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Two Closely Related Wheat Storage Proteins Follow a Markedly Different Subcellular Route in *Xenopus laevis* Oocytes

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α-Gliadins and γ-gliadins are two closely related wheat storage proteins that evolved from a common ancestral gene. However, synthesis of α-gliadins and γ-gliadins in *Xenopus laevis* oocytes revealed striking differences in their subcellular routing. The major portion of α-gliadin accumulated inside the oocyte, whereas most of the γ-gliadin was secreted. Disruption of the Golgi apparatus by monensin revealed that the major part of secretion of γ-gliadin is Golgi mediated. The difference in the subcellular route between α-gliadin and γ-gliadin may be attributed to differential transport from the endoplasmic reticulum to the Golgi apparatus, a process that is generally the rate-limiting step in protein secretion. Coinjection of the two mRNAs had no effect on their routing, indicating no interaction between them. Our results support the hypothesis that subcellular transport of gliadins in wheat endosperm occurs in two separate routes; one is Golgi mediated, and the other is not. We also show that the subcellular transport may be markedly affected by small structural variations within closely related storage proteins.

INTRODUCTION

The major storage proteins of wheat are alcohol-soluble prolamins called gliadins. The gliadins are monomeric proteins that migrate between 30 kD and 60 kD on SDS-PAGE and were first subdivided into four groups, α-, β-, γ-, and ω-gliadins, on the basis of migration on acidic PAGE (Woychik et al., 1961). Later, a classification reflecting biochemical similarities and chromosomal location divided gliadins into two categories, sulfur-poor gliadins (ω-gliadins) and sulfur-rich gliadins (α-, β-, and γ-gliadins) (Kreis et al., 1985).

The monomeric S-rich α-gliadins and γ-gliadins resemble each other in general structure. A computer comparison of their amino acid sequences showed that there is close to 70% similarity between the two proteins. Both proteins possess an amino-terminal signal peptide that is removed upon transport into the rough endoplasmic reticulum (RER). The amino-terminal part of each mature protein is composed of a region of tandem repeats preceded by a small unique prerepetitive region. The carboxy-terminal region is composed of unique amino acid sequences that are divided into proline-poor, polyglutamine, and proline-rich domains. These regions also contain 6 to 8 cysteine residues that can form intramolecular disulfide bonds (Kreis et al., 1985). α-Gliadins and γ-gliadins also possess similar secondary structures. These proteins contain a 30% to 35% β-turn structure, which is found mainly in the repeated domain (Tatham et al., 1985), as well as a 30% to 35% α-helix and a 10% to 20% β-sheet structure, which are associated mainly with the carboxy-terminal domain (Tatham and Shewry, 1985). The high stability of the helices is probably due to extensive hydrogen bonding, although disulfide bonds are clearly important. Thus, individual gliadin monomers are stabilized by intramolecular disulfide bonds and strong noncovalent forces (Tatham and Shewry, 1985).

Although the monomeric S-rich α-gliadins and γ-gliadins are similar in amino acid sequence and secondary structure and probably have evolved from a common ancestral gene, it is important to note their differences. In hexaploid wheat (genome AABBDD), the α-gliadin genes are located on chromosomes of homoeologous group 6, whereas those of the γ-gliadins are on chromosomes of homoeologous group 1 (Kasarda et al., 1976; Payne, 1987). This finding indicates that a translocation occurring at some time during wheat evolution separated a single locus of S-rich gliadins into two loci. Since this event, the two loci have evolved separately to create the α-gliadins and the γ-gliadins. Indeed, α-gliadins and γ-gliadins cross-react immunologically only weakly (Kim et al., 1988; R. Rubin and G. Galili, unpublished results) and possess several structural differences. The repeated amino acid sequence motifs are different and the repetitive domain of the γ-gliadin is larger. There are two polyglutamine regions in the α-gliadin but only one such region in the γ-gliadin. In addition, the γ-gliadin has 8 cysteine residues, whereas the α-gliadin has only 6.

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Wheat seed storage proteins, including α-gliadins and γ-gliadins, are synthesized on membrane-bound polysomes and are subsequently inserted into the endoplasmic reticulum (ER); however, the subcellular site of their aggregation and protein body formation in wheat is still a matter of controversy. Subcellular fractionation of developing wheat endosperms has indicated that the majority of wheat prolamins are deposited in protein bodies that are surrounded by RER membranes (Miflin et al., 1981). However, electron micrographs have demonstrated that the majority of wheat prolamins are deposited in protein bodies that are localized within the vacuoles (Kim et al., 1988). The role of the Golgi apparatus in the subcellular routing of wheat prolamins to the vacuoles is also not clear. Electron micrographs have shown the presence of Golgi apparati in developing wheat endosperm, suggesting their role in the deposition process (Campbell et al., 1981; Parker, 1982; Parker and Hawes, 1982; Kim et al., 1988). However, protein bodies in wheat have also been observed within RER membranes, suggesting that at least some prolamins aggregate within the RER and are presumably being transported as intact protein bodies from the RER to the vacuoles (Campbell et al., 1981; Parker, 1982). Based on these observations, Parker (1982) suggested that wheat storage proteins may be transported from the RER to protein bodies by two different routes; one route involves the transport of storage proteins by way of the Golgi to vacuoles and the second may lead directly from the RER to vacuoles, bypassing the Golgi.

In oats, the majority of the prolamin storage proteins are also localized in vacuoles. However, electron micrographs have indicated that in oats the prolamins aggregate directly within the RER and are then transported to vacuoles (Lending et al., 1989). Saigo et al. (1983) noted the presence of direct connections between the RER and smooth vacuolar membrane and speculated that direct transport of oat storage proteins from the RER to vacuoles may exist in addition to the Golgi-mediated transport.

