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The Rate of Phaseolin Assembly Is Controlled by the Glucosylation State of Its N-Linked Oligosaccharide Chains

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Many of the proteins that are translocated into the endoplasmic reticulum are glycosylated with the addition of a 14-saccharide core unit ($Glc_3Man_9GlcNAc_2$) to specific asparagine residues of the nascent polypeptide. Glucose residues are then removed by endoplasmic reticulum–located glucosidases, with diglucosylated and monoglucosylated intermediates being formed. In this study, we used a cell-free system constituted of wheat germ extract and bean microsomes to examine the role of glucose trimming in the structural maturation of phaseolin, a trimeric glycoprotein that accumulates in the protein storage vacuoles of bean seeds. Removal of glucose residues from the N-linked chains of phaseolin was blocked by the glucosidase inhibitors castanospermine and *N*-methyldeoxynojirimycin. If glucose trimming was not allowed to occur, the assembly of phaseolin was accelerated. Conversely, polypeptides bearing partially trimmed glycans were unable to form trimers. The effect of castanospermine on the rate of assembly was much more pronounced for phaseolin polypeptides that have two glycans but was also evident when a single glycan chain was present, indicating that glycan clustering can modulate the effect of glucose trimming on the rate of trimer formation. Therefore, the position of glycan chains and their accessibility to the action of glucosidases can be fundamental elements in the control of the structural maturation of plant glycoproteins.

INTRODUCTION

Translocation of nascent secretory polypeptides into the endoplasmic reticulum (ER) is often accompanied by the glycosylation of specific asparagine residues (N-glycosylation; Kornfeld and Kornfeld, 1985). This process occurs via the transfer of a preassembled oligosaccharide from a dolichol carrier to the side chain of an asparagine contained within the consensus sequence Asn-Xaa-Ser/Thr (where Xaa can be any amino acid other than proline). The composition of the oligosaccharide that is transferred to the acceptor asparagine ($Glc_3Man_9GlcNAc_2$) is conserved in evolutionarily distant organisms such as yeast, plants, and mammals (Staneloni et al., 1981; Kornfeld and Kornfeld, 1985; Herscovics and Orlean, 1993). Once attached to the polypeptide, this structure is modified by enzymes present in the different compartments of the secretory pathway. These modifications can give rise to a wide array of different structures, and the final outcome of the process depends on factors such as the interaction of the glycan with the protein matrix and the tissue- and organism-specific repertoire of modifying enzymes.

For a long time, it has been difficult to hypothesize a general function for N-glycosylation. N-linked glycans can affect protein solubility and stability and have been implicated in recognition events, but the degree of dependence from glycosylation is highly glycoprotein specific (Vitale et al., 1993; Helenius, 1994). Glycoprotein secretion can occur normally when glycan processing is inhibited (Lerouge et al., 1996), and the extensive modification of glycan chains that occurs in the Golgi apparatus is not even required for plant development and reproduction (von Schaewen et al., 1993). Therefore, it is clear that glycans often play a subtle role in the biosynthesis of plant glycoproteins.

The picture of the role of glycans is, however, changing thanks to the recent discovery of a link between the early processing events that occur in the ER and the function of ER resident proteins that are thought to assist with protein folding and assembly in this compartment (Helenius, 1994). The processing of the core oligosaccharide unit begins in the ER with the sequential removal of the three glucose residues: first, glucosidase I removes the outermost α-1,2–linked glucose, and then glucosidase II releases the two remaining α-1,3–linked glucose residues. Whereas the first of these α-1,3–linked glucose residues is rapidly cleaved, the resulting monoglucosylated oligosaccharide can persist for relatively long periods (Hori and Elbein, 1983; Suh et al., 1989). Both slow removal and glucose readdition are likely to contribute to the prolonged life of the monoglucosylated form. Indeed, analysis of the substrate specificity of glucosidase II

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from mung bean seedlings showed that $Glc_2Man_9GlcNAc_2$ is a better substrate than $Glc_1Man_9GlcNAc_2$ (Kaushal et al., 1990). In addition, a single glucose residue can be readded to unglucosylated chains by UDP-glucose:glycoprotein glucosyltransferase, an ER enzyme present in various organisms and tissues, including bean seeds (Parodi et al., 1984). An important feature of this enzyme is its ability to discriminate between native and unfolded proteins, the latter being much better substrates for the in vitro reglucosylation process (Trombetta et al., 1989; Sousa et al., 1992; Sousa and Parodi, 1995). The transient presence of monoglucosylated oligosaccharides is therefore a common feature in the biosynthesis of all glycoproteins, including those synthesized by plant cells.

Recent findings are starting to elucidate the role of these early modifications in the synthesis of glycoproteins. Although it appears that glucose trimming is not strictly required for glycoprotein secretion and cell viability (Stanley, 1984; Lerouge et al., 1996), the sequential removal of glucose residues from N-linked chains is a prerequisite for the correct folding of many glycoproteins (Helenius, 1994). In mammalian cells, glucose trimming regulates the interaction of newly synthesized polypeptides with two ER-located proteins (calnexin and calreticulin) that have a specificity for monoglucosylated oligosaccharides (Herbert et al., 1995; Peterson et al., 1995). Although the exact role of these proteins in protein folding and assembly has yet to be elucidated, it is likely that they function as molecular chaperones that specifically assist the biosynthesis of N-glycosylated proteins.

In this study, we examined the role of glucose trimming in the structural maturation of a plant glycoprotein. As a model system, we used phaseolin, the major storage protein in bean seeds. Phaseolin polypeptides are encoded by a small gene family and can be divided into two highly homologous classes, α and β (Slightom et al., 1985). Polypeptides belonging to both classes are synthesized as two glycoforms, bearing either one or two glycan chains (Bollini et al., 1983; Sturm et al., 1987b). The presence of two glycoforms is due to the inefficient utilization of one of the two potential glycosylation sites (Sturm et al., 1987b). Once translocated into the ER lumen, phaseolin polypeptides must fold and assemble in a trimeric structure before being transported to their final destination (Pedrazzini et al., 1994; Vitale et al., 1995). Glycosylation is not required for the assembly of phaseolin polypeptides but affects the rate at which trimers are formed. Analysis of the assembly state of newly synthesized polypeptides present in the ER of bean cotyledons shows that fully glycosylated polypeptides, bearing two oligosaccharide chains, assemble at a slower rate than do singly glycosylated or unglycosylated ones (Ceriotti et al., 1995a; Vitale et al., 1995). These differences in assembly rate could be due either to a direct effect of the glycan chains on the folding of the protein or to an indirect effect mediated by a differential interaction of phaseolin glycoforms with the ER folding machinery.

To gain insight into the glycan-mediated regulation of phaseolin assembly, we took advantage of the flexibility of an in vitro system that faithfully reproduces the folding and assembly processes. The results indicate that glucose trimming is a key event in the assembly of phaseolin polypeptides and that the observed differences in the rate of trimer formation are due primarily to the control exerted by a system that monitors the glucosylation state of newly synthesized proteins in the plant ER.

