oocyte homogenates and the corresponding media (Fig. 4).

The results reported in Fig. 4 indicate that after a 2-h pulse, the newly synthesized γ-zein and truncated forms (HbP and DC) remain stable until the 48-h chase (see oocyte lanes). Comparison by densitometric analysis between bands after 0-h and 48-h chases indicates that no degradation of either γ-zein or deleted proteins was observed during this period of time. The immunoprecipitated bands correspond to expected molecular weights of the translocated proteins, indicating that proteins accumulate without any discernible post-translational modification. Similar results were obtained after short periods of chase (2 h and 6 h, results not shown). After a 2-h pulse and a 24-h or 48-h chase, no secretion of polypeptide into the media was observed, even after long exposures of the fluorogram (Fig. 4, media lanes). Extracellular protein degradation could be excluded since a mixture of proteinase inhibitors was added in all experiments. In fact, this result was expected since no intracellular decrease of γ-zein or HbP and DC was detected over the course of the chase. We found that when the oocyte homogenates were spun briefly before immunoprecipitation, the recovery of proteins was much lower than without centrifugation. Most of the γ-zein, HbP and DC remained in the pellet even in extraction

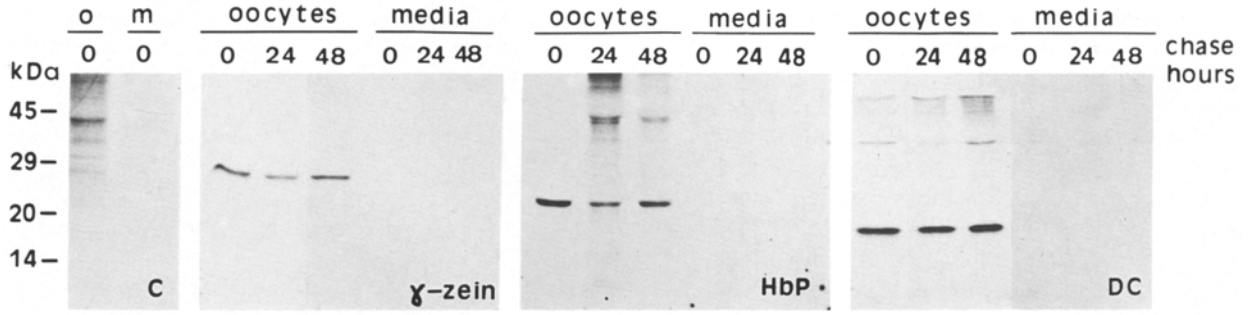

Fig. 4. Pulse-chase labelling experiments of oocytes expressing γ-zein, HbP or DC proteins. Oocytes were pulse-labelled for 2 h and chased for 0 h, 24 h and 48 h. Radiolabelled proteins were immunoprecipitated from cell homogenates (*o*, oocytes) or incubation media (*m*, media) equivalent to three oocytes. Analysis was by SDS–PAGE and fluorography. An overexposed fluorogram corresponding to non-injected oocytes is also shown (*C*). Positions of molecular weight markers are indicated on the *left*

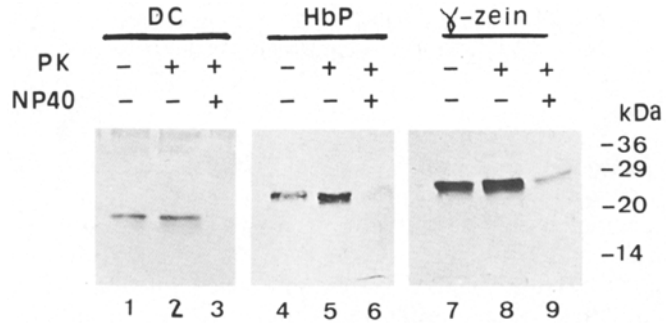

Fig. 5. Insertion of *DC*, *HbP* and *γ-zein* into microsomal membranes of injected oocytes. Oocytes collected 3 d after injection were homogenized and fractionated as described in *Materials and* methods. Accumulation of DC, HbP and γ-zein proteins in the microsomal fraction *(lanes 1, 4, 7)* as well their resistance to proteinase K (*PK*) in the absence *(lanes 2, 5, 8)* or in the presence of detergent (1% NP40) is shown. Positions of molecular weight markers are indicated on the *right*

buffers containing neutral detergent. This suggests that these proteins are present as associated forms in oocytes.

