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Plant and mammalian sorting signals for protein retention in the endoplasmic reticulum contain a conserved epitope

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We studied protein sorting signals which are responsible for the retention of reticuloplasmins in the lumen of the plant endoplasmic reticulum (ER). A non-specific passenger protein, previously shown to be secreted by default, was used as a carrier for such signals. Tagging with C-terminal tetrapeptide sequences of mammalian (KDEL) and yeast (HDEL) reticuloplasmins led to effective accumulation of the protein chimeras in the lumen of the plant ER. Some single amino acid substitutions within the tetrapeptide tag (-SDEL, -KDDL, -KDEI and -KDEV) can cause a complete loss of its function as a retention signal, demonstrating the high specificity of the retention machinery. However, other modifications confer efficient (-RDEL) or partial (-KEEL) retention. It is also shown that the efficiency of protein retention is not significantly impaired by an increased ligand concentration in plants. The efficiently retained chimeras (-KDEL, -HDEL and -RDEL) were shown to be recognized by a monoclonal antibody directed against the C-terminus of the mammalian reticuloplasmin protein disulfide isomerase (PDI). The recognized epitope is also present in several putative reticuloplasmins in microsomal fractions of plant and mammalian cells, suggesting that the antibodies recognize an important structural determinant of the retention signal. In addition, data are discussed which support the view that upstream sequences beyond the C-terminal tetrapeptide can influence or may be part of the structure of reticuloplasmin retention signals.

Key words: endoplasmic reticulum/epitope/passenger protein/retention signal/reticuloplasmin

Introduction

The endoplasmic reticulum (ER) of eukaryotic cells is the site of synthesis, maturation and assembly of both secreted proteins and those which remain in the endomembrane system. The major components of the ER lumen are a set of soluble proteins, collectively referred to as the reticuloplasmins (Koch, 1987; Macer and Koch, 1988). Maintenance of these proteins in the ER is a signal mediated process (Munro and Pelham, 1987) which separates them from the secretory proteins within the bulk flow (Wieland *et al.*, 1987) of the endomembrane system. The highly conserved sequence Lys-Asp-Glu-Leu (KDEL) present at the C-terminus of mammalian reticuloplasmins was shown to be necessary for their retention and appears to be sufficient to reduce the secretion of several secretory proteins (Munro and Pelham, 1987; Zagouras and Rose, 1989). A minor change in this sequence results in complete loss of retention (Zagouras and Rose, 1989) which suggests that a highly specific receptor is present. However, a number of closely related sequences are present at the C-terminus of reticuloplasmins and were shown to cause retention of modified passenger proteins (Mazzarella *et al.*, 1990; Andres *et al.*, 1990, 1991; Haugejorden *et al.*, 1991).

It has recently been proposed that retention of reticuloplasmins could depend on two complementary processes in mammalian cells (Booth and Koch, 1989). The first mechanism would involve a higher order structure within the ER lumen. Reticuloplasmins would form a gel-like matrix in combination with calcium ions within the ER lumen, surrounding a fluid phase that would be composed mainly of non-resident proteins. Vesicles that bud off the ER would preferentially contain the fluid phase, thus preventing excessive losses of reticuloplasmins by bulk flow. The signal mediated pathway (Munro and Pelham, 1987) could mediate the 'salvage' of the escaping reticuloplasmins within these vesicles, thus increasing the efficiency of the retention machinery.

Accumulating evidence strongly argues against the hypothesis of an ER resident membrane bound KDEL receptor. First, the high concentration of soluble reticuloplasmins within the lumen of the ER (Koch, 1987) implies that no ER membrane protein would be sufficiently abundant to function as an immobile KDEL receptor. It was also shown that the mobility of the luminal binding protein (BiP) in the ER lumen is not influenced by its KDEL sequence (Ceriotti and Colman, 1988). This indicates that reticuloplasmins do not associate with the ER membrane and can diffuse freely within the lumen of the ER. It has therefore been suggested that reticuloplasmins are continuously retrieved from a post-ER 'salvage' compartment (Munro and Pelham, 1987) to prevent excessive losses by passive migration. This was supported by targeting experiments with a lysosomal protein that was modified by the addition of the C-terminal KDEL sequence. The chimeric protein was efficiently retained in the ER but continued to be modified by an enzyme which is thought to be present in the first post-ER compartment (Pelham, 1988). The proposed mechanism requires a receptor which binds KDEL with high affinity in a salvage compartment and with low affinity in the ER. Rapid recycling of a receptor between the two compartments would guarantee the retention of proteins which are far more abundant than the receptor itself (Kelly, 1990). A putative receptor was recently identified in the salvage compartment

using anti-idiotype antibodies that mimic a KDEL-like structure (Vaux *et al.* 1990).

A retention signal similar to that described for mammalian cells has been identified and characterized in the yeast *Saccharomyces cerevisiae*, but the first amino acid of the tetrapeptide is histidine rather than lysine (Pelham *et al.*, 1988). The yeast protein retention system appears to be less efficient and more easily saturated by increased amounts of ligands than the mammalian retention machinery (Pelham *et al.*, 1988; Lewis *et al.*, 1990). Signal recognition is most probably highly specific in both systems since the yeast sequence does not confer retention in mammalian cells and vice versa (Pelham *et al.*, 1988). Two ER retention defective (ERD) yeast mutants have been isolated and the corresponding genes have been cloned (Hardwick *et al.*, 1990; Semenza *et al.*, 1990). One of these genes, *ERD2*, was shown to affect both the specificity and the capacity of the luminal ER protein retention system (Lewis *et al.*, 1990). An *ERD2*-like gene was isolated from human cells (Lewis and Pelham, 1990) but direct evidence for the presence of a KDEL binding site on the corresponding gene product is lacking. Moreover, the gene product appears to be clearly distinct from the previously proposed mammalian salvage receptor (Vaux *et al.*, 1990). It remains to be shown whether eukaryotic cells contain several distinct salvage receptors with similar functions, or whether both types of proteins are needed to form a functional salvage receptor (Warren, 1990).

In contrast to mammalian cells, very little is known about protein transport in the endomembrane system of plants. The presence of a default pathway for protein secretion in plants has been demonstrated (Denecke *et al.* 1990) but discrete targeting signals for intracellular locations within the endomembrane system have only been studied for vacuolar proteins (reviewed by Chrispeels, 1991). Several genes coding for proteins that reside in the lumen of the plant ER have been cloned and sequenced. The putative auxin receptor of maize (Hesse *et al.*, 1989; Inohara *et al.*, 1989; Tillman *et al.*, 1989) and sulfhydryl endopeptidases of *Vigna mungo* (Akasofu *et al.*, 1989) and *Phaseolus vulgaris* (Tanaka *et al.*, 1991) contain the C-terminal KDEL peptide. In contrast, the tobacco homologue of the mammalian luminal binding protein (BiP) contains the tetrapeptide HDEL (Denecke *et al.*, 1991). The introduction of the KDEL sequence to a vacuolar glycoprotein led to the detectable accumulation of the mutant protein in the nuclear envelope of cells from transgenic tobacco seeds (Herman *et al.*, 1990). However, most of the detected protein was present in the storage vacuoles and it was not clear whether the remaining fraction was retained due to malfolding of the modified protein or due to the specific impact of a functional retention signal.