RESULTS

Translocation and Glycosylation of β-Type Phaseolin in Microsomes

We recently described a cell-free system that permits the study of early processing events involved in the synthesis of plant secretory proteins (Ceriotti et al., 1995b). The system consists of a wheat germ extract supplemented with microsomal membranes obtained from developing bean cotyledons. When phaseolin mRNA was translated in this system, we detected four major, labeled products in the 44- to 50-kD range after SDS-PAGE analysis (Figure 1A, lane 1). Their mobilities were consistent with the ones expected for (1) signal-processed, unglycosylated (β_0), (2) untranslocated (pβ, not clearly resolved from β_0), (3) singly glycosylated (β_1), or (4) fully glycosylated (β_2) phaseolin. The same four polypeptides were synthesized under several different translation conditions, although the ratio between them was dependent on a variety of factors, including the concentration of synthetic mRNA and the amount of microsomes added to the system (data not shown). Treatment of the translation reaction with proteinase K (Figure 1A, lane 2) resulted in the selective degradation of pβ polypeptides, whereas β_0, β_1, and β_2 polypeptides were protected from proteolytic attack. This demonstrates that β_0, β_1, and β_2 were translocated into microsomes. Proteinase K treatment in the presence of detergent resulted in the degradation of all polypeptides and the formation of a stable cleavage product of ~25 kD (Figure 1A, arrow). The fact that this product was not observed upon digestion in the absence of detergent indicates that it is derived from one or more of the translocated polypeptides.

To confirm that β_1 and β_2 polypeptides correspond to singly and fully glycosylated forms of phaseolin, translation was performed in the presence of the tripeptide (acetyl)-Asn-Tyr-Thr-(amide), which has been shown to compete for the transfer of oligosaccharide chains to potential glycosylation sites in dog pancreatic microsomes (Lau et al., 1983). Inclusion of the tripeptide resulted in the complete inhibition of the synthesis of β_1 and β_2 polypeptides (Figure 1B) and in the corresponding increase of the β_0 form (β_0 cannot be distinguished from pβ in Figure 1B, but the two bands can be distinguished in a shorter exposure of the same gel). Con-

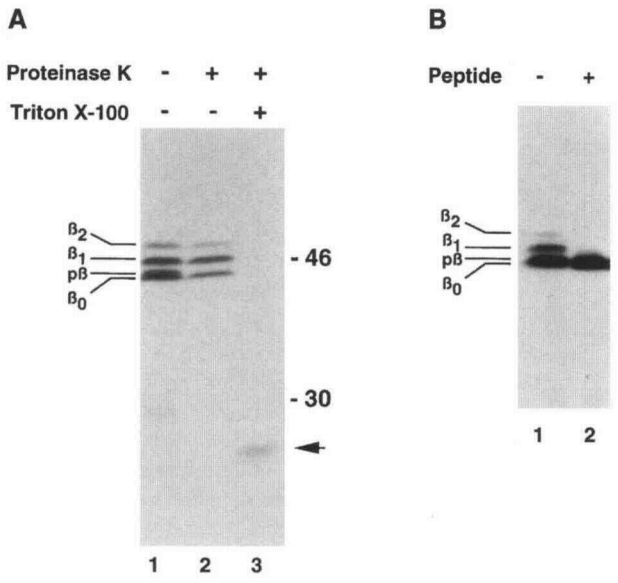

Figure 1. Translocation and Glycosylation of Phaseolin Polypeptides.

(A) Synthetic mRNA coding for β-type phaseolin was translated for 1 hr in the presence of microsomes. Oxidized glutathione was not included in the translation reaction. After translation, aliquots of the reaction were treated with (+) or without (−) proteinase K, either in the absence (−) or presence (+) of Triton X-100. Samples were analyzed by SDS-PAGE and fluorography. The arrow indicates the position of the protease-resistant fragment that is generated in the presence of proteinase K and Triton X-100. The positions of molecular mass markers in kilodaltons are indicated at right.

(B) Translation (25 μL) was performed as described in **(A)**, except that either 2 μL of DMSO (−, lane 1) or 2 μL of 2.5 mM (acetyl)-Asn-Tyr-Thr-(amide) peptide in DMSO (+, lane 2) was included in the translation reaction. Samples were analyzed by SDS-PAGE and fluorography.

version of β_1 and β_2 polypeptides to the β_0 form was also observed when translation products were treated with endo-β-*N*-acetylglucosaminidase H, an enzyme that removes high-mannose N-linked chains from glycoproteins (data not shown). Taken together, these data indicate that in the presence of microsomal membranes, a large fraction of the in vitro–synthesized phaseolin polypeptides is translocated into the microsomes, where the signal peptide is efficiently cleaved. The translocated protein is then in part glycosylated at either one or both of the potential glycosylation sites, giving rise to the two glycoforms that have been observed in vivo (Bollini et al., 1983).

Assembly of Phaseolin in Microsomes

We investigated whether the assembly of phaseolin polypeptides occurred in this in vitro translation system. Phaseolin mRNA was translated for 30 min in the presence of ^{3}H-leucine. An aliquot of the translation reaction was removed, and the rest was then incubated in the presence of unlabeled leucine. After solubilization of membranes, the translation products were separated by sedimentation velocity centrifugation in sucrose gradients. The distribution of monomeric and trimeric phaseolin in these gradients is well characterized (Ceriotti et al., 1991, 1995a; Vitale et al., 1995). Fractions recovered from the sucrose gradients were analyzed by SDS-PAGE and fluorography (Figure 2A). Even at the end of the pulse, the majority of the untranslocated phaseolin (pß) was recovered in the pellet fraction (P in Figure 2A), which indicates that the lack of translocation and/or the presence of the signal peptide is not compatible with proper folding of this protein. The size and the composition of these phaseolin-containing aggregates were not investigated further. Conversely, no translocated protein was found to aggregate and sediment at the bottom of the gradient. At the end of the pulse, unglycosylated, translocated phaseolin (β_0) was recovered mainly in the monomeric form. A fraction of these polypeptides assembled during the chase, confirming that the presence of glycan chains is not required for phaseolin trimerization. Although the assembly of β_1 polypeptides was completed within the first 30 min of the chase, trimerization of β_2 phaseolin occurred more slowly and was still incomplete at the end of a 90-min chase period. This marked difference in the assembly rate between β_1 and β_2 polypeptides closely resembles the situation found in vivo (Ceriotti et al., 1991; Vitale et al., 1995). The relatively slow assembly rate of β_0 phaseolin is most likely due to the presence of a transient folding defect that hampers the glycosylation and slows the assembly of these polypeptides (see below).

In vitro treatment of native, trimeric phaseolin with proteolytic enzymes results in the generation of polypeptides with a molecular mass of 20 to 30 kD. These polypeptides are resistant to further proteolysis (Deshpande and Nielsen, 1987). To establish whether the assembly of in vitro–synthesized polypeptides results in the formation of a nativelike structure, monomeric and trimeric phaseolin recovered from sucrose gradients were treated with trypsin and analyzed by SDS-PAGE. The results of this analysis are shown in Figure 2B. Monomeric and trimeric polypeptides clearly differed in their susceptibility to proteolysis. Although monomers were rapidly degraded, trimers were converted into fragments in the 23- to 24-kD range. These results suggest that in vitro–synthesized phaseolin is assembled in a trimeric structure that closely resembles the one generated in vivo.