We are interested in studying whether all prolamins in wheat are transported to vacuoles by way of a Golgi-mediated mechanism. Because wheat prolamins are not glycosylated and one cannot follow their Golgi-mediated transport by testing for Golgi-specific additions of sugar moieties, we selected *Xenopus laevis* oocytes as a heterologous system for the synthesis of individual or combinations of wheat prolamin storage proteins. *X. laevis* oocytes have been shown to translate foreign mRNAs, including plant mRNAs, quickly and efficiently (Colman, 1984). Both native mRNAs and mRNAs transcribed in vitro from cloned genes are translated. Moreover, cleavage of the signal peptide as well as subcellular transport of many foreign proteins have been shown to be performed correctly within the oocyte (Colman et al., 1981; Colman, 1984; Colman et al., 1984). Proteins for export are transported from the ER by way of the Golgi into secretory vesicles and are secreted into the extracellular environment (Colman and Morser, 1979). Because oocytes lack a storage vacuole, proteins destined for this organelle are generally transported to the Golgi and then secreted from the oocytes by bulk flow (for review, see Pfeffer and Rothman, 1987; Rose and Doms, 1988; Rothman, 1989). Indeed, when legume globulin mRNA was microinjected, the proteins, which in legume seeds accumulate in storage vacuoles, were found in the medium (Bassuner et al., 1983; Vitale et al., 1986). In contrast, maize zein storage proteins, which in seeds accumulate within the ER, aggregate within the oocytes (Hurkman et al., 1981). Because they are exported from the oocytes, vacuolar proteins that are transported by way of the Golgi can be distinguished easily from proteins that are deposited in ER-derived vesicles and do not reach the Golgi.

In this paper, we report that wheat α-gliadins and γ-gliadins experience markedly different subcellular transport in *Xenopus* oocytes. Whereas the majority of the γ-gliadins are secreted by a Golgi-mediated manner, this is not the case with the α-gliadins. Potential implications of these results on the subcellular localization of gliadins in developing wheat endosperms are discussed.

RESULTS

Synthesis of α-Gliadins and γ-Gliadins in *Xenopus* Oocytes

Plasmids for in vitro run-off transcription reactions were constructed as shown in Figure 1. *X. laevis* oocytes were injected with α-gliadin or γ-gliadin mRNA synthesized from the linearized templates. Proteins from these oocytes and uninjected controls were labeled with ^{35}S-methionine for 48 hr, and alcohol-soluble proteins from the cells' content and the incubation medium were analyzed by SDS-PAGE and fluorography. Gliadins are known to be alcohol soluble, whereas most oocyte proteins are insoluble in aqueous alcohol. An analysis of the proteins is shown in Figure 2. Control oocytes and medium contained almost no detectable alcohol-soluble proteins (Figure 2, lanes 1 and 2), whereas alcohol extracts of mRNA-injected oocytes revealed one major protein band (Figure 2, lanes 3 and 5). Each band migrated between 30 kD and 40 kD, as expected for proteins belonging to the α-gliadin and γ-gliadin families. After 48 hr of labeling, alcohol-soluble α-gliadins and γ-gliadins were also detected in the medium (Figure 2, lanes 4 and 6). Their identity as α-gliadins and γ-gliadins was confirmed by immunoprecipitation (data not shown). The α-gliadin in the medium migrated more slowly than that in the oocyte (Figure 2, cf. lanes 3 and 4).

Protein Routing in *Xenopus* Oocytes

From the data in Figure 2, it seemed that the amount of secretion of the γ-gliadin was much higher than that of the

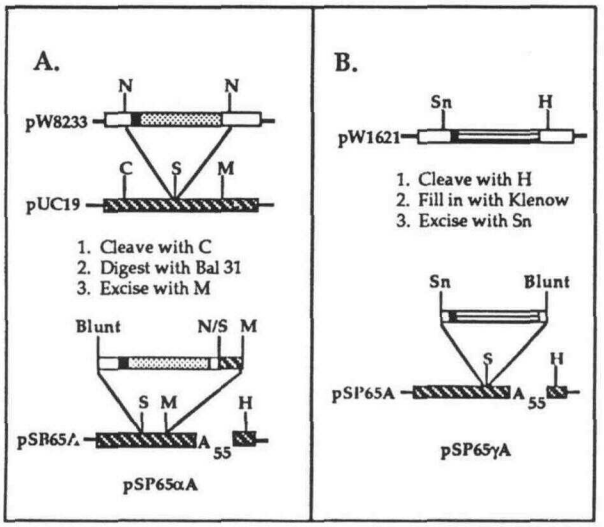

Figure 1. Subcloning of Gliadin Genes into pSP65A.

(A) pW8233 (Rafalski et al., 1984) was digested with NcoI to release the α-gliadin gene. The ends were filled in using the Klenow fragment and the gene was inserted into the SmaI site of pUC19. The resulting plamid was cleaved with SacI and digested with Bal31 nuclease, and the gliadin gene was excised with BamHI. The fragment was inserted into pSP65A digested with SmaI and BamHI to form pSP65αA.

(B) pW1621 (Sugiyama et al., 1986) was cleaved with HindIII, the end filled in using the Klenow fragment, and the γ-gliadin excised by digestion with SnaBI. The fragment was inserted into the SmaI site of pSP65A, creating pSP65γA.