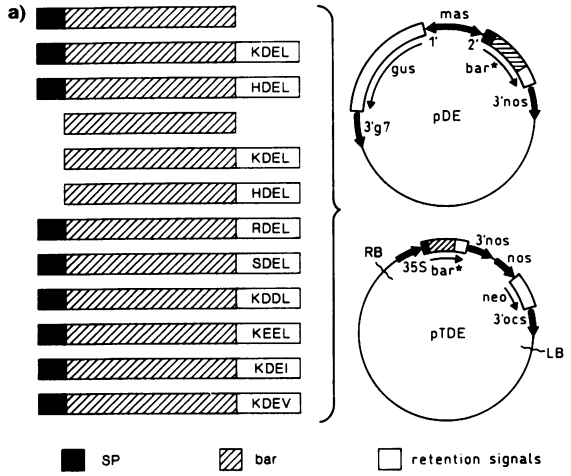
This work describes a functional analysis of ER retention signals in plants using a passenger protein that can be characterized enzymatically in order to exclude possible malfolding. It is shown that protein retention in the plant ER is mediated by C-terminal sequences which give rise to a common structural determinant as determined by monoclonal antibodies. The fact that this epitope is conserved in several abundant microsomal proteins of plants and mammals as well as the observation that the retention mechanism cannot easily be saturated by increased amounts of heterologous ligands suggests that there is a high similarity between the plant and mammalian protein retention machinery.

Results

Phosphinothricin acetyl transferase (PAT) is a suitable passenger protein for the analysis of retention signals

For the functional analysis of ER retention signals in plant cells, a model system was required which would allow a specific function in protein retention to be assigned to a small C-terminal part of a protein. Since transport of proteins in the endomembrane system is known to be influenced by the state of folding and the overall structure of the proteins, targeting experiments using a passenger protein should allow the impact of a specific signal to be distinguished from changes in the overall protein structure that result from the modification.

The enzyme phosphinothricin acetyl transferase (PAT) was used as a passenger molecule since it has been demonstrated that PAT hybrid proteins containing C-terminal fusions remain enzymatically active (Botterman *et al.*, 1991). This suggests that the PAT C-terminus is an extended structure and we assumed that addition of tetrapeptides to the C-terminus of this protein would not disturb its general three-dimensional structure. Indications for this were obtained by demonstrating that enzymatic parameters for the tetrapeptide-tagged PAT enzymes remain unaffected (see Materials and methods). Furthermore, PAT is secreted from tobacco



b)

PAT	HDVGFWQLDFSLPVPPRPVLPVTEI**KDEL**
BLP4	PIITAVYQKSGGAPGGESGASEDDD**HDEL**
ABP1	DWSMPHTAAVLKFPFVWDEDCFEAA**KDEL**
SH-EP	CGIAMMASYPIKNSSDNPTGSLSS**PKDEL**

Fig. 1. Schematic representation of chimeric gene constructions with signal sequences and retention signals. (**a**) The fragment encoding the PR1b signal peptide (Denecke *et al.*, 1990) is presented in black, the *bar* sequence encoding phosphinothricin acetyl transferase (PAT) is shaded and the different tetrapeptide sequences are indicated in the single letter code. The chimeric structural *bar* gene in the plasmids pDE and pTDE is represented by bar*. The coding regions of the β-glucuronidase and neomycin phosphotransferase II genes are indicated by 'gus' and 'neo'. The promoters and their direction of transcription are represented by heavy arrows. 'mas' represents the dual mannopine synthase promoter (1' and 2'), 'nos' represents the nopaline synthase promoter and 35S the CaMV 35S promoter. The 3' untranslated regions from the T-DNA gene 7 (3'g7), nopaline synthase (3'nos) and octopine synthase (3'ocs) are indicated by heavy arrows downstream of the coding regions. RB and LB represent the right and left T-DNA borders. (**b**) C-terminal amino acid sequence from PAT-KDEL, tobacco BiP (BLP4), the maize auxin binding protein (ABP1) and the mung bean sulfhydril endopeptidase (SH-EP). The C-terminal tetrapeptides and the proline residues are indicated in bold.

protoplasts if targeted to the ER lumen (Denecke *et al.*, 1990). A protein that is secreted by default offers an ideal model system for studying transport signals since it is very unlikely to contain competing targeting information.

A schematic overview of chimeric structural genes, coding for several PAT derivatives, is shown in Figure 1. The *bar* coding region was modified by additing a sequence encoding a signal peptide at the 5′ end (Denecke *et al.*, 1990) and by inserting several tetrapeptide-encoding DNA fragments between the last codon and the stop codon. The resulting C-terminal amino acid sequence contains four additional amino acids immediately downstream of the last residue (Ile) of PAT (Figure 1b). These coding regions (*bar**) were inserted downstream of the TR-DNA derived *mas* 2′ promoter and provided with the 3′ non-coding region of the nopaline synthase gene (3′nos). The plasmids for transient gene expression designated as pDE also contain an internal marker gene coding for cytoplasmic β-glucuronidase (GUS) which is used to determine the efficiency of DNA transfer and the percentage of non-specific leakage of proteins. The latter is due to mechanical disruption of a significant fraction of the protoplasts during the cell culture after the gene transfer (Denecke *et al.*, 1990). The vectors for *Agrobacterium tumefaciens* mediated plant transformations (pTDE) contain the chimeric structural genes coding for the different PAT derivatives under control of the CaMV 35S promoter and a kanamycin resistance gene as a dominant marker for the selection of stable transformants (Figure 1). The different *bar** structural genes were also inserted in vectors that allow the regulated expression in *Escherichia coli* and *S.cerevisiae* (see Materials and methods).

Yeast and mammalian ER retention signals prevent the secretion of PAT in tobacco protoplasts

We analysed whether the C-terminal sequences of mammalian (KDEL) or yeast (HDEL) reticuloplasmins can reduce the rate of PAT secretion in tobacco leaf protoplasts using a transient expression system (Denecke *et al.*, 1990). The intra- and extracellular protein levels of PAT and its modified forms were analysed relative to the levels of an internal marker in the cell suspensions 30 h after electric field mediated DNA transfer. Osmotic lysis of protoplasts was used to fractionate the intracellular portion partially into a cytoplasm-enriched fraction and a microsomal-enriched fraction. Figure 2 illustrates that the presence of these tetrapeptides at the PAT C-terminus results in reduced PAT secretion into the culture medium (CM). This is accompanied by an accumulation of PAT derivatives in the membrane-enriched fraction (ME) and an overall increase in the total amount of the modified marker proteins compared with the secreted form (compare lanes ssPAT with ssPAT-KDEL and ssPAT-HDEL). The increased levels of the retained PAT derivatives are unlikely to be the result of a higher protein synthesis rate, since the tetrapeptides have no influence on the synthesized PAT levels when the signal sequence for translocation is absent (compare lanes PAT with PAT-KDEL and PAT-HDEL).

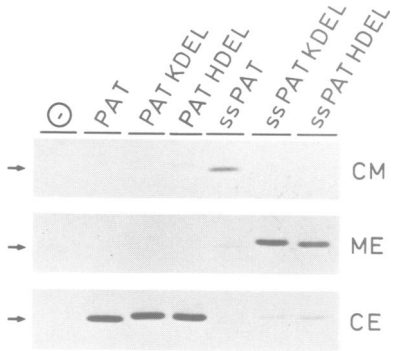


Fig. 2. Intra- and extracellular localization of PAT derivatives in electroporated protoplast suspensions. Western blot of concentrated culture medium (CM), membrane enriched (ME) and cytoplasmic enriched (CE) cell fractions using a polyclonal antiserum directed against PAT. Identical proportions of each fraction were loaded. The negative control is indicated as (−) and consists of fractions of a cell suspension electroporated with a plasmid containing only the internal marker gene encoding GUS. DNA concentrations were standardized in order to obtain identical GUS activities in each electroporated protoplast suspension. Abbreviations for the different chimeric proteins are given in each lane. The PR1b signal sequence is given by ss; PAT refers to phosphinothricin acetyl transferase while KDEL and HDEL refer to tetrapeptide sequences given in the one letter code. The arrows indicate the position of purified PAT on the gels.