No trimers could be detected if wild-type phaseolin was synthesized in the absence of microsomes or if the system was supplemented with a synthetic mRNA coding for a mutated phaseolin that we have previously shown to be assembly defective when expressed in tobacco protoplasts (data not shown). We conclude that folding and assembly of glycosylated phaseolin polypeptides are faithfully reproduced in vitro. Both singly and fully glycosylated polypeptides are

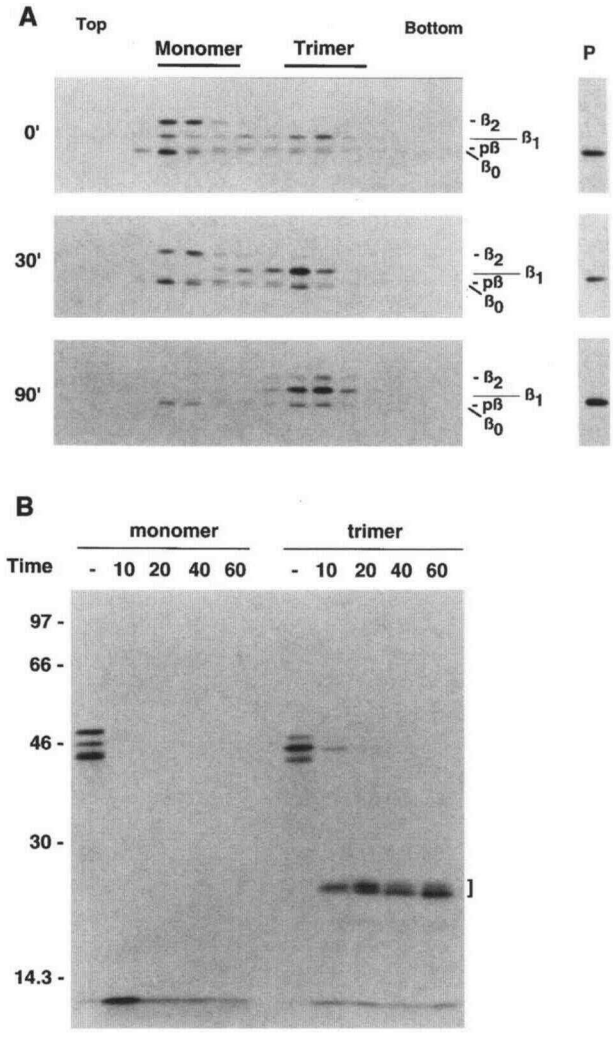

Figure 2. *Translocated Phaseolin Polypeptides Assemble into a Nativelike Trimeric Structure.*

(A) Synthetic mRNA coding for β-type phaseolin was translated in vitro in the presence of microsomes. After 30 min, the sample was chased with excess unlabeled leucine, and aliquots of the translation were taken at the indicated times (0, 30, and 90 min [0′, 30′, and 90′]). Translation products were then separated by sedimentation velocity centrifugation on sucrose gradients, and gradient fractions were analyzed by SDS-PAGE and fluorography. Only the central part of the gradient containing phaseolin polypeptides is shown. Top and bottom indicate the orientation in which fractions from the gradients were loaded on the gel. Material sedimented at the bottom of the gradient was recovered in SDS-PAGE sample buffer and identically analyzed (P). See Methods for details.

(B) Phaseolin polypeptides were synthesized in vitro and fractionated on sucrose gradients as described in **(A)**. Monomeric phaseolin from the 0 time chase sample and trimeric phaseolin from the 90-min chase sample were treated for the indicated time (minutes, as given above the gel) with 250 μg/mL trypsin and then analyzed by SDS-PAGE and fluorography. The bracket at right indicates the fragmentation products produced by digestion of trimeric phaseolin. The positions of molecular mass markers in kilodaltons are indicated at left.

assembly competent, and their relative rate of trimerization is consistent with the one observed in vivo.

Removal of Terminal Glucose Residues Occurs in Microsomes

We next decided to determine whether glycan processing was involved in phaseolin structural maturation. In living cells, the remodeling of glycan chains begins with the cleavage of three glucose residues. To establish whether removal of glucose residues occurs in isolated bean microsomes, phaseolin mRNA was translated in the presence of different concentrations of the glucosidase inhibitors castanospermine (CST) and *N*-methyldeoxynojirimycin (m-DNJ), and the translation products were analyzed by SDS-PAGE and fluorography. The inclusion of CST (Figure 3A) or m-DNJ (Figure 3B) had a clear effect on the mobility of phaseolin glycoforms; this is an indication that inhibition of glucose trimming had occurred. A series of partially trimmed products, migrating

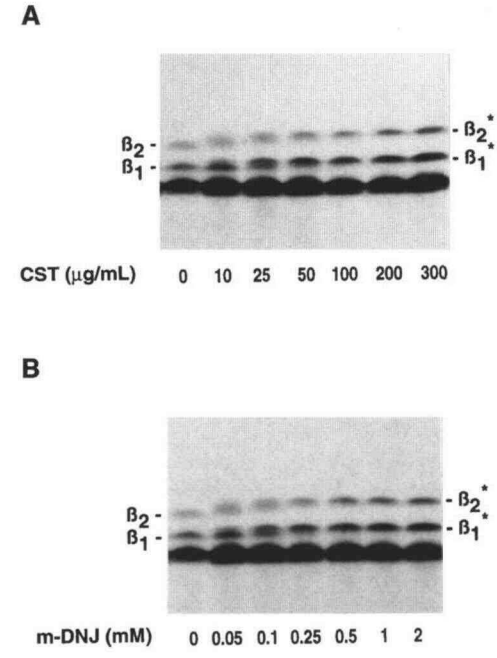

Figure 3. Inhibition of Glucose Removal from Phaseolin N-Linked Chains.

Phaseolin mRNA was translated for 2 hr in either the absence or presence of a glucosidase inhibitor. Translation products were analyzed by SDS-PAGE and fluorography. β_1* and β_2* indicate the positions of the untrimmed forms of singly glycosylated and fully glycosylated phaseolin, respectively.

(A) The indicated concentration of CST was included in the translation reaction.

(B) The indicated concentration of m-DNJ was included in the translation reaction.

as a diffuse band between the fully trimmed and the untrimmed forms, was evident in the case of β_2 polypeptides synthesized in the presence of intermediate concentrations of glucosidase inhibitors. The inclusion of 1-deoxymannojirimycin (DMM), a mannosidase inhibitor that is known to be inactive toward mung bean glucosidase I (Szumilo et al., 1986), did not have any effect on the mobility of phaseolin glycoforms, even when tested at a concentration (5 mM) that is known to be sufficient to completely inhibit the Golgi apparatus–mediated processing of phaseolin glycans in bean cotyledons (Vitale et al., 1989; data not shown in Figure 3, but see Figure 4A, lane 4). All of these inhibitors of glycan processing had a slight stimulatory effect on the incorporation of radiolabeled leucine.

These results indicate that the removal of glucose residues from newly synthesized phaseolin occurs in isolated bean microsomes. This observation is consistent with the reported ER localization of glucosidase activity in developing bean cotyledons (Sturm et al., 1987a).

Inhibition of Glucose Trimming Accelerates the Assembly of Phaseolin

To examine the role of glycan processing in the assembly of phaseolin, synthetic mRNA was translated for 1 hr in the absence or presence of various glycosidase inhibitors. The effect of CST on the assembly of a mutated phaseolin (Δ1-2) lacking both glycosylation sites was also analyzed. At the end of translation, one aliquot of the reaction was analyzed on SDS–polyacrylamide gels, whereas the rest was used to evaluate the state of assembly of phaseolin polypeptides by sedimentation velocity centrifugation in sucrose gradients.

The results of the analysis of total translation products are shown in Figure 4A. Inclusion of CST (200 μg/mL) or m-DNJ (2 mM) resulted in decreased mobility of β_1 and β_2 polypeptides, indicating that inhibition of glucose trimming had occurred (compare lane 1 with lanes 2 and 3 in Figure 4A). However, no effect on the mobility of phaseolin glycoforms was detected when DMM (5 mM) was present during translation (compare lanes 1 and 4 in Figure 4A). The two glycosylated phaseolin polypeptides were absent in the case of the glycosylation mutant, and the main translation product was a polypeptide corresponding to signal-cleaved unglycosylated phaseolin (Figure 4A, lanes 5 and 6).