Hatched boxes represent polylinker sequences; white boxes represent noncoding regions of genes; black boxes represent regions coding for signal peptides; dot-filled boxes represent α-gliadin coding sequences; and boxes with horizontal lines represent γ-gliadin coding sequences. Restriction enzymes: M, BamHI; H, Hind III; N, NcoI; S, SmaI; C, SacI; Sn, SnaBI.

α-gliadin. (Each lane in Figure 2 contains the equivalent of protein from one oocyte, except the lane showing the medium from oocytes with the α-gliadin mRNA, which contains the equivalent of two and one-half oocytes.) To measure the time course of accumulation and secretion of the α-gliadins and γ-gliadins, oocytes injected with synthetic α-gliadin or γ-gliadin mRNAs were labeled for 1 day to 3 days with ^{35}S-methionine. Gliadins were alcohol extracted from the oocytes and the medium, and their radioactivity was determined. The results are shown in Figure 3. Both the α-gliadins and γ-gliadins were detected within the oocytes and the medium 1 day after labeling; however, there was a marked difference in their rate of secretion. The majority of the γ-gliadin was secreted, reaching 70% of the counts after 3 days, but only about 5% of α-gliadin was found outside the oocyte.

Xenopus oocytes are known to process signal peptides of foreign proteins in a very efficient manner (Larkins et al., 1979). It was possible, however, that the low level of α-gliadin secretion was due to improper recognition and processing of its signal peptide and inefficient sequestration into the membrane of the RER. This possibility was tested in two ways. First, the α-gliadin synthesized in oocytes was compared with that synthesized in the reticulocyte lysate in vitro system. As shown in Figure 4A, the α-gliadin synthesized in oocytes migrated slightly faster than that synthesized in vitro. The decrease in molecular weight of the gliadin synthesized in the oocytes is due to

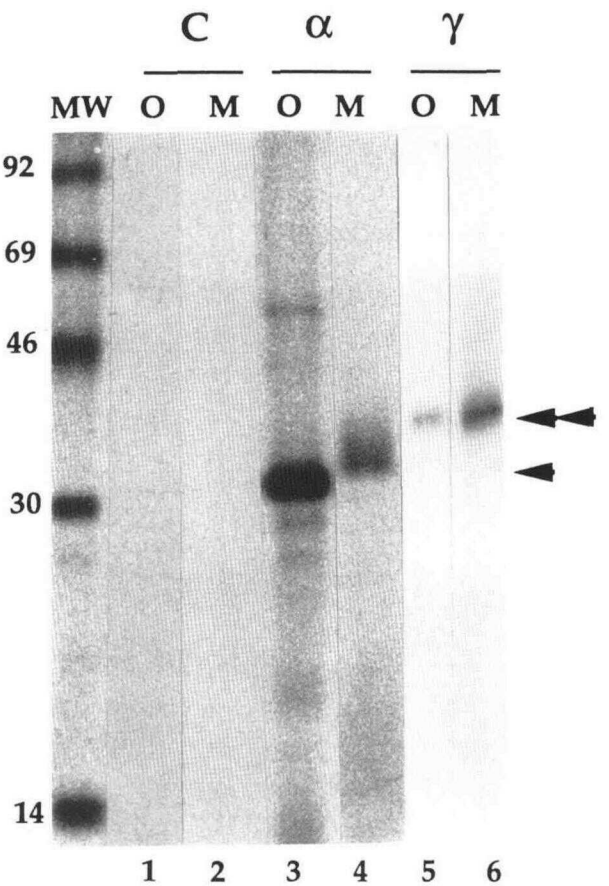

Figure 2. Expression of α-Gliadin and γ-Gliadin in *X. laevis* Oocytes.

Oocytes were injected with either α-gliadin or γ-gliadin SP6 mRNAs and left overnight to permit messenger recruitment. The oocytes were incubated with ^{35}S-methionine for 48 hr and then harvested. Alcohol extracts of homogenized oocytes (O) or medium (M) were run on 15% SDS-PAGE and fluorographed. C, control. Single-headed arrow represents α-gliadin and the double-headed arrow represents γ-gliadin. Molecular weights (MW) are shown (in kilodaltons).

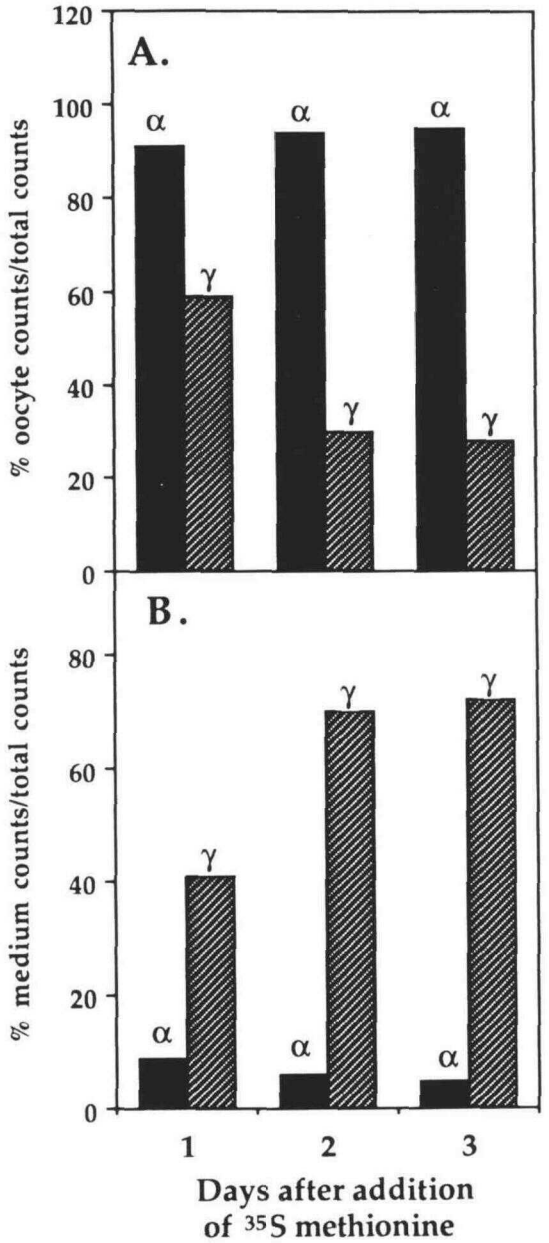

Figure 3. Accumulation of α-Gliadin and γ-Gliadin in Oocytes and Medium.