The γ-zein is located in ER-derived PBs together with α- and β-zeins of maize endosperm cells (Ludevid et al. 1984). We have previously demonstrated that microsomes isolated from maize endosperms containing endogenous polysomes, synthesize, translocate and process γ-zein polypeptides (Torrent et al. 1986). To establish whether truncated γ-zeins were correctly processed and incorporated in membrane-bound organelles, microsomes from oocytes were prepared using discontinuous sucrose gradients. Oocytes were injected with γ-zein, HbP and DC transcripts and, after 3 d incubation, microsomal preparations were analyzed after proteinase-K digestion with or without detergent (Fig. 5). Immunoblots were labelled with anti-γ-zein antiserum. The DC, HbP and γ-zein polypeptides were all able to accumulate in dense structures (Fig. 5, lanes 1, 4, 7) and be incorporated within membranes, since they are protected from proteinase-K digestion (lanes 2, 5, 8). As expected, they were digested after membrane solubilization with detergent (lanes 3, 6, 9). No appreciable amounts of γ-zein, HbP or DC proteins were detected either in the supernatant fraction or in the pellet of the gradients (not shown). Therefore, the bulk of γ-zein, as well as its truncated forms, is located in PB-like vesicles, where it is protected from proteolytic digestion.

Discussion

There is a growing consensus that no specific sorting signal is required to route secretory proteins from the ER to the cell surface. If true, it follows that organelle-resident proteins must contain specific retention signals (Pfeffer and Rothman 1987). We have used *Xenopus* oocytes to investigate which part(s) of the γ-zein is responsible for its retention in the ER of endosperm cells. Amphibian oocytes have been described as being a good heterologous system to study targeting of storage proteins (Hurkman et al. 1981; Simon et al. 1990; Ceriotti et al. 1991). Here we show that when γ-zein polypeptides were expressed in *Xenopus* oocytes, they were synthesized and accumulated at high levels in membrane-bound organelles, thereby demonstrating their resistance to proteolytic digestion. Earlier studies have show that α-zeins – the major storage proteins of maize – and modified lysine-rich zeins assemble in *Xenopus* oocyte vesicles, forming dense structures like the maize PBs (Wallace et al. 1988). However, there is no homology between α-zein and γ-zein polypeptides of maize prolamins (Shewry and Tatham 1990). The α-zein, insoluble in aqueous media, consists mainly of a long repetitive domain containing blocks of 20 hydrophobic amino acids (Messing 1987). The γ-zein is a Cys-rich prolamine of 28 kDa with a high Pro-rich repeat, in which Pro forms clusters of three residues, and with a Cys-rich C-terminal domain related to the domains of γ- and α-gliadins and B1 hordeins (Shewry and Tatham 1990). Because γ-zein is soluble in aqueous media containing reducing agents and it is located in PBs, it has been suggested that α-zein aggregates could be maintained by a network of γ-zein cross-linked by disulphide bonds (Ludevid et al. 1984). In this study, we have investigated the role of Cys-rich domains in γ-zein aggregation and retention inside *Xenopus* oocytes. Deletion of the Pro-X domain and Cys-rich domain can damage the native disulphide bonds and change the protein solubility. However, we found that expression of

these transcripts in oocytes resulted in the effective retention of truncated γ-zeins. Furthermore, the latter were able to assemble within vesicles only slightly less dense than maize PBs. In experiments using three-step gradients of 20, 55 and 65% sucrose (results not shown), microsomes containing γ-zein, HbP and DC sedimented at 55% sucrose, whereas maize PBs sedimented at 65% sucrose (Ludevid et al. 1984). Our results suggest that structural motifs other than intra or inter disulphide bonds are required for protein aggregation and retention inside oocytes.