The distribution of phaseolin polypeptides along sucrose gradients is shown in Figure 4B. In the absence of inhibitors, the assembly status of newly synthesized β_1 and β_2 polypeptides was markedly different. Although fully glycosylated polypeptides were recovered in monomeric form, singly glycosylated ones were completely assembled into trimers. The inclusion of DMM during translation did not affect the assembly status of β_2 phaseolin. Conversely, in the presence of the glucosidase inhibitors CST and m-DNJ, these polypeptides were recovered mainly in trimeric form at the end of the 1-hr translation period.

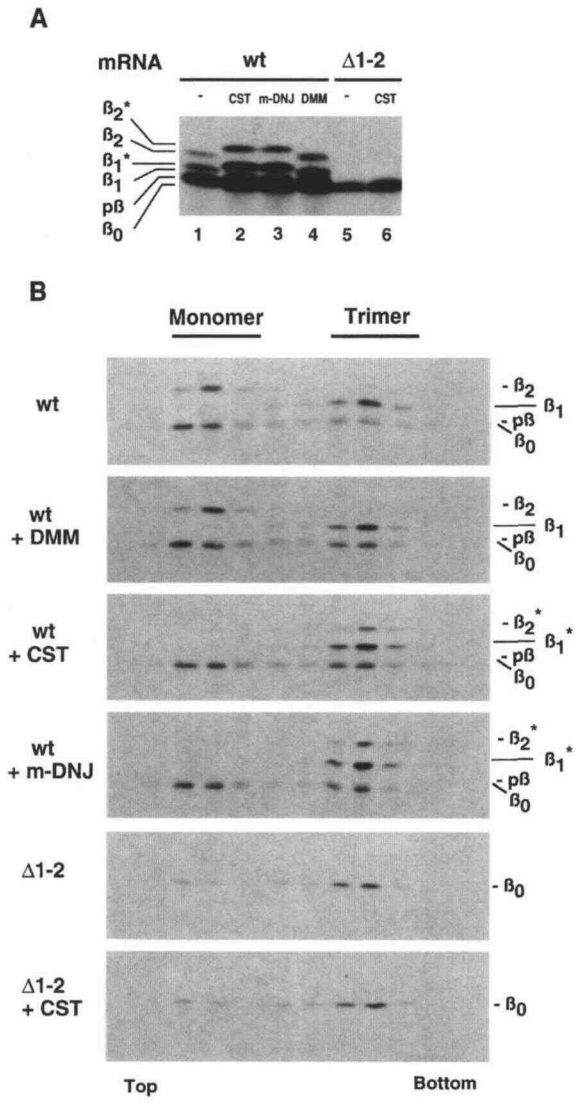

Figure 4. Assembly of Phaseolin Polypeptides in the Presence of Glycosidase Inhibitors.

Synthetic mRNAs coding for wild-type (wt) or mutated (Δ1-2) phaseolin were translated for 1 hr in the absence or presence of the indicated inhibitors of glycan processing: CST, 200 μg/mL; m-DNJ, 2 mM; DMM, 5 mM. β_1* and β_2* indicate the positions of the untrimmed forms of β_1 and β_2 phaseolin, respectively.

(A) Total translation products synthesized in the absence of glycosidase inhibitors (−, lanes 1 and 5) or in the presence of CST (lanes 2 and 6), m-DNJ (lane 3), or DMM (lane 4) were analyzed by SDS-PAGE and fluorography.

(B) Translation products synthesized in the absence or presence (+) of the indicated glycosidase inhibitors were fractionated by sedimentation velocity centrifugation on sucrose gradients. The gradients were fractionated from the top, and each fraction was analyzed by SDS-PAGE and fluorography. Top and bottom indicate the orientation in which fractions from the gradients were loaded on the gel.

Most of the Δ1-2 polypeptides were recovered in trimeric form both in the absence and presence of CST (Figure 4B). Also in the case of this mutant, the untranslocated polypeptides aggregated and sedimented at the bottom of the gradient (data not shown). The almost complete assembly of Δ1-2 phaseolin indicates that the lack of glycan chains per se does not hamper trimer formation. This is consistent with results from in vivo experiments (Ceriotti et al., 1995a; Vitale et al., 1995) and suggests that the concurrent lack of glycosylation and slow assembly of some of the in vitro–translocated wild-type polypeptides should be ascribed to their incorrect folding (see also Figure 2A). Indeed, the use of certain glycosylation sites has been shown to be dependent on the folding of the newly synthesized polypeptide (Holst et al., 1996).

The effect of CST on wild-type phaseolin was further investigated using a pulse–chase protocol. Radiolabeled phaseolin was synthesized for 30 min in either the absence or presence of CST. Unlabeled leucine was then added, and aliquots were taken at 0, 30, and 90 min of the chase. Translation products were analyzed either immediately or after fractionation by sedimentation velocity centrifugation in sucrose gradients. An analysis of total translation products (Figure 5A) shows that in the absence of CST, β_2 phaseolin undergoes a processing event that causes a small but reproducible increase in electrophoretic mobility (compare the mobility of β_2 in lanes 1 to 3 of Figure 5A). No change in migration during the chase was evident if CST was included in the reaction (Figure 5A, lanes 4 to 6).

The distribution of phaseolin polypeptides in sucrose gradients after sedimentation velocity centrifugation is shown in Figure 5B. The assembly of β_2 polypeptides was slow in the absence of CST but did occur between 30 and 90 min of the chase. Thus, assembly of trimers coincided with the mobility shift observed in Figure 5A. The inclusion of CST greatly accelerated the assembly of this phaseolin glycoform, and in its presence, trimerization was almost complete at the end of the first 30 min of the chase. Similarly, inclusion of CST in the translation mixture also stimulated the assembly of β_1, although the effect was not as pronounced as for β_2 (compare the distribution of β_1 in the 0′ −CST and 0′ +CST gradients of Figure 5B). Together, these results show that inhibition of glucose trimming accelerates the assembly of both phaseolin glycoforms. It is therefore likely that generation of polypeptides bearing partially or fully trimmed chains constitutes a rate-limiting step in the assembly of this glycoprotein.

Polypeptides with Partially Trimmed Chains Are Not Competent for Assembly

The effect of glucosidase inhibitors on the rate of phaseolin assembly suggests that once glucose trimming has begun, processing beyond a critical stage is required for the protein to proceed along the folding–assembly pathway. To test this