Oocytes and medium were harvested after 1 day to 3 days incubation in ^{35}S-methionine, and TCA-precipitable counts of alcohol extracts of oocytes and medium samples were determined.
(A) The percentage of total counts in the oocyte was determined. The results of two separate experiments were averaged.
(B) The percentage of total counts in the medium was determined. The results of two separate experiments were averaged.

removal of its signal peptide. The presence of the α-gliadin within membranes was assayed based on its resistance to proteinase-K digestion. Figure 4B demonstrates that α-gliadin synthesized in oocytes was resistant to proteinase-K digestion, but this resistance was abolished upon addition of 1% Triton X-100 to the reaction mixture (Figure 4B). Moreover, the resistance to proteinase-K digestion was due to internalization within membranes and not to

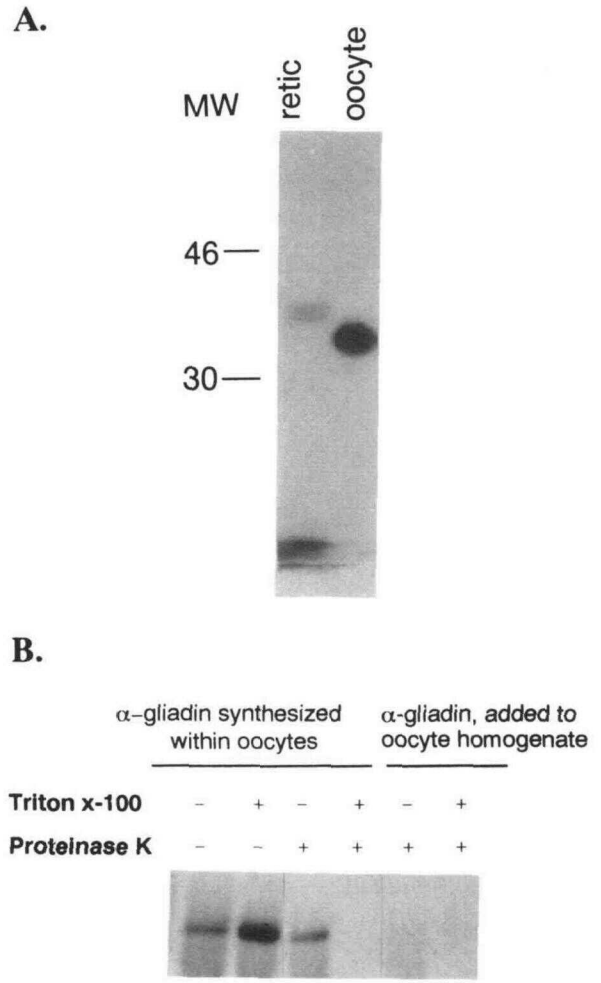

Figure 4. Recognition of the Signal Peptide and Insertion of the α-Gliadin into Membranes of *X. laevis* Oocytes.

(A) α-Gliadins synthesized in the reticulocyte lysate in vitro system and synthesized in vivo by the oocytes were run on 10% SDS-PAGE and fluorographed. Molecular weights (MW) are shown (in kilodaltons).
(B) Protease treatment (with Triton X-100 and proteinase K) of membrane homogenates from oocytes injected with α-gliadin mRNA (left four lanes) and membrane homogenates of noninjected oocytes to which purified α-gliadin was added (right two lanes).

hydrophobic interaction with the membrane because purified α-gliadin, added to a homogenate of noninjected oocytes, was sensitive to proteinase-K digestion even in the absence of the detergent (Figure 4B, right two lanes).

It has been shown that interaction among secretory proteins may play a role in their secretion. For instance, immunoglobulin heavy chains remain in the ER and are not secreted unless assembled with the light chain (Bole et al., 1986). To determine whether interaction between α-gliadins and γ-gliadins could affect their accumulation in the oocytes, the two mRNAs were coinjected into the oocyte. The results are shown in Figure 5. Accumulation of the α-gliadin in these oocytes far exceeded that of the γ-gliadin, indicating that each protein is routed independently.

Effect of Tunicamycin on Synthesis and Routing

Tunicamycin is an antibiotic that blocks *N*-linked glycosylation by inhibiting the formation of the donor sugar (Tkacz and Lampen, 1975). Tunicamycin treatment is also known to induce the synthesis of several ER proteins, such as the immunoglobulin heavy chain binding protein (BiP), which interacts with secreted proteins (Olden et al., 1979). Because wheat gliadins are not *N*-glycosylated (Shotwell and Larkins, 1988), it was interesting to test whether tunicamycin would affect the rate of secretion of the α-gliadins and γ-gliadins. Such an effect could only be attributed to induction of ER proteins by this compound. The results of tunicamycin treatment are shown in Figures 6A and 6B, lanes 3 and 4. Treatment with tunicamycin noticeably reduced the level of secretion of the γ-gliadin, compared with the control (Figure 6B, cf. lanes 1 and 2 and lanes 3 and 4). The effect of tunicamycin on the secretion of α-gliadin could not easily be determined because of the low level of α-gliadin secretion in untreated oocytes (Figure 6A, cf. lanes 1 and 2 and lanes 3 and 4). Tunicamycin had no major effect on the level of synthesis of either the α-gliadin or γ-gliadin. It is also interesting to note that tunicamycin had no effect on the apparent increase in molecular weight of α-gliadins in the medium (Figure 6A, lane 4). This result is expected because α-gliadins lack *N*-glycosylation sites.