PAT-KDEL or PAT-HDEL accumulate in the ER lumen

To obtain sufficient amounts of homogenous biological material for further analysis of the intracellular fate of PAT derivatives, transgenic tobacco plants were generated that contain chimeric genes designed for the synthesis and translocation of either PAT, PAT-KDEL or PAT-HDEL to the ER lumen. Similarly to the transient expression (Figure 2), PAT was efficiently secreted in protoplasts prepared from leaves of plants synthesizing ssPAT while no significant secretion could be observed in the two PAT derivatives ssPAT-KDEL and ssPAT-HDEL (unpublished data).

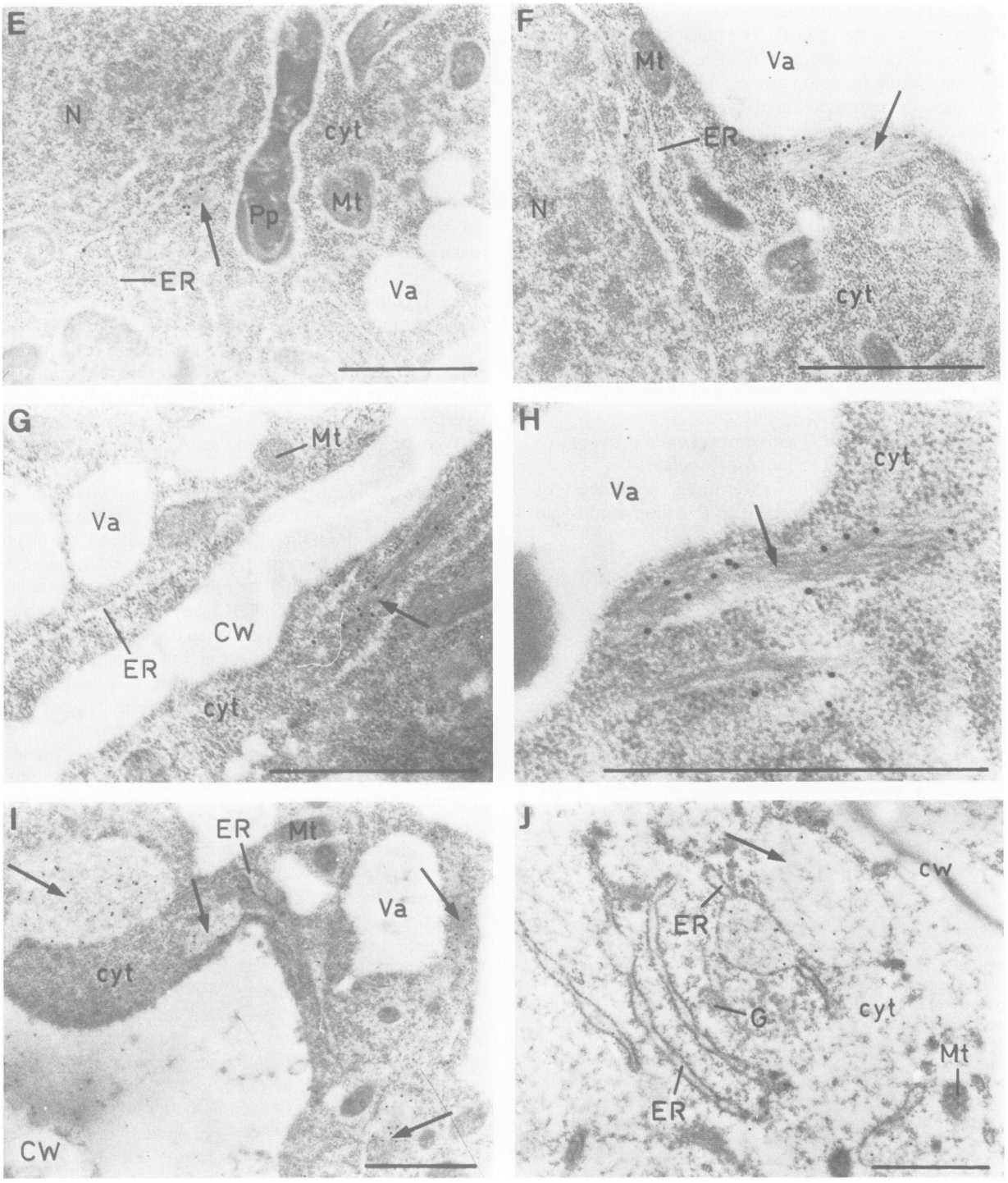
In order to identify the compartment in which the PAT derivatives accumulate intracellularly, plant tissue sections were labelled immunologically and analysed by electron microscopy. Cells from the root meristem and the associated elongating zone proved to be most suitable for this analysis due to the high amounts of ER membranes and the absence of large vacuoles. The experiments demonstrated that PAT-KDEL was localized in the ER and in the nuclear envelope (Figure 3A−C). No significant label was found in root meristem sections of untransformed plants (Figure 3D).

Organelle structures containing large amounts of PAT-KDEL clustered over a small surface were also detected (Figure 3E−H). The fibrous appearance of their luminal content and their connection with the rough ER might reflect the aggregation of large amounts of heterologous protein in the ER lumen due to its high concentration. These structures could correspond to the more dilated or vesicularized cisternae of the rough ER that were observed in sections of elongating cells (Figure 3I−J), except that they remain condensed in the meristem. Structures that resemble the Golgi apparatus (as in Figure 3D and J) were devoid of gold particles. In tissue sections of PAT-secreting plants, only weak labelling of the ER was observed (J.Denecke and R.De Rycke, unpublished data). The apparent absence of PAT in other parts of the endomembrane system or in the cell wall is possibly due to the low stability of PAT (see next section), resulting in PAT concentrations below the detection limit in our experiments. In summary, the data demonstrate the accumulation of PAT-KDEL in the ER lumen, but no

conclusion can be drawn concerning the presence or absence of PAT-KDEL in post-ER compartments.

Retention of PAT in the ER prevents the degradation of PAT in a post-ER compartment

It has been shown previously that several marker enzymes including PAT failed to be produced in high amounts if translocated to the ER lumen, in spite of their high stability in the protoplast culture medium (Denecke *et al.*, 1990). It was concluded that cellular post-transcriptional events limit either the synthesis or the intracellular stability of the encoded proteins. Figure 2 demonstrated that PAT derivatives retained in the ER accumulate to higher levels than secreted PAT. Similar results were obtained when the levels of PAT protein and the corresponding steady state levels of mRNA were analysed in stably transformed plants. Table 1 (upper panel) shows the protein:mRNA ratios for five independent transformants for each chimeric gene (ssPAT, ssPAT-KDEL and ssPAT-HDEL). The protein:mRNA ratio for the retained PAT derivatives was found to be ~10-fold higher. On the other hand, protein:mRNA ratios did not vary significantly for the three types of proteins when the signal peptide was missing (unpublished data). The differences are