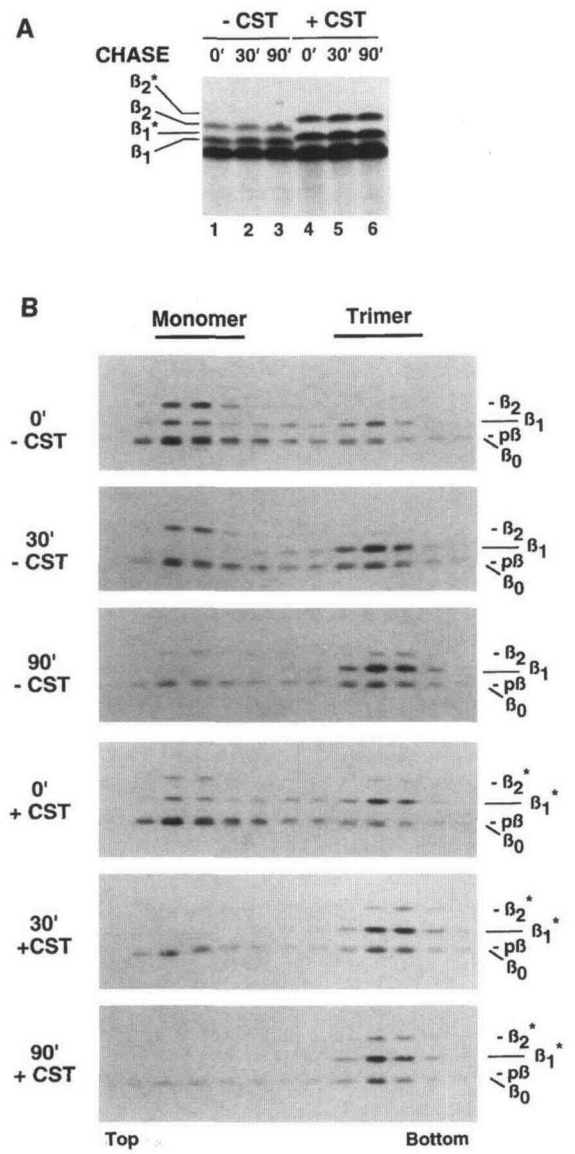

Figure 5. Pulse–Chase Analysis of Phaseolin Assembly in the Absence and Presence of CST.

Radiolabeled phaseolin was synthesized in vitro for 30 min in either the absence (−CST) or presence (+CST) of 200 μg/mL CST. The translation reaction was then chased with excess cold leucine, and aliquots were removed at 0, 30, and 90 min (0′, 30′, and 90′). β_1* and β_2* indicate the position of the untrimmed forms of β_1 and β_2 phaseolin, respectively.

(A) Translation products present at different times of chase were analyzed by SDS-PAGE and fluorography.

(B) The translation products were fractionated by sedimentation velocity centrifugation on sucrose gradients. Gradients were fractionated from the top, and polypeptides present in the gradient fractions were analyzed by SDS-PAGE and fluorography. Top and bottom indicate the orientation in which fractions from the gradients were loaded on the gel.

possibility, phaseolin mRNA was translated for 30 min in the absence of CST to allow some trimming of glucose residues to occur. At this point, unlabeled leucine was added, and the reaction mixture was divided into two parts. CST was added to one of the two parts to inhibit further glucosidase action. Samples were removed at different times and analyzed either directly or after fractionation in sedimentation velocity gradients.

The analysis of the total translation products is shown in Figure 6A. As previously shown in Figure 5A, β_2 was subjected during the chase to a processing event that resulted in a small increase in electrophoretic mobility (Figure 6A, lanes 1 to 3). This processing was blocked by CST (compare lane 3 with lanes 4 and 5 in Figure 6A), demonstrating that it indeed consists in the removal of one or more glucose residues.

The distribution of in vitro–synthesized polypeptides after sedimentation velocity centrifugation on sucrose gradients is shown in Figure 6B. The fraction of β_2 polypeptides that trimerized was clearly lower when CST was present during the chase (compare the distribution of β_2 in the 90′ −CST and 90′ +CST gradients of Figure 6B). Therefore, whereas β_2 assembly was accelerated when glucose trimming was not allowed to occur, the stabilization of one or more partially trimmed intermediates slowed trimer formation (compare the assembly state of β_2 in the 90′ +CST gradients at bottom in Figures 5B and 6B). The addition of CST at the end of the pulse had no discernible effect on β_1 mobility (Figure 6A) and did not inhibit subsequent assembly (Figure 6B).

To determine whether the time-dependent increase in mobility of β_2 polypeptides was somehow related to the trimerization process, the monomeric and trimeric forms of phaseolin recovered from sucrose gradients were run in adjacent lanes. The results presented in Figure 6C show that in the absence of CST, β_2 undergoes a small increase in electrophoretic mobility during the chase; they also reveal

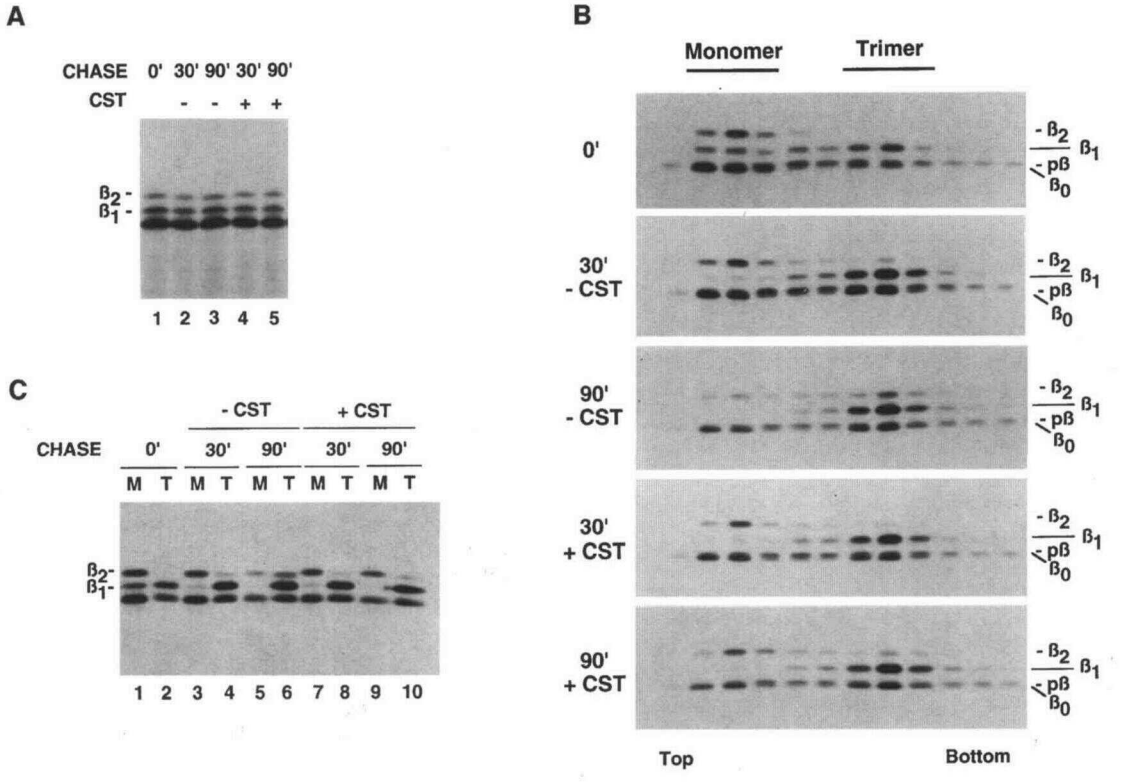

Figure 6. Post-Translational Addition of CST Blocks Phaseolin Assembly.

Radiolabeled phaseolin was synthesized in vitro for 30 min in the absence of CST (0′ chase), and the translation reaction was then chased for 30 or 90 min (30′ or 90′) in either the absence (−CST) or presence (+CST) of 300 μg/mL CST.

(A) Analysis by SDS-PAGE and fluorography of total translation products.

(B) In vitro–synthesized polypeptides were fractionated by sedimentation velocity centrifugation on sucrose gradients. Gradients were fractionated from the top, and fractions were analyzed by SDS-PAGE and fluorography. Top and bottom indicate the orientation in which fractions from the gradients were loaded on the gel.