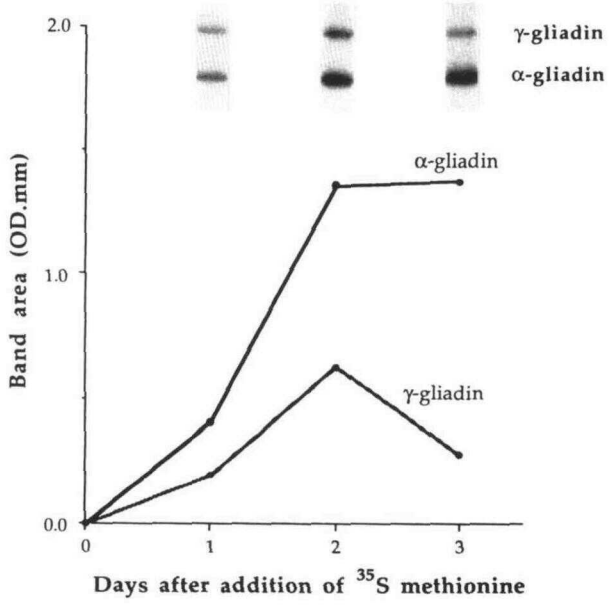

Figure 5. Accumulation of α-Gliadins and γ-Gliadins after Coinjection of Their mRNAs.

α-Gliadin and γ-gliadin SP6 mRNAs were injected together and oocytes processed after 1 day to 3 days incubation in ^{35}S-methionine. The results of fluorography of alcohol extracts are shown at the top. A plot of band intensity measured by an optical densitometer is shown below.

Effect of Monensin on Routing

The major effect of treatment of cells with monensin is to disrupt the Golgi apparatus. Monensin induces a dose-dependent reduction in the intracellular transport of newly synthesized secretory macromolecules. Nonsecreted materials accumulate in the Golgi apparatus. Moreover, longer exposure to monensin results in Golgi saturation and upstream accumulation of material in the ER (Ledger and Tanzer, 1984). The results of treatment of injected oocytes with monensin are seen in Figures 6A and 6B, lanes 5 to 8. Monensin did, indeed, block almost all secretion of γ-gliadin from the oocyte (Figure 6B, lane 6). The low secretion level of the α-gliadin made it difficult to detect quantitative effects of monensin (Figure 6A, lanes 5 to 8). The monensin sensitivity of the secretion of wheat γ-gliadins indicates that a conventional, Golgi-related pathway is involved in their secretion.

Folding of α-Gliadin and γ-Gliadin in the Oocyte

It was previously shown that correct formation of disulfide bonds within proteins including wheat γ-gliadins is catalyzed by an ER enzyme called protein disulfide isomerase (PDI) (Freedman, 1984; Bulleid and Freedman, 1988). This folding process plays an important role in protein secretion (Mains and Sibley, 1983; Bole et al., 1986; Gething et al., 1986). The possibility existed, therefore, that the retention of most of the α-gliadins within the oocytes might have been due to defective disulfide bond formation. To see whether both the α-gliadins and γ-gliadins, separately and in combination, were able to form intramolecular disulfide bonds inside the oocyte, the extracted proteins were fractionated on SDS-PAGE in the presence and absence of β-mercaptoethanol (β-ME). It is known that disulfide-bonded gliadins migrate faster in these gels than their reduced counterparts (Bulleid and Freedman, 1988). The results

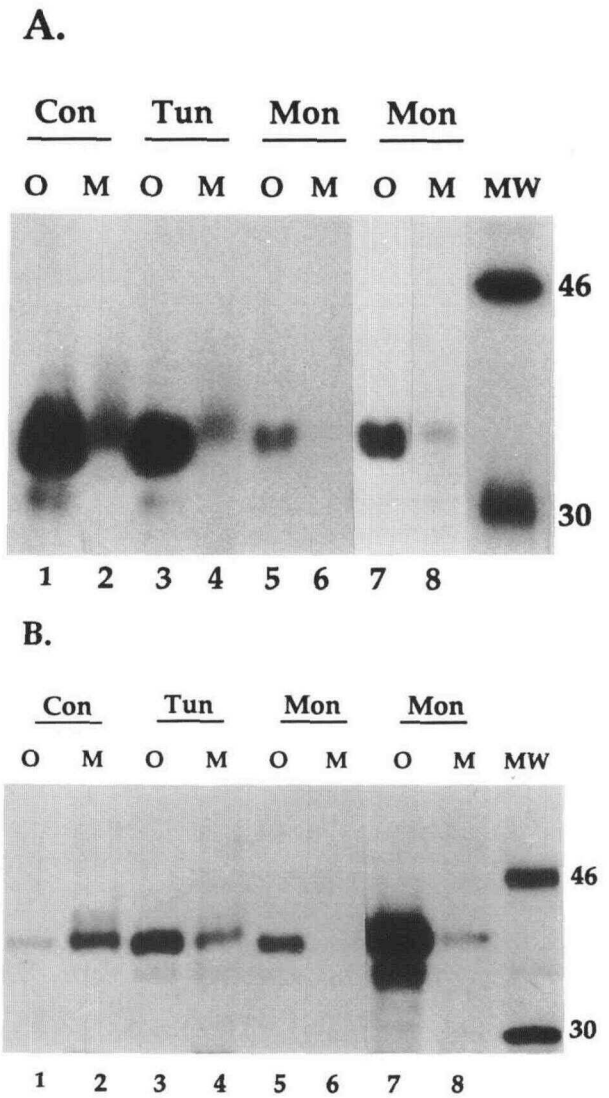

Figure 6. *Tunicamycin* and Monensin Treatment of Oocytes Injected with α-Gliadin or γ-Gliadin.