We cannot exclude the exit of γ-zein, HbP and DC from the ER compartment and their accumulation in a pre/post Golgi secretory vesicles. Since γ-zein is not glycosylated, this makes it difficult to investigate that route. However, exit from the ER seems unlikely for the following two reasons. (i) Proteins of the ER appear to be more unstable in a post-ER compartment, e.g. KDEL-containing proteins are highly degraded when the tetrapeptide is modified or removed (Denecke et al. 1992); ER-retention-defective yeast strains only secrete BiP (luminal binding protein) in low amounts, the bulk of this protein having been degraded (Denecke et al. 1991). By contrast neither γ-zein, nor HbP and DC were degraded during the 48-h chase and remained stable even after 5 d of oocyte incubation. (ii) Exit from the ER should lead to protein secretion according to the bulk-flow theory. Vacuolar storage proteins such as phaseolin (Bassuner et al. 1983) and wheat γ-gliadin (Simon et al. 1990) when expressed in *Xenopus* oocytes were secreted into the medium; they have vacuolar sorting signals which are not recognized by animal cells. There is evidence from our results that no traces of γ-zein and its truncated forms were secreted. This suggests that they are retained in the ER of *Xenopus* oocyte cells.

The question remains why γ-zein is retained in the ER. The γ-zein (Prat et al. 1985) does not contain KDEL (the C-terminal consensus sequence present in some of ER-resident proteins) (Munro and Pelham 1987). In maize, γ-zein is synthesized in membrane-bound polysomes and translocated co-translationally across membranes (Torrent et al. 1986). A maize signal-recognition particle mediates this process (Campos et al. 1988). In *Xenopus* oocytes, γ-zein, HbP and DC are similarly retained, indicating that the retention signal, if it exists, is present in all polypeptides. The N-terminal Pro-rich repetitive region and the 17-amino-acid C-terminal tail common in the three proteins could be responsible for this retention. We present evidence that these regions could act as functional retention signals even when present in a different structural context - without the linker region (Pro-X) in HbP and lacking the 94 amino acids of the Cys-rich domain in DC. Whether or not the size of the repeat can be critical is unknown. Two plant storage proteins – 17 kDa zein of maize (Prat et al. 1987) and γ-coixin of Job's Tears *(Coix lachryma-jobi)* seeds (Leite et al. 1991) – are highly homologous to γ-zein but contain a shorter repeat (three to five units). Both are located inside ER-derived protein bodies. Conformational studies of a synthetic peptide of the eight units of Pro-Pro-Pro-Val-His-Leu reveal that this polypeptide adopts an extended-helix-conformation of the poly-proline II type (Rabanal et al. 1993). An interesting aspect of this conformation is that it contains an amphipatic feature, with histidine residues on one side and hydrophobic amino acids on the other.

It seems more likely to us that ER localization of some plant storage proteins will be explicable in terms of protein-protein and/or protein-membrane binding. Thus, we can speculate that the repeat region or the C-terminal hydrophobic tail could either interact with membranes and/or self-assemble to form large aggregates that would be unable to diffuse to the sites of transport-vesicle formation. Chaperones such as PDI (protein disulphide isomerase; Bulleid and Freedman 1988) or BiP, recently identified in maize (Fontes et al. 1991), could provide the primary protein-binding site. It is known that misfolded, unassembled or incompletely oligomerized proteins are not able to exit from the ER and thus remain in this organelle until eventual degradation (Hurtley and Helenius 1989). Thus, misfolding could be the cause of aggregation and retention of γ-zein and truncated forms within the ER. Research is in progress to define the role of the repeat region and the C-terminal tail in γ-zein retention.

We thank C. Romera (Centre de Investigació i Desenvolupament, CSIC, Barcelona, Spain) for purifying and preparing the γ-zein antibody and I. Alvarez (Centre de Investigació i Desenvolupament, CSIC, Barcelona, Spain) for many helpful discussions. This research was supported by grants from the Comisión Interministerial de Ciencia y Tecnología (CICYT), Bio-90 (to P.P) and BIO 92/186 (to M.D.L.). M.I.G. and J.M.C. are recipient of a postgraduate fellowship from the Formación del Personal Investigador (FPI).