(C) Monomeric (M) and trimeric (T) phaseolin from the sucrose gradients shown in **(B)** was analyzed by SDS-PAGE and fluorography.

that trimers contain only the fastest migrating form (lanes 1 to 6). When CST was included in the chase (Figure 6C, lanes 7 to 10), the processing as well as assembly of β_2 polypeptides were greatly inhibited. Therefore, β_2 assembly appears to be tightly linked to a processing event that involves the removal of one or more glucose residues. All together, these data indicate that once a partially glucose-trimmed form of monomeric β_2 phaseolin is generated, additional glucosidase action is required for trimer formation.

The Presence of Both Glycan Chains Determines the Slower Assembly of β_2 Polypeptides

In wild-type phaseolin, singly glycosylated β_1 polypeptides have a glycan at position 252, whereas β_2 polypeptides are glycosylated at Asn-252 and Asn-341 (Sturm et al., 1987b). The difference in the assembly rate between these glycoforms could be due either to the extra glycan at position 341 or to a cooperative action of the two oligosaccharide chains.

To distinguish between these possibilities, we examined the assembly of a mutated phaseolin (N252S) in which the site at position 252 was destroyed. These polypeptides can only be glycosylated on Asn-341, giving rise to a novel glycoform that differs from wild-type β_1 phaseolin in the position but not in the number of glycan chains. RNAs coding for wild-type or N252S phaseolin were translated for 30 min in the absence or presence of CST. The pattern of total translation products is shown in Figure 7A. Translation of N252S mRNA resulted in the synthesis of a single glycosylated polypeptide (β_1) in addition to untranslocated (pβ) and translocated but unglycosylated (β_0) products (Figure 7A, lane 4). Although pβ and β_0 polypeptides cannot be distinguished at the exposure shown in Figure 7A, examination of films exposed for a shorter period of time revealed that compared with the wild type, a larger fraction of translocated phaseolin is not glycosylated in the N252S mutant. This finding is consistent with the inefficient utilization of Asn-341 in native phaseolin (Sturm et al., 1987b). The slower mobility of glycosylated N252S phaseolin in the presence of CST confirms that glucose trimming also can be efficiently inhibited in the case of this mutant (compare lanes 3 and 4 in Figure 7A).

The oligomerization state of wild-type and N252S polypeptides was then analyzed by sedimentation velocity centrifugation on sucrose gradients (Figure 7B). Even in the absence of CST, some trimerization of β_1 polypeptides occurred both in the case of wild-type and N252S phaseolin. Conversely, β_2 polypeptides were fully recovered in monomeric form if CST was not present during translation. This indicates that both glycans must be present to determine the rate of assembly that was observed in the case of β_2 polypeptides.

After sedimentation velocity centrifugation, β_0 phaseolin was clearly separated from the untranslocated pβ polypeptides, which were almost completely recovered at the bottom of the gradient (not shown in Figure 7B, but see Figure 2). A much larger fraction of unglycosylated β_0 polypeptides was trimeric in the case of N252S phaseolin than in the case of the wild-type protein (compare the distribution of β_0 in the wild-type and N252S gradients without CST in Figure 7B). The correct folding and fast assembly of β_0 N252S polypeptides were expected, because for most of them, the lack of

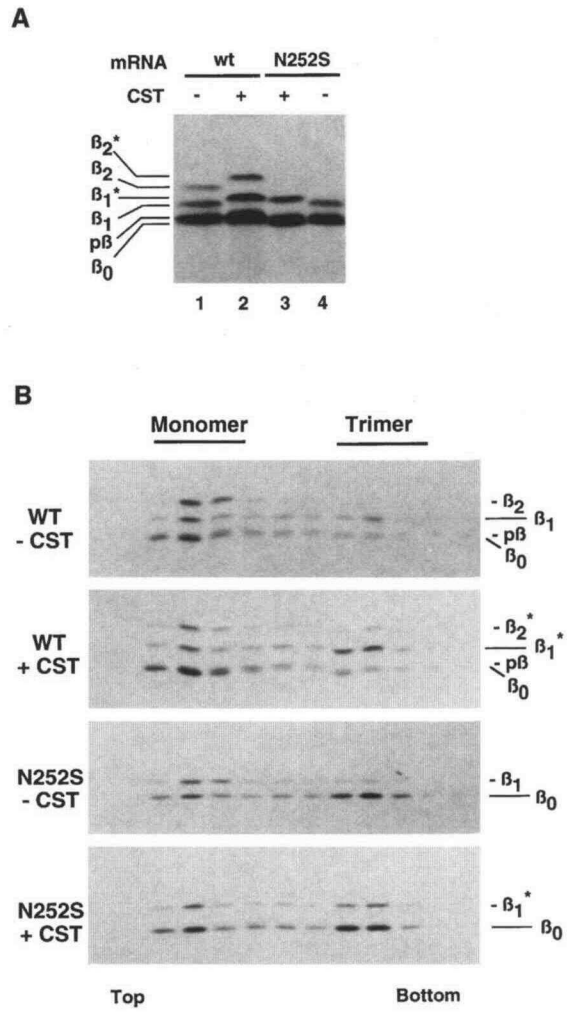

Figure 7. Role of Individual Glycans in Phaseolin Assembly.

Synthetic mRNAs coding for wild-type (wt) or N252S phaseolin were translated for 30 min in either the absence (−CST) or presence (+CST) of 200 μg/mL CST. β_1* and β_2* indicate the position of the untrimmed forms of β_1 and β_2 phaseolin, respectively.

(A) Analysis by SDS-PAGE and fluorography of total translation products.

(B) In vitro–synthesized polypeptides were fractionated by sedimentation velocity centrifugation on sucrose gradients. Gradients were fractionated from the top, and fractions were analyzed by SDS-PAGE and fluorography. Top and bottom indicate the orientation in which fractions from the gradients were loaded on the gel.

glycan chains was not due to misfolding but to the absence of the efficiently glycosylated site at position 252. Therefore, the effect of CST on correctly folded unglycosylated (β_0) and singly glycosylated (β_1) phaseolin can be directly compared in the N252S gradients of Figure 7B. The trimerization of the glycosylated but not of the unglycosylated N252S polypeptides was stimulated by CST, confirming that the effect of this drug is dependent on the presence of glycan chains. In addition, these results demonstrate that the assembly of the novel glycoform bearing a single glycan chain at position 341 is also controlled by the trimming of glucose residues.

DISCUSSION

Glucose Trimming Controls the Rate of Phaseolin Assembly

The main conclusions that can be drawn from this work concern the role of glucose trimming in the structural maturation of plant glycoproteins. We show that the rate of assembly of phaseolin polypeptides is markedly influenced by the glucosylation state of their oligosaccharide chains. The exact determination of the trimming step that regulates phaseolin assembly is hampered by the small amount of material that is synthesized in the cell-free system. However, previous studies have shown that the two outermost glucose residues of animal and plant glycoproteins are rapidly removed, whereas monoglucosylated oligosaccharides represent a relatively long-lived processing intermediate (Hori and Elbein 1983; Helenius, 1994). Therefore, it is also likely that in the cell-free system, the concerted action of glucosidase I and glucosidase II rapidly generates polypeptides bearing monoglucosylated oligosaccharides. In this model, trimming beyond the monoglucosylated state would be the major rate-limiting step in phaseolin assembly (Figures 8A and 8B). When glucose trimming is not allowed to occur (or when glycosylation is inhibited), the assembly of phaseolin is not slowed by the transient exposure of monoglucosylated chains, and trimer formation is faster (Figures 8C and 8D).