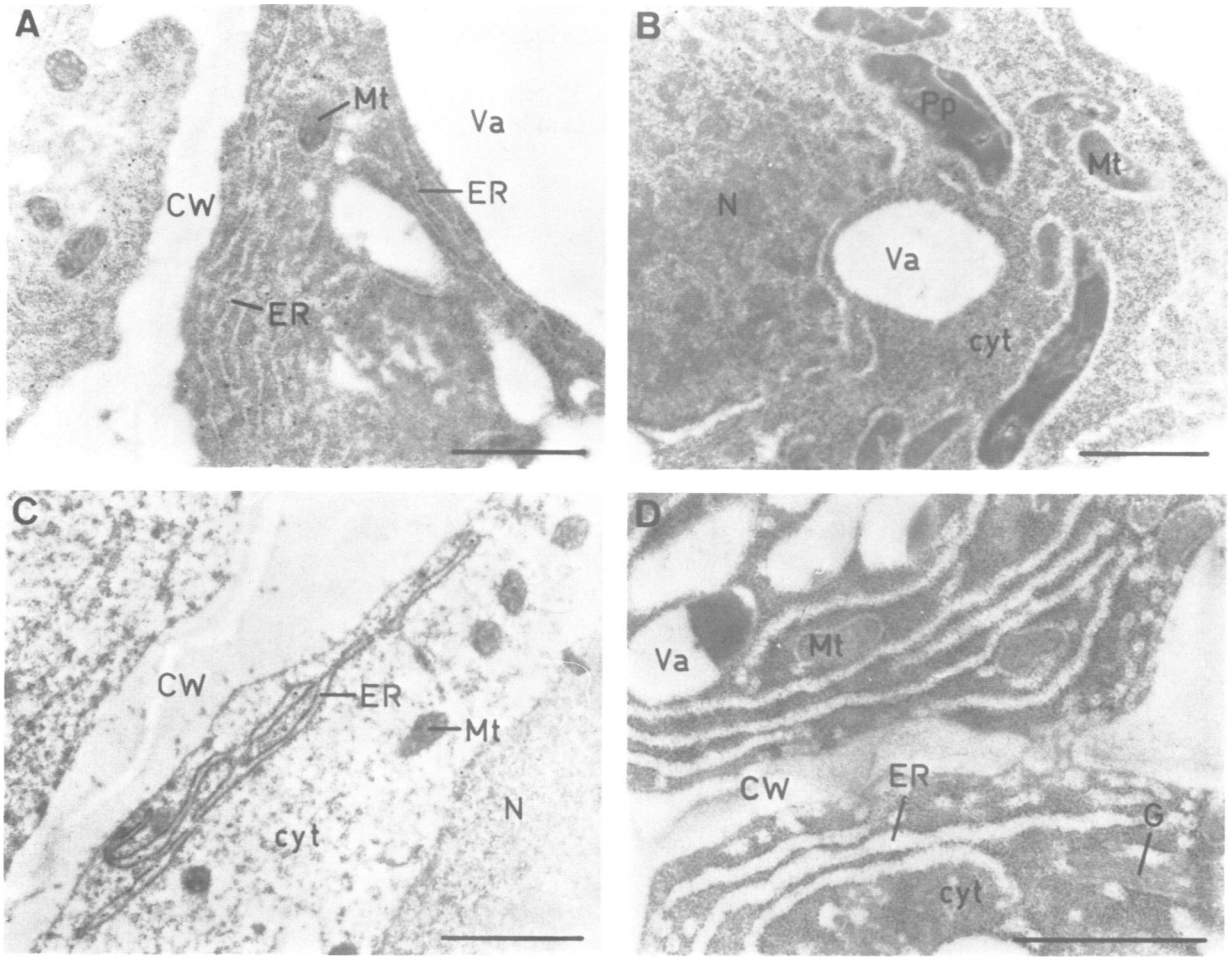


Fig. 3. Immunocytochemical localization of PAT derivatives in transgenic plant tissues. The figure shows electron micrographs of immunogold labelled root tissue sections of transformed (**A–C**; **E–J**) and untransformed (**D**) plants. The plants express a gene coding for the synthesis and ER translocation of PATKDEL and contain high levels of the transgene product (~0.5% of total leaf protein in A, B, E, F and I and ~1% of total leaf protein in C, G, H and J). N, nucleus; ER, endoplasmic reticulum; G, Golgi; Va, vacuole; Mt, mitochondrion; Pp, proplastid; CW, cell wall; The arrows indicate the structures in which PAT-KDEL accumulates. Bars represent 1 μm.

therefore unlikely to be the result of characteristics of the modified proteins or due to a different protein synthesis rate.

To analyse whether secretory PAT is degraded rapidly, a set of transient gene expression experiments was performed. We took advantage of the fact that, early after gene transfer, heterologous gene product levels closely reflect the *de novo* synthesis rate directed by the introduced genes, whereas later, a steady state level is approached (Denecke *et al.*, 1989). We compared the total intra- and extracellular PAT enzyme activity directed by three different genes at two different time points after gene transfer (Table I, lower panel). The experiments show that cytosolic PAT and endoplasmic PAT-KDEL accumulate to a greater extent than secretory PAT. However, the differences are hardly detectable early after gene transfer, suggesting that the rate of protein synthesis is almost equal in the three cases. A 3-fold reduced copy number of the gene coding for cytoplasmic PAT simulates a 3-fold reduced synthesis rate and causes a similar reduction of the PAT level at both time points after gene transfer. This allows us to conclude that the lower accumulation of secretory PAT must be the result of a reduced stability. It is conceivable that PAT degradation in the post-ER compartment is overcome by the introduction of a functional retention signal that prevents the protein from passing through this compartment.

The protein retention mechanism is highly specific

To analyse the requirements of protein retention in plants, several derivatives of the KDEL signal were introduced at the C-terminus of PAT as illustrated in Figure 1 and transient expression assays with tobacco protoplasts were performed. Samples were taken 10 h after gene transfer to avoid large differences in expression levels and to allow measurement of the percentage of secreted protein in the range of linear extracellular accumulation (Denecke *et al.*, 1990). Cell extracts and concentrated culture media were analysed for both protein quantity and enzyme activity (Figure 4a and b). PAT retention is almost complete if the first amino acid of the tetrapeptide is positively charged (KDEL, HDEL or RDEL). However, no significant retention is observed when serine is introduced at this position (SDEL). The latter sequence is present at the C-terminus of the putative *Plasmodium falciparum* BiP (Peterson *et al.*, 1988) and might serve as a retention signal in this organism. Replacement of the aspartic acid residue on the second position by the closely related glutamic acid (KEEL) results

Table I. PAT accumulation in function of targeting signals

Plasmid	Independent transformants				
ssPAT	1.0	1.4	0.9	2.4	2.8
ssPATKDEL	17	12	7	13	15
ssPATHDEL	10	8	14	11	7

Plasmid	3 hours		20 hours	
	GUS	PAT	GUS	PAT
PAT	3.7	6.2	98	100
PAT(1/3)	1.1	1.9	56	42
ssPAT	3.9	5.9	100	34
ssPATKDEL	3.4	5.7	89	92

Upper panel: Stable transformants. The ratio (given in relative units) between PAT activities and the corresponding mRNA levels determined for leaf tissues of five independent transgenic plants for each of the chimeric genes (ssPAT, ssPATKDEL and ssPATHDEL). The protein:mRNA ratio in one of the PAT secreting plants was arbitrarily set as 1 relative unit (first lane, ssPAT). Lower panel: Transient gene expression. Total (extra and intracellular) enzyme activity were measured 3 and 20 h after gene transfer. All activities were at least 10-fold higher than the detection limit and are given as percentages of the activity from the sample with the highest activity (in the case of both GUS and PAT). Lanes are indicated as in Figure 2. (1/3) refers to a 3-fold diluted copy number.

only in partial retention of PAT while replacement by related amino acids at the third (KDDL) or last position (KDEI, KDEV) results in a complete loss of the retention. Measurement of the extra- and intracellular GUS activities shows that the non-specific leakage is comparable in all cases (Figure 4b, lower panel). Similar transient expression experiments performed with protoplasts derived from other species indicate that *Nicotiana tabacum* shares the observed retention specificity for these PAT derivatives with the closely related species *Solanum tuberosum* and the more distantly related species *Brassica napus* (unpublished data).