References

Argos, P., Pedersen, K., Marks, M.D., Larkins, B.A. (1982) A structural model for maize zein proteins. J. Biol. Chem. **257**, 9984–9990

Bassuner, R., Huth, A., Manteuffel, R., Rapoport, T.A. (1983) Secretion of plant storage globulin polypeptides by *Xenopus* laevis oocytes. Eur. J. Biochem. **133**, 321–336

Borgese, N., Gaetani, S. (1980) Site of synthesis of rat liver NADH-cytochrome b reductase, an integral membrane protein. FEBS Lett. **112**, 216–220

Boronat, A., Martinez, M.C., Reina, M., Puigdomènech, P., Palau, J. (1986) Isolation and sequencing of a 28 kD glutelin-2 gene from maize. Common elements in the 5′ flanking regions among zein and glutelin genes. Plant Sci. **47**, 95–102

Braakman, I., Helenius, J., Helenius, A. (1992) Manipulating disulfide bond formation and protein folding in the endoplasmic reticulum. EMBO J. **41**, 1717–1722

Bulleid, N.J., Freedman, R.B. (1988) Defective co-translational formation of disulphide isomerase deficient microsomes. Nature **335**, 649–651

Campos, N., Palau, J., Torrent, M., Ludevid, M.D. (1988) Signal recognition particles are present in maize. J. Biol. Chem. **263**, 9646–9650

Ceriotti, A., Pedrazzini, E., Fabbrini, M.S., Zoppe, M., Bollini, R., Vitale, A. (1991) Expression of the wild-type and mutated vacuolar storage protein phaseolin in *Xenopus* oocytes reveals relationships between assembly and intracellular transport. Eur. J. Biochem. **202**, 959–968

Chrispeels, M.J. (1991) Sorting of proteins in the secretory system. Annu. Rev. Plant Physiol. Plant Mol. Biol. **42**, 21–53

Chrispeels, M.J., Higgins, M.J., Spencer, T.J.V. (1982) Assembly of storage protein oligomers in the endoplasmic reticulum and