The block of assembly imposed by the presence of partially trimmed chains is most likely mediated by the interaction with ER folding factors. In mammalian cells, the glucosylation state of glycoproteins can affect the interaction of folding and assembly intermediates with the ER resident proteins calnexin and calreticulin (Hammond et al., 1994; Peterson et al., 1995), and recognition of monoglucosylated chains has been shown to be involved in substrate binding to calnexin (Herbert et al., 1995; Ware et al., 1995). According to the current model, the enzyme UDP-glucose: glycoprotein glucosyltransferase monitors the folding process and reglucosylates not-yet-folded polypeptides, thus extending their interaction with calnexin and/or calreticulin (Helenius, 1994). Indeed, the assembly of phaseolin appears to be subjected to a regulation similar to the one that operates in the case of influenza hemagglutinin, a viral membrane protein whose folding is assisted by these chaperones (Herbert et al., 1996).

Plant homologs of calnexin and calreticulin have been identified (Huang et al., 1993; Menegazzi et al., 1993; Chen et al., 1994; Denecke et al., 1995; Napier et al., 1995), and the presence of protein complexes containing calreticulin has been reported (Denecke et al., 1995). However, the role of calnexin and calreticulin in the structural maturation of plant glycoproteins has yet to be established, as well as an involvement of glycan chains in the interaction with other proteins.

A Molecular Basis for the Differential Trimming of Phaseolin Glycoforms

The stimulatory effect of CST on the assembly of both singly glycosylated and fully glycosylated polypeptides indicates that a similar mechanism must regulate the assembly of the two glycoforms. It is therefore likely that the faster assembly of β_1 polypeptides is due to faster trimming beyond the critical glucosylation state. This would also explain why the post-translational addition of CST can block the assembly of β_2 but not of β_1 polypeptides: if this latter glycoform is more rapidly converted beyond the critical stage of trimming, it can escape the block of assembly exerted by the post-translational addition of CST (Figures 8A and 8B).

Why should removal of glucose residues occur at different rates in the case of β_1 and β_2 polypeptides? Previous studies have shown that whereas the single glycan present on β_1 phaseolin is extensively modified during transit through the Golgi apparatus, the two glycans of β_2 polypeptides are not subjected to any Golgi-mediated processing event (Sturm et al., 1987b). Therefore, the glycan at position 252 becomes complex only when Asn-341 is unoccupied. An interaction between these two glycans is not surprising because the two glycosylated asparagine residues are not far from each other in the folded protein (Lawrence et al., 1990). If removal of glucose residues is similarly restricted when both glycans are present, this could justify a difference in the rate of trimming (and hence assembly) of β_1 and β_2 polypeptides. In an alternative explanation, processing of the glycan on Asn-341 would be intrinsically much slower than processing of the glycan on Asn-252. However, we believe this is unlikely because both singly glycosylated N252S phaseolin and wild-type β_1 polypeptides assemble at a faster rate than do β_2 polypeptides. An interference between the two oligosaccharide chains (rather than the presence of a glycan on Asn-341 per se) must therefore be at the basis of the slow assembly of fully glycosylated phaseolin.

Although our data strongly suggest that the presence of both glycans slows the rate of the final step of glucose trimming, other interpretations cannot be formally excluded. For instance, the presence of two glycans may be required for the stable interaction with a bivalent chaperone.

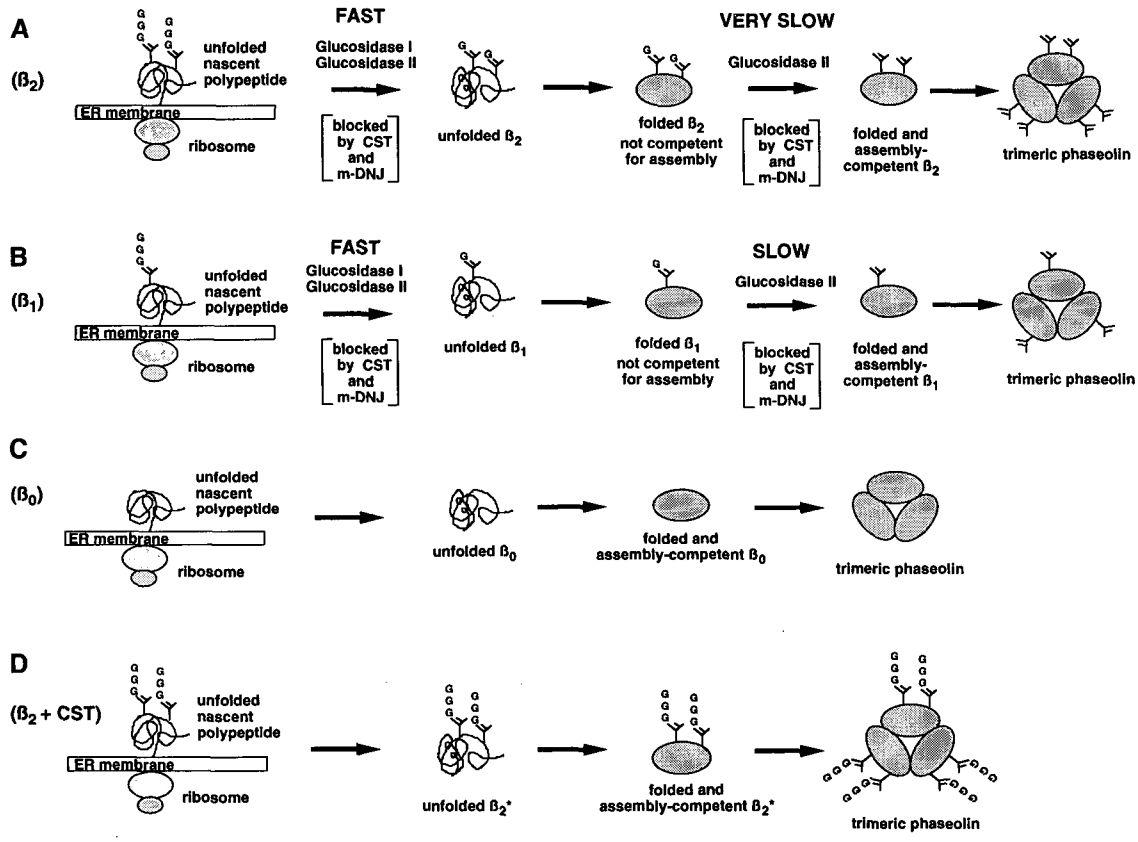

Figure 8. Model Illustrating the Steps Involved in the Assembly of Phaseolin Glycoforms.

The relative rates of the steps involving glucosidase action are indicated.

(A) A phaseolin polypeptide is translocated into the ER and glycosylated with two glycan chains, generating a β_2 polypeptide. Trimming of the first two glucose residues is fast and produces polypeptides bearing monoglucosylated chains. Although folding is rapid, assembly cannot occur until glucosidase II has removed the innermost glucose residue. Possibly, this step, which is intrinsically slow, is made even slower by the interaction between the two glycan chains. Polypeptides bearing unglycosylated chains can then assemble into trimers.

(B) A phaseolin polypeptide is translocated into the ER and glycosylated with a single glycan chain, generating a β_1 polypeptide. Subsequent steps are as in **(A)**, but removal of the last glucose is proposed to be not as slow as in the case of polypeptides bearing two glycan chains. The overall rate of assembly is faster than that which occurs in **(A)**.

(C) If both glycosylation sites are destroyed, unglycosylated polypeptides are synthesized (β_0). Once folding of the monomer is completed, assembly is fast because glucosidase action is not required to produce assembly-competent subunits.

(D) A phaseolin polypeptide is synthesized in the presence of CST, translocated into the ER, and glycosylated with two glycan chains. Because CST is present during and after translation, no glucose trimming occurs, and β_2* (untrimmed) polypeptides are generated. The innermost glucose is never exposed, and the assembly of folded subunits can occur without delay, as in the case of unglycosylated polypeptides.