The experiments suggest that the plant retention machinery has a different specificity from that of mammalian yeast and cells. In contrast to our observations, it has been found that KDEI can be functional in mammalian cells (Andres *et al.*, 1991). It has also been shown that KDEL does not function in yeast and that HDEL does not confer retention in mammalian cells (Pelham *et al.*, 1988). In order to obtain more information about potential differences in the retention specificities between organisms, we analysed the PAT derivatives in the yeast *S.cerevisiae*. Based on previous studies and the assumption that the targeting information resides entirely in the four C-terminal amino acids (Pelham *et al.*, 1988), PAT-KDEL was expected to be secreted while PAT-HDEL should be retained. Surprisingly, none of the derivatives (including PAT-HDEL) showed significant intracellular accumulation or reduced secretion compared with unmodified PAT. Data for the stationary growth phase are shown in Figure 4c. Similar data were obtained in other growth conditions, both exponential and stationary growth phases and different transgene expression levels. The relatively slow secretion of PAT corresponds well with previous observations in plant cells (Denecke *et al.*, 1990). Like the situation in plant cells, the observed expression levels of secretory PAT were at least 10-fold lower than those of cytoplasmic PAT, but no significant increase in the total PAT activity was observed for any of the derivatives. The

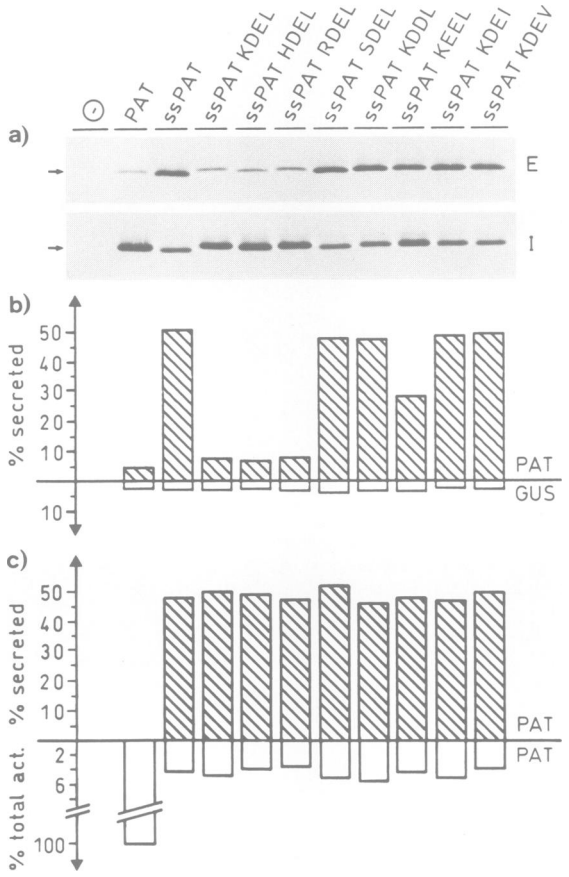


Fig. 4. Analysis of the retention directed by mutant C-terminal tetrapeptide sequences in plant and yeast cells. Cells were separated from the culture medium and equal proportions were analysed. Lanes are identical for (a), (b) and (c) and are indicated as in Figure 2. (**a**) Western blot of concentrated culture medium (E) and cell extracts (I) using a polyclonal antiserum directed against PAT. DNA standardization using the internal marker GUS was done as described in the legend to Figure 2. The arrow indicates the position of purified PAT. (**b**) Percentage of enzymatic activity in the culture medium of PAT, its derivatives and the cytoplasmic control GUS versus the total enzymatic activity in each cell suspension. (**c**) The percentage of secreted PAT activity (upper panel) and the percentage of total enzymatic PAT activity compared with the culture with cytosolic PAT (lower panel) in suspensions of *S.cerevisiae* in the stationary growth phase. Overnight precultures in YEB−glucose were used to inoculate 100 ml cultures of YEB−galactose medium to OD_{600} 0.02, which were incubated for 24 h.

secretory properties and the normal enzymatic activity of the PAT derivatives suggests that the proteins are not malfolded.

A common epitope is present on different modified but functional retention signals

To obtain more information on the structural determinant required for reticuloplasmin retention in plant cells, we analysed whether the retained PAT derivatives share a common epitope with mammalian reticuloplasmins. We used monoclonal antibodies (hybridoma cell line '1D3' in Vaux *et al.*, 1990) directed against a synthetic peptide comprising the last 12 amino acids of rat protein disulfide isomerase (PDI), an abundant reticuloplasmin containing a C-terminal KDEL peptide. It has previously been shown that the interaction of PDI and calreticulin with the monoclonal antiserum depends on the C-terminal residues (Vaux *et al.*,

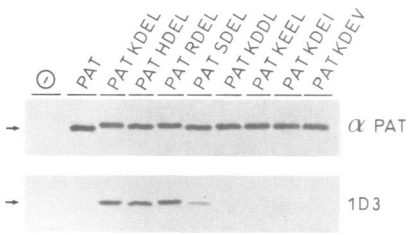


Fig. 5. Affinity of a monoclonal anti-KDEL antibody for different PAT derivatives. Extracts were prepared from *E.coli* strains producing the respective PAT chimeras. Aliquots containing identical PAT enzyme activities were analysed by Western blotting using either a polyclonal anti-PAT antiserum (αPAT) or a monoclonal anti-peptide antiserum (1D3). The arrows indicate the position of purified PAT.

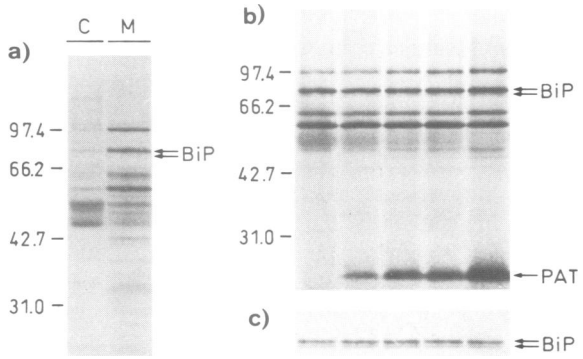


Fig. 6. Affinity of a monoclonal anti-KDEL antibody for plant microsomal proteins. (**a**) Cytoplasmic (C) and microsomal (M) enriched fractions were analysed by Western blotting using the 1D3 antiserum. Numbers refer to the molecular weight of the size markers (in kDa). The positions that correspond to tobacco BiP gene products are indicated by arrows. (**b**) Western blot (1D3 antiserum) of microsomal fractions from protoplasts prepared from leaves of different transgenic tobacco plants containing a gene coding for PAT-HDEL. Lanes are organized according to increasing amounts of PAT-HDEL protein in the transformants (0.01 – 1% of total leaf protein). Numbers refer to the molecular weight of the size markers (in kDa). The positions that correspond to tobacco BiP gene products and to purified PAT are indicated by arrows. (**c**) Western blot of the same extracts as in (b) but analysed with polyclonal anti-BiP antiserum. Lanes are identical to the lanes in (b). The positions that correspond to tobacco BiP gene products are indicated by arrows.

1990). These authors also demonstrated that antibodies raised against the 1D3 derived anti-idiotype immunoglobulins exhibit affinity to a broad range of reticuloplasmin C-termini, which suggests at least a low affinity of 1D3 antibodies to reticuloplasmins other than PDI and calreticulin. Since PAT is unlikely to contain PDI specific epitopes, 1D3 immunoglobulins were expected to be a useful reagent for the analysis of the retained PAT derivatives.

To standarize the amounts of protein analysed, samples of partially purified PAT derivatives with equal enzymatic activities were analysed by Western blots. Polyclonal antisera directed against PAT revealed equal amounts of PAT derivatives in each lane (Figure 5), confirming that the different modifications did not alter the specific enzymatic activity of the carrier protein. The monoclonal 1D3 antibodies showed a clear affinity for the PAT derivatives that contain the tetrapeptides KDEL, HDEL or RDEL (Figure 5). These chimeric proteins were efficiently retained in the plant ER (Figure 4a). Weak reactivity was also observed with the secreted derivatives PAT-SDEL and PAT-KDEI, but not with the partially retained PAT-KEEL. This suggests that the 1D3 antibodies recognize an epitope which is closely related but not identical to the structure that is important in retention signalling in plants.