processing of the polypeptides in the protein bodies of developing cotyledons. J. Cell. Biol. **93**, 306–313
Colman, A. (1984) Translational of eukaryotic messenger RNA in *Xenopus* oocytes and expression of exogenous DNA in *Xenopus* oocytes. In: Transcription and translation: A practical approach, pp. 271–302, Ames B.D., Higgins S.J., eds. IRL Press, Oxford
Denecke, J., Botterman, J., Deblaere, R. (1990) Protein secretion in plant cells can occur via a default pathway. Plant Cell **2**, 51–59
Denecke, J., Goldman, M.H.S., Demolder, J., Seurinck, J., Botterman, J. (1991) The tobacco luminal binding protein is encoded by a multigene family. Plant Cell **3**, 1025–1035
Denecke, J., De Rycke, R., Botterman, J. (1992) Plant and mammalian sorting signals for protein retention in the endoplasmic reticulum contain a conserved epitope. EMBO J. **11**, 2345–2355
Fontes, E.B.P., Shank, B.B., Wrobel, R.L., Moose, S.P., OBrian, G.R., Wurtzel, E.T., Boston, R.S. (1991) Characterization of an immunoglobulin binding protein homolog in the maize floury-2 endosperm mutant. Plant Cell **3**, 483–496
Freedman, R.B. (1989) Protein disulfide isomerase: Multiple roles in the modification of nascent secretory proteins. Cell **57**, 1069–1072
Hurkman, W.J., Smith, L.D., Richter, J., Larkins, B. (1981) Subcellular compartimentalization of maize storage proteins in *Xenopus* oocytes injected with zein messenger RNAs. J. Cell Biol. **87**, 292–299
Hurtley, S.M., Helenius, A. (1989) Protein oligomerization in the endoplasmic reticulum. Annu. Rev. Cell Biol. **5**, 277–307
Kim, W.T., Franceschi, V.R., Kirshnan, H.B., Okita, T.W. (1988) Formation of wheat protein bodies: Involvement of the Golgi apparatus in gliadin transport. Planta **176**, 173–182
Krishnan, H.B., Franceschi, V.R., Okita, T.W. (1986) Immunochemical studies on the role of the Golgi complex in protein body formation in rice seeds. Planta **169**, 471–480
Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680–685
Larkins, B.A., Hurkman, W.J. (1978) Synthesis and deposition of zein protein bodies of maize endosperm. Plant Physiol. **62**, 256–263
Leite, A., De Freitas, F.A., Yunes, J.A., Arruda, P. (1991) Nucleotide sequence of a cDNA clone encoding γ-coixin from *Coix lacryma-jobi* seeds. Plant Physiol. **97**, 1604–1605
Ludevid, M.D., Torrent, M., Martinez-Izquierdo, J.A., Puigdomenech, P., Palau, J. (1984) Subcellular localization of glutelin-2 in maize (*Zea mays* L.) endosperm. Plant Mol. Biol. **3**, 227–234
Ludevid, M.D., Martinez-Izquierdo, J.A., Armengol, M., Torrent, M., Puigdomenech, P., Palau, J. (1985) Immunological relations between glutelin-2 and low molecular weight zein-2 proteins from maize *(Zea mays)* endosperm. Plant Sci. **41**, 41–48
Marquart, T., Helenius,A. (1992) Misfolding and aggregation of newly synthesized proteins in the endoplasmic reticulum. J. Cell Biol. **117**, 505–513
Messing, J. (1987) In: Genetic engineering, vol. 6, pp. 1–46, Rigby, P.W., ed. Academic Press, London
Munro, S., Pelham, H.R.B. (1987) A C-terminal signal prevents secretion of luminal ER proteins. Cell **48**, 988–997
Pfeffer S.R., Rothman J.E. (1987) Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. Annu. Rev. Biochem. **56**, 829–852
Prat, S., Cortadas, J., Puigdomenech, P., Palau, J. (1985) Nucleic acid (cDNA) and amino acid sequences of the maize endosperm protein glutelin-2. Nucl. Acid Res. **13**, 1493–1504
Prat, S., Perez-Grau, L., Puigdomenech, P. (1987) Multiple variability in the sequence of a family of maize endosperm proteins. Gene **52**, 41–49
Rabanal, F., Ludevid, M.D., Pons, M., Giralt, E. (1993) CD of proline-rich polypeptides: Application to the study of the repetitive domain of maize glutelin-2. Biopolymers, in press
Rubin, R., Levanony, H., Galili, G. (1992) Evidence for the presence of two different types of protein bodies in wheat endosperm. Plant Physiol. **99**, 718–724
Sambrook, J., Fritsch, E., Maniatis, T. (1989) In: Molecular cloning: A laboratory mannual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
Shewry, P.R., Tatham, A.S. (1990) The prolamin storage proteins of cereal seeds: structure and evolution. Biochem. J. **267**, 1–12
Simon, R., Altschuler, Y., Rubin, R., Galili, G. (1990) Two closely related wheat storage proteins follow a markedly different subcellular route in *Xenopus laevis* oocytes. Plant Cell **2**, 941–950
Stiefel, V., Perez-Grau, L., Albericio, F., Giralt, E., Ruiz-Avila, L., Ludevid, M.D., Puigdomenech, P. (1988) Molecular cloning of cDNAs encoding a putative cell wall protein from *Zea mays* and immunological identification of related polypeptides. Plant Mol. Biol. **11**, 483–493
Torrent, M., Poca, E., Campos, N., Ludevid, M.D., Palau, J. (1986) In maize, glutelin-2 and low molecular weight zeins are synthesized by membrane-bound polyribosomes and translocated into microsomal membranes. Plant Mol. Biol. **7**, 393–403
Vitale, A., Sturm, A., Bollini, R. (1986) Regulation of processing of a plant glycoprotein in the Golgi complex: a comparative study using *Xenopus* oocytes. Planta **169**, 108–116
Wallace, J.C., Galili, G., Kawata, E.E., Cuellar, R.E., Shotwell, M.A., Larkins, B.A. (1988) Aggregation of lysine-containing zeins into protein bodies in *Xenopus* oocytes. Science **240**, 662–664