Oocytes were injected with α-gliadin or γ-gliadin and treated with tunicamycin or monensin as described in Methods. Oocytes and medium were incubated with ^{35}S-methionine for 48 hr and then harvested. Alcohol extracts of the homogenized oocytes (O) and medium (M) were analyzed by 10% SDS-PAGE and fluorographed.

(A) α-Gliadin.

(B) γ-Gliadin.

Tun, tunicamycin; Mon, monensin; Con, control. Lanes 7 and 8 represent overexposures of lanes 5 and 6. Molecular weights (MW) are shown (in kilodaltons).

are shown in Figure 7. The migration of α-gliadins and γ-gliadins extracted in the absence of β-ME was slightly faster than the migration of proteins extracted under reducing conditions, showing that intramolecular disulfide bonds were formed in both proteins.

DISCUSSION

Although wheat storage proteins have been studied extensively during the last several decades, their subcellular transport and deposition in protein bodies are still a matter of controversy. Recent electron microscopy studies have shown that the majority of wheat storage proteins are deposited in clusters of protein bodies that are localized in vacuoles (Kim et al., 1988). However, two different transport routes have been suggested: one is Golgi mediated and one bypasses the Golgi (Parker, 1982).

Unfortunately, the important question of whether all wheat storage proteins are transported by way of the Golgi cannot be studied directly in wheat endosperms because wheat prolamins are not glycosylated and, therefore, are not modified within the Golgi apparatus. More-

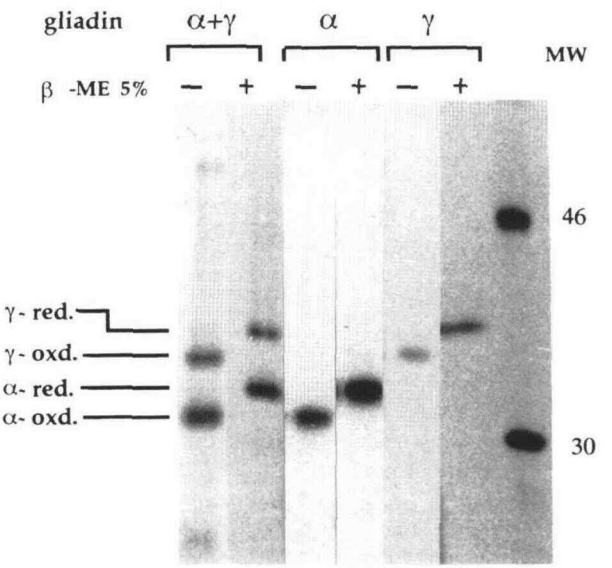

Figure 7. Folding of the α-Gliadins and γ-Gliadins within the Oocytes.

Oocytes were microinjected with α-gliadin, γ-gliadin, or α-gliadin and γ-gliadin SP6 mRNAs and labeled with ^{35}S-methionine for 48 hr. Alcohol-soluble gliadins were dissolved in SDS buffer containing (+) or lacking (−) β-ME and then fractionated on 10% SDS-PAGE. Detection of the radioactive bands was by fluorography. Molecular weights (MW) are shown (in kilodaltons). Red., reduced protein; oxd., oxidized protein.

over, it is very difficult to follow the dynamics of the transport of prolamins by examination of electron micrographs of developing wheat endosperms because the micrographs generally represent a steady-state situation in which the majority of the proteins are already localized within the vacuoles.

We selected *X. laevis* oocytes as a heterologous system to study wheat prolamin transport by way of the Golgi for three main reasons: (1) By using this system, we could study the transport of individual wheat prolamins, which we could not do with developing wheat endosperms; (2) *X. laevis* oocytes have been shown to perform correct subcellular transport for a variety of foreign animal and plant proteins, including proteins that remain within the RER and that are transported by way of the Golgi, indicating that the general process of protein transport has been highly conserved during evolution (Colman, 1984); and (3) one can follow Golgi-mediated transport of vacuolar proteins in *Xenopus* oocytes even if the proteins are not glycosylated. It is generally accepted that the transport of proteins from the RER occurs in several steps. First, the proteins are transported from the RER to the Golgi. This transport is critically dependent on structure, on correct folding of the secretory proteins, and on their interactions with integral ER proteins (for review, see Rose and Doms, 1988). In the Golgi apparatus, proteins are then sorted for transport to lysosomes or vacuoles or for secretion. Although transport of proteins from the Golgi to lysosomes or vacuoles requires specific peptide signals on the transported proteins, secretion occurs by a default pathway (for reviews, see Pfeffer and Rothman, 1987; Rothman, 1989). Because oocytes contain RER and Golgi apparati but lack vacuoles, it is expected that vacuolar proteins that are transported by way of the Golgi will be secreted eventually to the medium by a default pathway. Such proteins could be physically separated from proteins that are not transported to the Golgi and are localized in the RER within the oocytes.