Plant and mammalian retention signals contain a conserved epitope

A close similarity between retention signals in plants and mammalian cells is expected, since well retained proteins in plants are recognized by an antibody that recognizes the mammalian signal. The weak reactivity with PAT-KDEI may reflect the functionality of KDEI in mammalian cells (Andres *et al.*, 1991). On the other hand, the fact that 1D3 antibodies have no affinity to the C-terminal part of yeast BiP (Vaux *et al.*, 1990) may reflect the presence of a structurally less related retention signal in yeast. Our observations encouraged us to use these antibodies for the identification of plant reticuloplasmins. This would confirm the presence of a conserved epitope between reticuloplasmins of plants and mammals, while the use of PAT as a carrier already excluded the interference of reticuloplasmin specific epitopes (Figure 5). Figure 6a shows the interaction of 1D3 monoclonals with several proteins that copurify with microsomal fractions (M) of germinating tobacco seeds. One of the reactive proteins has a mobility on denaturing gels similar to that of a gene product from one of the members of the tobacco BiP gene family. The purified protein reacts both with 1D3 immunoglobulins and the polyclonal anti-tobacco BiP serum, which matches the observation that the cloned members of the tobacco BiP family contain the C-terminal HDEL tetrapeptide (Denecke *et al.*, 1991). Several reactive proteins with 1D3 antigenicity similar to that of the retained PAT derivatives were purified and revealed to contain significant amino acid sequence similarities with other known reticuloplasmins in mammalian cells (J.Denecke and B.Ek, in preparation). Mammalian reticuloplasmins are recognized by 1D3 immunoglobulins with a similarly low affinity to the putative plant homologues, except for mammalian PDI which is bound with higher affinity (unpublished data).

The efficiency of protein retention is not significantly influenced by the ligand dosage.

We analysed whether the plant retention system can be saturated by introducing increasing amounts of heterologous reticuloplasmins. Transgenic plants containing genes coding for either PAT-KDEL or PAT-HDEL were screened in order to obtain a subpopulation with a large range of heterologous passenger protein levels. In the plants with the highest levels, PAT derivatives represented ~1% of total leaf protein (see Materials and methods). However, the percentage of secreted PAT-HDEL after 30 h protoplast culture varied between 2 and 4%, independently of the levels of transgene expression. This is comparable to the non-specific leakage due to cell disruption observed in previous experiments (Denecke *et al.*, 1990) and suggests that the plant retention machinery is not easily saturated.

Figure 6b shows an analysis of microsomal extracts from five different transformants containing variable amounts of PAT-HDEL (see figure legend). The 1D3 monoclonal antibodies detected both the putative plant reticuloplasmins and PAT-HDEL, providing an estimate for the relative

abundance of the heterologous protein compared with other proteins with similar epitopes. The presence of artificially introduced ligands does not significantly alter the composition of putative reticuloplasmins in microsomal preparations. A control experiment with polyclonal antibodies directed against tobacco BiP (Denecke *et al.*, 1991) using the same extracts demonstrates that BiP protein levels are unaffected by increasing amounts of the transgene product (Figure 6c). Similarly, the amount of tobacco BiP in the culture medium was unaffected by the level of intracellular PAT-HDEL. Attempts to identify physiological conditions (including the calcium ionophore A23187) under which protein retention is less efficient or experiments to impair the partial retention of PAT-KEEL by increasing concentrations of competing PAT-KDEL in transient expression experiments were both unsuccessful (J.Denecke, unpublished observations).

Discussion

A model system for the analysis of retention signals

The cytoplasmic enzyme PAT proved to be a suitable carrier molecule for the analysis of ER retention signals. Addition of several tetrapeptides at its C-terminus resulted in the effective retention of the hybrid proteins in the ER of plant cells. This is in contrast with other experiments with carrier molecules where only partial retention was observed (Zagouras and Rose, 1989; Herman *et al.*, 1990). Either the lack of competing targeting signals for other destinations or specific features of the C-terminal portion of PAT enable the hybrid proteins to acquire a structure that corresponds to a functional retention signal. Two lines of evidence indicate that the observed retention was unlikely to be due to malfolding of the carrier protein. First, addition of the tetrapeptides to the C-terminus of PAT did not have a significant effect on the enzymatic parameters (see Materials and methods). Secondly, overproduction of ER retained PAT derivatives in transgenic plants did not lead to enhanced steady state levels of BiP. Enhanced BiP levels can be regarded as an indication for the presence of malfolded proteins in the ER (Kozutsumi *et al.*, 1988). We concluded that the PAT derivatives were retained due to a discrete structural determinant which is normally absent in PAT but which does not disturb the overall protein structure if present.

The bulk of the retained PAT derivatives were shown to be localized in the ER lumen, but the most distal location could not be determined. In the light of the 'two step' model (Booth and Koch, 1989), it is probable that the described PAT chimeras are retained largely due to the signal mediated mechanism. PAT does not contain acidic regions as do calreticulin or other reticuloplasmins and it is unlikely that a heterologous protein would specifically participate in the formation of a gel matrix. However, large amounts of such PAT derivatives in the ER lumen could disturb the above mentioned gel matrix locally. This may cause the observed dilated or vesicular structures of the rough ER. It is also possible that the synthesis and translocation of large amounts of ligands in the transformants may cause a general hypertrophy of the ER.

An interesting aspect of our results is the fact that PAT is degraded in a post-ER compartment but appears to be more stable when retained in the ER. These results correspond well with the observation that ER retention defective (ERD) yeast strains secrete only low amounts of BiP in spite of an increase in the *de novo* synthesis (Hardwick *et al.*, 1990; Semenza *et al.*, 1990). This suggests that BiP might be degraded more quickly if it is not properly retained in the ER. The question remains why PAT is degraded if it is allowed to migrate further than the ER and whether this has any relationship to the slow secretion of this protein. Since PAT has been shown to be stable in the culture medium (Denecke *et al.*, 1990), degradation must occur intracellularly.

It was recently observed that the tunicamycin mediated inhibition of invertase secretion in carrot suspension cultures is due to rapid breakdown of the unglycosylated protein (Faye and Chrispeels, 1989). The authors suggested that degradation would occur during the last stages of secretion due to the simultaneous presence of proteases in the secretory vesicles. The unglycosylated forms of invertase could be more susceptible to proteolysis. However, an alternative explanation for the tunicamycin inhibited secretion of invertase would be that a significant fraction of the unglycosylated invertase is transported to the vacuoles, followed by a rapid degradation. The low rate of PAT secretion could thus merely be the result of a few escaping proteins that are transported to the cell surface by default, while the majority of PAT is degraded. It is possible that secretory proteins contain specific structural features to escape such a degradative pathway or to improve the rate of their secretion. It would also be unlikely that a cytoplasmic protein as PAT would match such requirements, even if correct folding and export out of the ER lumen is achieved. The slow secretion of PAT and the observation that PAT degradation is overcome, when the protein is artificially retained in the ER, are in accordance with this view.

What determines the structure of retention signals?