G, glucose residue.

Other factors, apart from the rate of glucose trimming, are also likely to be involved in the regulation of phaseolin assembly. We have previously shown that monomeric phaseolin is found in association with the ER chaperone BiP and that the complex dissociates before or upon trimerization (Vitale et al., 1995). It should also be noted that inhibition of glucose removal greatly reduces but does not completely eliminate the difference between the rates of assembly of β_1 and β_2 phaseolin. This might reflect either an intrinsic difference in the folding of the two glycoforms or a residual ability of the ER folding machinery to discriminate between singly glycosylated and fully glycosylated polypeptides, even in the absence of the fundamental contribution provided by the exposure of critical glucose residues.

The Possible Roles of Glucose Trimming in the Structural Maturation of Phaseolin

Inhibition of glucose trimming often results in gross misfolding and retention in the ER (Helenius, 1994). However, the

folding of some glycoproteins is not absolutely dependent on the action of ER glucosidases (Vassilakos et al., 1996), and glucosidase-deficient cell lines have been isolated (Stanley, 1984). It has also been reported recently that glycoprotein secretion from plant cells is not affected by CST (Lerouge et al., 1996). Because incorrectly folded proteins are normally retained in the ER, this finding indicates that many plant glycoproteins can fold even in the presence of this drug. Therefore, it is apparent that the function of glucose residues can be at least in part superseded by other mechanisms that allow folding of glycoproteins to occur with an efficiency that is compatible with cell viability. Consistent with this view, the expression of the ER chaperone BiP is markedly increased in a glucosidase II–deficient cell line (Balow et al., 1995).

The significance of the glucose-mediated control of phaseolin assembly remains to be elucidated. Our results show that polypeptides bearing partially trimmed glycans are not competent for assembly. Therefore, the rate of glucose trimming might impose a certain time span during which the interaction of newly synthesized polypeptides with other ER proteins (including other subunits) is greatly restricted. During this period, intrasubunit folding would be allowed to occur without being disturbed by the interference of other proteins that are trying to reach their final conformation in the crowded environment of the ER lumen. Interaction between newly synthesized polypeptides might otherwise lead to aggregation and irreversible misfolding (Kiefhaber et al., 1991).

Phaseolin belongs to the class of proteins that can fold even when no glucose residues are removed from the glycan chains. However, trimming of phaseolin glycans might become important under certain physiological conditions. It has been shown that CST has a dual effect on the folding of influenza virus hemagglutinin in microsomes: the rate of this process is stimulated, whereas its efficiency is reduced (Herbert et al., 1996). The detrimental effect of CST on the efficiency of folding is amplified at relatively elevated temperatures. Therefore, it is possible that the trimming of glucose residues constitutes a key event in a folding pathway that although normally dispensable, becomes important when the establishment of stress conditions would otherwise lead to an impairment of phaseolin structural maturation. An exhaustive analysis of phaseolin assembly in microsomes under different conditions (temperature, energy charge, redox potential, and the like) should help to clarify the role of early glycan processing in the structural maturation of plant glycoproteins.

METHODS

Materials

Developing cotyledons of common bean (*Phaseolus vulgaris* cv Greensleeves) at midmaturation stage were obtained from plants grown in a greenhouse under a 12-hr-light/12-hr-dark cycle. The cell-free translation system (wheat germ extract, amino acid mixture lacking leucine, KOAc, and RNasin) was purchased from Promega (Madison, WI). L-4,5-^{3}H-leucine (4.4 to 7.0 Tbq/mmol and 185 MBq/mL) was obtained from Amersham International. Oxidized glutathione was purchased from Fluka (Buchs, Switzerland). Castanospermine (CST) and 1-deoxymannojirimycin (DMM) were purchased from Boehringer Mannheim. *N*-methyldeoxynojirimycin (m-DNJ), trypsin, and soybean trypsin inhibitor were purchased from Sigma. Proteinase K was from Bethesda Research Laboratories. (Acetyl)-Asn-Tyr-Thr-(amide) peptide was a kind gift from Glenn Matthews (University of Birmingham, UK).

Site-Directed Mutagenesis

Standard molecular cloning techniques were used for DNA manipulation (Sambrook et al., 1989). Glycosylation sites (Asn-252-Leu-Thr-254 and Asn-341-Phe-Thr-343) were mutagenized, converting residue 252 from Asn (AAC) to Ser (TCC) and residue 343 from Thr (ACT) to Phe (TTT), generating pSP6N252S and pSP6T343F, respectively. Site-directed mutagenesis was performed using the method of Kramer et al. (1984) with a Boehringer Mannheim site-directed mutagenesis kit and the mutagenic oligonucleotides TCTCAGTCAGGGATCCAAATTCG and TACCGAAACCAAAGAAATTCACG. The two mutations were then combined by replacing a HindIII-StyI fragment of pSP6T343F with the equivalent fragment of pSP6N252S, thereby generating pSP6Δ1-2.

In Vitro Transcription

Synthetic mRNAs coding for wild-type phaseolin or glycosylation mutants were produced by in vitro transcription of pSP6βPHSLwt (Ceriotti et al., 1991), pSP6N252S, and pSP6Δ1-2. In vitro transcription of linearized plasmids was performed as previously described (Ceriotti et al., 1995b).

In Vitro Translation and Analysis of Translation Products

Nuclease-treated microsomes were prepared from developing bean cotyledons, as previously described (Ceriotti et al., 1995b). Translation of synthetic mRNA was performed using the following translation mixture: 25 μL of wheat germ extract, 1 μL of RNasin (40 units per mL), 4 μL of 1 mM amino acid mixture without leucine, 3.2 μL of 1 M KOAc, 4.8 μL of L-4,5-^{3}H-leucine, 4 μL of 75 mM oxidized glutathione, 4 μL of nuclease-treated microsomes (50 A_{280} units per mL), and 4 μL of synthetic mRNA (50 ng/μL). Translations were performed at 25°C. For the chase, 4 μL of 10 mM leucine was added. Protease protection of translocated products and enzymatic deglycosylation were performed as described previously (Ceriotti et al., 1995b). For sucrose gradient analysis, an aliquot of the translation reaction was diluted eightfold with ice-cold dilution buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 6 mM $MgCl_2$, 3 mM ATP, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride), incubated for at least 30 min on ice, and loaded on a 12-mL 5 to 25% linear sucrose gradient (Ceriotti and Colman, 1990). Gradients were spun at 39,000 rpm for 20 to 22 hr at 20°C in a SW40 rotor (Beckman Instruments, Fullerton, CA) and fractionated from the top using an Auto Densi-Flow apparatus (Labconco, Kansas City, MO). Material sedimented at the bottom of the gradient was directly recovered in SDS–PAGE

sample buffer. Aliquots from the translation reaction, from each gradient fraction, and from the pellet were then analyzed by SDS-PAGE (15% acrylamide and 0.075% bisacrylamide in the separating gel) using the system of Laemmli and Favre (1973). Gels were treated for fluorography as described by Bonner and Laskey (1974).

Analysis of the Protease Sensitivity of Phaseolin Molecules

In vitro–synthesized phaseolin was fractionated on sucrose gradients, and aliquots of the gradient fractions were treated in the presence of trypsin (250 μg/mL) at 37°C. Digestion was terminated at different times by the addition of 500 μg/mL soybean trypsin inhibitor. Untreated and trypsin-treated samples were then analyzed by SDS-PAGE and fluorography.

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