Injection of the α-gliadin and γ-gliadin mRNAs into *Xenopus* oocytes led to relatively high levels of synthesis of the two proteins, which could be easily detected in fluorographs. Both proteins were inserted into the RER membranes and were folded by disulfide bond formation (a process that was previously shown for γ-gliadin to be catalyzed by the enzyme PDI; see Bulleid and Freedman, 1988). However, the subsequent subcellular transport of the α-gliadins and γ-gliadins was markedly different. The γ-gliadin was mostly secreted in a Golgi-mediated manner, which is sensitive to monensin treatment, whereas a very small amount of the α-gliadin reached the extracellular environment (Figures 2, 3, 5, and 6). In fact, about 95% of the α-gliadin was retained within the oocytes even 5 days after protein labeling, which was the maximal period tested (data not shown). Retention of most of the α-gliadin was not due to improper cleavage of its signal peptide and insertion into the RER membranes (Figure 4). It is, therefore, reasonable to assume that this retention is a function of the mature protein. We are currently investigating this hypothesis.

Although α-gliadin secretion was very slow, we believe that the protein was actively transported out of the oocytes and was not merely leaking into the medium. Besides the data showing recognition and cleavage of the α-gliadin signal peptide, there was additional evidence for active transport. We could not detect any protein in the medium of oocytes that were injected with mRNA of other nonsecreted proteins such as maize storage proteins (data not shown). Also, the fact that α-gliadin present in the medium was of higher molecular weight than α-gliadin in the oocytes indicates additional processing along the transport pathway.

The differences in transport of the two gliadins cannot be explained by their selective synthesis or degradation. *Xenopus* oocytes incorporate amino acids from the medium very rapidly, and the labeling periods of over 6 hr used in our study are considered a pulse-chase-type labeling (Colman, 1984). Therefore, one can follow the selective synthesis and degradation of proteins. We observed comparable levels of α-gliadins and γ-gliadins, and the amount of gliadin protein inside the oocyte was comparable with the amount of gliadin secreted into the medium. In addition, the medium contained a mixture of protease inhibitors that was previously shown to inhibit secreted oocyte proteases (Burmeister et al., 1984; see Methods).

The markedly different transport of the α-gliadins and γ-gliadins is particularly interesting because these closely related proteins possess close to 70% amino acid similarity and a very similar secondary structure configuration (Tatham and Shewry, 1985; Tatham et al., 1985). This finding shows that transport of the gliadins from the RER to the Golgi may be affected by small structural motifs that may be different in the α-gliadins and γ-gliadins. We are currently studying methods to identify these motifs. Our data (Figure 5) also show that α-gliadins and γ-gliadins are routed independently and do not seem to interact.

Several possibilities may explain the differential transport of the α-gliadins and γ-gliadins in the oocytes:

(1) Wheat prolamins are hydrophobic in nature. The differential transport of the α-gliadins and γ-gliadins could be caused by differences in their solubility in the RER, which is believed to be a high ionic environment.

(2) The α-gliadin may have assembled within the RER into large aggregates that cannot be transported to the Golgi. It has been previously shown that some wheat α-gliadins but not γ-gliadins can aggregate by way of noncovalent interactions (Kasarda, 1980).

(3) The transport of the α-gliadins and γ-gliadins could have been due to their differential recognition by ER proteins that play a role in their transport.

Although at present we cannot favor any of the above explanations for the differential transport of the α-gliadins

and γ-gliadins in the oocytes and there is no evidence for differential packaging of α-gliadins and γ-gliadins in wheat, we believe that these results are of potential significance and may provide some clues about protein body formation in wheat seeds. The results of this study support the previous suggestion of Parker (1982) that there is more than one subcellular route for wheat storage proteins. Although a large proportion of some proteins, mainly γ-gliadins, may be transported by way of the Golgi, a large proportion of other proteins, mainly α-gliadins, may aggregate within the ER and presumably do not reach the Golgi. The α-type proteins probably stay in ER-derived vesicles within the oocytes. In wheat, these proteins presumably aggregate into protein bodies within ER-derived vesicles and are then transported to vacuoles by a route that bypasses the Golgi.

Evidence suggesting the presence of more than one route for storage proteins has also been reported for other cereals. In oats, where the prolamins are also localized within vacuoles, electron micrograph studies have indicated that at least some of these proteins aggregate within the RER (Lending et al., 1989). Moreover, Saigo et al. (1983) noted the presence of direct connections between RER and smooth vacuolar membranes in electron micrographs of developing oat endosperms and speculated that a direct transport between the RER and vacuoles was a possible pathway for protein body formation in oats.

Because wheat gliadins are not glycosylated, our results with tunicamycin indicate that the subcellular transport of the γ-gliadin is controlled by cellular factors. Tunicamycin is known to induce ER proteins such as the glucose-regulated protein GRP-78 (Olden et al., 1979), also called BiP, which has been shown to interact with secreted proteins and also has been implicated in protein retention within the ER (Pelham, 1986). Although BiP has been identified in the ER of *Xenopus* oocytes (Ceriotti and Colman, 1988) and we have also recently detected it in developing wheat endosperm (S. Giorini and G. Galili, unpublished results), its role in the transport of wheat gliadins has yet to be demonstrated experimentally. It is important to note that the role of tunicamycin in secretion of nonglycosylated proteins is not general because tunicamycin was also shown to have no effect on the secretion of other nonglycosylated proteins, such as barley amylases, from *X. laevis* oocytes (Simon and Jones, 1988).