Our results indicate that protein retention in the plant ER can be mediated by a small set of related C-terminal peptide sequences. C-terminal tagging with KDEL, HDEL and RDEL are sufficient to cause efficient PAT retention while KEEL confers partial retention. The tetrapeptides KDEL and HDEL were found at the C-termini of three distinct plant reticuloplasmins (Akasofu *et al.*, 1989; Hesse *et al.*, 1989; Inohara *et al.*, 1989; Tillman *et al.*, 1989; Denecke *et al.*, 1991; Tanaka *et al.*, 1991), implying that they possibly function *in vivo*. RDEL and KEEL are also shown to function in mammalian cells (Mazzarella *et al.*, 1990; Andres *et al.*, 1990). Several single amino acid substitutions within the tetrapeptide tag (SDEL, KDDL, KDEI and KDEV) can completely disrupt the retention signal in plant cells. This suggests that the retention machinery is highly specific, in spite of the fact that more than one sequence can act as a retention signal. ER retention signals in plants were characterized immunologically using a monoclonal antiserum directed against the mammalian sorting signal. The fact that retained PAT derivatives and putative plant reticuloplasmins were bound with similar affinity demonstrates that the plant ER retention signal was well reconstituted in the carrier protein. This also implies that there is remarkable conservation between plant and mammalian signals. However, the observed specificity differs from those observed in other eukaryotes. For defined passenger proteins, KDEL cannot confer retention in yeast, while HDEL fails to function in mammalian cells (Pelham *et al.*, 1988). In contrast to plants, KDEI appears to form

an efficient retention signal in mammalian cells (Andres *et al.*, 1991). Unfortunately, the data cannot be compared directly since different passenger proteins were used in each case.

It is possible that plants contain several receptors with different specificities, or that one receptor binds to different sequences. The latter is likely to be the case in the *Kluyveromyces lactis* retention machinery (Lewis *et al.*, 1990). However, upstream sequences could affect the structure of the tetrapeptide. The range of sequences that lead to a functional retention signal in plant cells might be different if upstream sequences in PAT are changed. In this context it is remarkable that the PAT C-terminal sequence is clearly distinct from the general acidic C-termini of known reticuloplasmins, including tobacco BiP (Denecke *et al.*, 1991). The PAT C-terminus is characterized by a large number of proline residues (Figure 1b). However, a non-acidic C-terminus also precedes the KDEL peptide in the luminal enzyme sulfhydryl endopeptidase of *V.mungo* (Akasufo *et al.*, 1989) and *P.vulgaris* (Tanaka *et al.*, 1991). This protein contains three sites for N-linked glycosylation close to its C-terminus. The sugar residues could mediate exposure of the KDEL peptide at the surface of the protein, to ensure the recognition by a receptor.

In one particular sequence context, different tetrapeptides may give rise to a similar structural determinant that is recognized by the same retention machinery. The interaction with the monoclonal antiserum 1D3, originally raised against a KDEL containing peptide, demonstrated that there was a common epitope in the PAT chimeras that were effectively retained within the ER lumen of tobacco cells. The weak interaction with the secreted PAT-KDEI may be due to the fact that KDEI confers protein retention in mammalian cells and that 1D3 immunoglobulins were raised against the mammalian ER retention signal.

Identical tetrapeptides may also fold differently if the sequence context varies. This is illustrated by the fact that PAT-HDEL fails to be retained in the yeast *S.cerevisiae* in spite of the presence of the HDEL tetrapeptide, which is believed to be both necessary and sufficient for the retention of *S.cerevisiae* BiP (Pelham *et al.*, 1988). Furthermore, the 1D3 antiserum lacks detectable affinity for an HDEL containing peptide comprising the last 13 amino acids of the yeast BiP terminus (Vaux *et al.*, 1990), while it has significant affinity for PAT-HDEL and tobacco BiP (containing HDEL). These data can be explained if the functionality of the HDEL tetrapeptide is context dependent. The yeast retention machinery might recognize a different structural determinant which is not matched by PAT-HDEL or tobacco BiP. This might also explain the partial complementation of *S.cerevisiae kar2* by tobacco BiP (Denecke *et al.*, 1991). If the tobacco ER retention signal were non-functional in *S.cerevisiae*, tobacco BiP would be secreted or mistargeted to the vacuole and degraded. The latter would explain the presence of degradation products of tobacco BiP, found in the transformed yeast strains (Denecke *et al.*, 1991). However, it cannot be excluded that our results obtained with the PAT derivatives are biased by unknown differences in the strain used in our experiments compared to previous studies (Pelham *et al.*, 1988).

Independent evidence for the context dependence of ER retention signals was recently obtained by analysing a series of tetrapeptide sequences in the context of two different reticuloplasmins (Haugejorden *et al.*, 1991). In several hybrid proteins, addition of KDEL led to undetectable (Pelham *et al.*, 1988) or incomplete retention (Zagouras and Rose, 1989; Herman *et al.*, 1990). This could be due to upstream sequences that are incompatible with correct KDEL folding or due to masking of the KDEL sequence by other parts of the protein. It remains to be shown whether specific features of PAT are responsible for the lack of retention in yeast, which apparently do not disturb retention signalling in plants. Yeast salvage receptors might recognize a different epitope which is also not recognized by the 1D3 antiserum. The C-terminal residues of yeast BiP could thus form a structure that is distinct from that of PAT-HDEL or tobacco BiP, in spite of the identity of the last four amino acids. We propose, therefore, that upstream sequences beyond the C-terminal tetrapeptide can influence or may be part of the structure that forms the retention signal in reticuloplasmins of eukaryotic cells.

Analogy with the mammalian retention machinery

The 1D3 antiserum directed against the C-terminal portion of mammalian PDI recognizes preferentially PDI in mammalian microsomes (Vaux *et al.*, 1990). This demonstrates that the recognized epitope is not completely identical to the retention signal as such. However, antibodies raised against the 1D3 derived anti-idiotype immunoglobulins exhibit affinity to a broad range of reticuloplasmin C-termini (Vaux *et al.*, 1990). This suggests a low affinity of 1D3 antibodies to other reticuloplasmins which is probably masked by the preference for PDI. The 1D3 antiserum binds to the retained PAT derivatives and to several microsomal plant proteins. The recognized epitope on PDI must therefore be closely related to the structure that is required for retention of reticuloplasmins in plants. Specific interactions of this antiserum with plant microsomal proteins may reflect the presence of such a structure and thus their classification as reticuloplasmins. The sequence similarity of these proteins with known mammalian reticuloplasmins provides further arguments for this view (J.Denecke and B.Ek, in preparation). The absence of the PDI preference in plant extracts is probably due to the low degree of PDI sequence conservation between different species (LaMantia *et al.*, 1991). We conclude that a distinct structural feature of the retention signal in plant reticuloplasmins resembles that of the corresponding signal in mammalian cells.

We also demonstrated that the plant protein retention system cannot be easily saturated by increased amounts of ligands. For both the artificially introduced ligand and other reticuloplasmins, no significant reduction of the retention efficiency could be observed even when the heterologous ligand was severely overproduced. A reliable test of the saturability depends on an accurate knowledge of the molarity of soluble ligands, which can only be estimated in this case. However, our data are in sharp contrast to the easily saturated protein retention system in *S.cerevisiae* (Pelham *et al.*, 1988; Lewis *et al.*, 1990) and correspond more to observations made for mammalian cells, which suggest a ligand dosage regulated retention system (Vaux *et al.*, 1990). However, the results obtained contradict the observation that stable overproduction of two glucose regulated proteins only leads to the detectable secretion of the overproduced protein but not of other reticuloplasmins (Dorner *et al.*, 1990). If protein retention depended on a

common signal through the recognition of one receptor, enhanced secretion of all reticuloplasmins would be expected. These authors suggested that the ER retention mechanism might be mediated by more specific interactions than just KDEL recognition. If this were true, several receptors with different specificities would be required in order to guarantee efficient retention of all the members of the group of reticuloplasmins. The question that remains to be solved in the future is which sequences beyond the tetrapeptide are responsible for the structure of the retention signal.

Materials and methods

Plasmid constructs

All DNA manipulations were done according to established procedures. The *E.coli* strain MC1061 (Casadaban and Cohen, 1980) was used for plasmid preparation.