The α-gliadin in the medium migrated more slowly than that from the oocytes (Figure 2, lanes 3 and 4). The reason for this is not understood, but the alteration in molecular weight should have occurred close to or during secretion because the slow migrating band was never observed with α-gliadin extracted from the oocytes. The fact that the slower migrating α-gliadin was not isolated from the oocytes even after treatment with monensin (Figure 6A, lane 8) further demonstrates that the alteration of the protein is a late or post-Golgi event. As expected, this slowly migrating α-gliadin was not due to *N*-glycosylation, and tunicamycin did not prevent its formation (Figure 6A, lane 4). Because butanol and urea extraction had no effect on the migration of the α-gliadin, it is not likely to be due to noncovalent interactions with lipids or other compounds. This slowly migrating γ-gliadin may be due to *O*-glycosylation or to some other covalent interactions between this hydrophobic protein and oocyte factors that occur at the time of secretion. Whether a similar association of α-gliadin also occurs in wheat endosperm remains to be studied.

METHODS

Plasmid Constructions

To clone the α-gliadin gene behind the SP6 in vitro transcription promoter (Figure 1A), plasmid pW8233 (Rafalski et al., 1984) was digested with NcoI to release a 1024-bp fragment containing the α-gliadin coding sequence and 5′- and 3′-flanking sequences. The ends of the fragment were filled in using the Klenow fragment and the gene was inserted into the SmaI restriction site of pUC19. The resulting plasmid was cleaved with SacI 5′ to the gene and digested with Bal31 to remove an undesired upstream 5′ ATG. The digested α-gliadin gene was excised with BamHI and ligated to pSP65A (Galili et al., 1988) cleaved with SmaI and BamHI to form pSP65αA (Figure 1A). The size of the 5′ leader sequence was determined to be 48 bp by DNA sequence analysis.

For construction of pSP65γA (Figure 1B), the plasmid pW1621 (Sugiyama et al., 1986), containing a cloned wheat γ-gliadin gene, was digested with HindIII at a site located 105 bp downstream from the coding sequence. This end was filled in using the Klenow fragment. The 5′ end was then cleaved at an SnaBI site located 23 bp upstream to the coding sequence. The blunt-ended fragment was subcloned into the SmaI site of pSP65A, creating pSP65γA (Figure 1B). Previous DNA sequencing of pW1621 revealed that this γ-gliadin lacks 1 of the 8 conserved cysteines generally present in γ-gliadins. Resequencing pW1621 in our laboratory (Y. Altschuler, unpublished observation) has shown this result to be a sequencing error and that the γ-gliadin coded by pW1621 does possess 8 cysteine residues.

In Vitro Transcription and Translation

pSP65A plasmids containing gliadin genes were linearized by digestion with HindIII and capped mRNA was transcribed in vitro according to the method of Krieg and Melton (1984) using SP6 polymerase and the cap analog G(5′)ppp(5′)G. The reaction mix was incubated for 2 hr at 40°C and then extracted with phenol:chloroform before ethanol precipitation.

In vitro translation of the synthetic transcripts was obtained using a rabbit reticulocyte lysate system following the method of Giveon and Soreq (1984). The final concentrations of K^+ and Mg^{2+} were 100 mM and 1.45 mM, respectively.

Microinjection of Oocytes

Ovarian lobes were removed from *Xenopus laevis* females anesthetized by hypothermia. Oocytes were defolliculated by incubation with gentle shaking in OR2 containing 1 mg/mL collagenase

for 1 hr to 2 hr. Five to 10 stage VI oocytes were injected with 50 nL of mRNA at a concentration of 0.1 μg/μL with or without 40 μg/mL tunicamycin. Oocytes were then incubated in excess OR2 medium (Kawata et al., 1988) supplemented with 100 μg/mL penicillin and streptomycin and 2.5 mM pyruvate. In some experiments, tunicamycin was added to 2 μg/μL or monensin to 6 μM. Oocytes were incubated overnight to permit mRNA recruitment. Protein labeling was performed the next day by replacing the excess OR2 medium with 50 μL to 100 μL of the same medium per group of oocytes supplemented with 0.5 mCi/mL ^{35}S-methionine (>800 Ci/mmol), 2 mM 6-aminocaproic acid, 0.1 mM phenylmethylsulfonyl fluoride, 10 mg/mL lima bean trypsin inhibitor, and 0.5 mM leupeptin (Burmeister et al., 1984).

Analysis of Synthesized Proteins

Oocytes were homogenized in 50 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and incubated on ice for 5 min before centrifugation in an Eppendorf microcentrifuge at 4°C for 15 min. The supernatant was discarded and the pellet resuspended in 70% ethanol plus or minus 1% β-ME. After extraction at 60°C for 30 min, the suspension was centrifuged at room temperature for 15 min, and one-tenth of the supernatant was used for measuring TCA-precipitable counts (Giveon and Soreq, 1984). The remainder of the supernatant was acetone precipitated. The medium samples were immediately brought to 70% ethanol and the above procedure was followed. The acetone-precipitated pellets were dissolved in SDS sample buffer and run on 10% or 15% SDS-PAGE (Laemmli, 1970). The gels were fluorographed with EN^{3}HANCE (DuPont-New England Nuclear) according to the manufacturer's instructions.

Immunoprecipitation was carried out according to the method described in Colman (1984). Protein A-Sepharose was used instead of *Staphyllococcus aureus* envelopes.

Protease Treatment of Oocyte Membranes

Preparation of oocyte membrane homogenates and protease treatments were performed according to Wallace et al. (1988) using proteinase-K at a concentration of 0.5 mg/mL.

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