The plasmid pOP443 (Velten *et al.*, 1984) containing the dual mannopine synthase (*mas*) promoter was used for the construction of vectors for transient gene expression in plant protoplasts. The internal marker gene was constructed by inserting a filled *NcoI*–*EcoRI* fragment containing a chimeric GUS coding sequence provided with the signals for 3′ end formation and polyadenylation of the T-DNA gene 7 (3′g7) into the filled *Bam*HI and the *Eco*RI site of pOP443 resulting in the chimeric gene *mas*1′–*gus*–3′*ocs*. A hybrid coding sequence containing the signal sequence of the pathogenesis related protein 1b and the *bar* coding sequence (described in Denecke *et al.*, 1990) was provided with the fragment of the *nos* gene containing the 3′ end formation and polyadenylation signals (3′*nos*). This fragment was inserted as a filled *NcoI*–*Hin*dIII fragment into the filled *Cla*I and the *Hin*dIII site of the pOP443 derivative, downstream from the *mas* 2′ promoter. The resulting plasmid (pDE222) contains two different chimeric genes. A *bar* coding region without the signal sequence (Denecke *et al.*, 1990) was used to construct a gene coding for cytosolic PAT (pDE221).

Both plasmids contain a *Bgl*II site that overlaps the last codon of the *bar* coding region and an *Xba*I site that overlaps the stop codon. The two restriction sites were used for the insertion of tetrapeptide-encoding DNA fragments. These fragments were generated by partially hybridizing 15mer oligonucleotides which resulted in the formation of sticky ends.

For stable plant transformation, we replaced the *mas* 2′ promoter with the CaMV 35S promoter and inserted the chimeric genes into the polylinker of the plant transformation vector pDE1001. The latter plasmid is derived from pGSC1700 (Cornelissen and Vandewiele, 1989) by the insertion of a *Bam*HI fragment containing the chimeric kanamycin resistance gene p*nos*–*neo*–3′*ocs* from pLGV*neo*1103 (Hain *et al.*, 1985) into the *Bgl*II site of pGSC1700.

For the production of PAT derivatives in *S.cerevisiae*, we inserted the chimeric coding regions from pDE222 and derivatives as *NcoI*(blunted)–*Hin*dIII fragments between the *Sma*I and *Hin*dIII sites of pEMBLyex4 (Cesareni and Murray, 1987).

For the production of PAT derivatives in *E.coli*, we replaced the *Ava*I–*Bgl*II fragment in plasmid pGSFRTC1 (Botterman *et al.*, 1991) by the *Ava*I–*Bam*HI fragment of derivatives of pDE222 containing the tetrapeptide-encoding DNA fragments.

Purification and characterization of PAT derivatives

The different PAT derivatives were synthesized under control of the inducible lambda P_R promoter using the *E.coli* strain NF1. The transformed strains containing the plasmid pGSFRTC1 and its derivatives were grown and extracted as described (Botterman *et al.*, 1991). Crude extracts were enriched for PAT and its derivatives by ammonium sulfate fractionation (fraction between 45 and 60%). The pellet was resuspended in a buffer containing 20 mM Tris pH 7.2 and 2 mM EDTA, applied onto a Sephacryl S200 HR column (90×2 cm) and eluted with the same buffer. Fractions containing high PAT enzyme activity were combined. Equal amounts of enzyme activity were loaded onto a 12.5% polyacrylamide gel and analysed by Western blotting. A polyclonal PAT antiserum detected identical amounts of protein in each lane. The Michaelis constants (K_m) for the substrate phosphinothricin (PPT), determined for PAT and the different modified enzymes according to Thompson *et al.* (1987), ranged between 0.11 and 0.14 mM PPT (compare with Botterman *et al.*, 1991).

Transient expression and sample preparation

Protoplasts were prepared, electroporated and kept in culture as described (Denecke *et al.*, 1989). 10 ml cell suspensions containing 3×10^5 cells per ml (counted 2 h after electroporation) of culture medium were incubated for different times and analysed. After separation of the cells by flotation at 80 *g*, the culture medium was collected and concentrated on Centricon 10 membranes (Amicon) to a final volume of 500 μl. The cells were washed in 250 mM NaCl, pelleted at 80 *g* and resuspended in 250 μl of PAT extraction buffer (Denecke *et al.*, 1989). After sonication (10 s, 5 μm amplitide), the sample was divided into two equal parts (125 μl) and an equal volume of PAT or GUS extraction buffer was added. After vortexing, cell debris was removed by centrifugation for 10 min at 16 000 *g* at 4°C. 15 μl of concentrated culture medium or PAT extract were applied on a 12.5% SDS–polyacrylamide gel and analysed by Western blotting.

Plasmid DNA dilutions were made in order to obtain equal activities of the internal marker GUS in electroporation experiments. GUS activities were used as internal standards to determine the percentage of non-specific leakage by cell mortality.

Immunocytochemical labelling

Root tips of *in vitro* grown tobacco plants were washed briefly in 10 mM sodium phosphate buffer (pH 7.2) to remove the agar and immediately fixed for 4 h at 22°C and under constant vacuum in a mixture of 2.5% formaldehyde and 0.3% glutaraldehyde in 10 mM sodium phosphate buffer. Washing, dehydration and embedding of the root tissues in LR white resin (Polysciences, Warrington, PA) and preparation of ultrathin sections were done as described previously (De Clercq *et al.*, 1990). The sections were incubated for 2 h at 22°C in a drop of PAT antiserum diluted 1:100 in 10 mM phosphate buffer (pH 7.2) supplemented with 150 mM NaCl, 0.05% Tween 20 and 0.1% (w:v) bovine serum albumin. Washing, protein A–gold labelling and final contrasting with 2% uranyl acetate were performed as described (De Clercq *et al.*, 1990).

Cell fractionation and analysis

One gram of seeds from *N.tabacum* 'Petit Havana' SR1 plants (Maliga *et al.*, 1973) were germinated in shaking liquid cultures at 24°C in culture medium containing MS salts, 10 g/l glucose and 0.5 g/l MES and brought to pH 5.8. Germinating seeds were collected 4 days after the start of imbibition. Cells were disrupted in 5 ml fractionation buffer (12% sucrose, 100 mM Tris–HCl pH 7.9, 2 mM $MgCl_2$, 5 mM $CaCl_2$) and cell debris was removed by centrifugation at 500 *g*. Microsomal fractions were separated from soluble cytoplasmic proteins by gel filtration of 4 ml supernatant over a Sepharose 4B column (2×20 cm, 4°C) as described by Stinissen *et al.* (1985). The turbid fraction (5 ml) was centrifuged for 2 h at 150 000 *g* in an SW50.1 rotor. The pellet was resuspended in 500 μl of fractionation buffer. The soluble fraction was combined and concentrated on a Centricon 10 membrane (Amicon) to a final volume of 500 μl. 15 μl of each fraction was applied onto a 9% SDS–polyacrylamide gel and analysed by Western blotting.

Protoplasts from transgenic tobacco leaves were prepared and cultivated for 24 h according to Denecke *et al.* (1989). 10^7 cells were concentrated by flotation on the culture medium, washed and pelleted in 250 mM NaCl by centrifugation at 80 *g*. The cells were resuspended in 5 ml fractionation buffer and disrupted by passing through a 25 G syringe. After removal of cell debris by centrifugation at 500 *g*, microsomes were separated from soluble cytoplasmic proteins by centrifugation for 2 h at 150 000 *g* in an SW50.1 rotor at 4°C. The pellet was resuspended by gentle sonication (10 s, 5 μm amplitude) in 500 μl of fractionation buffer. 15 μl of this fraction was applied on 9% SDS–polyacrylamide gel and analysed by Western blotting. The samples were diluted 10-fold in the case of analysis with anti-tobacco BiP antiserum.

Northern analysis and RNA quantification

Total leaf RNA was prepared according to Dean *et al.* (1985). Slot blot quantification of PAT mRNA was performed as described by Denecke *et al.* (1990). Hybridizations with a riboprobe comprising the PAT coding sequence were done according to Amersham protocols and hybridization signals were quantified by liquid scintillation counting. Relative levels of mRNA were calculated after normalization using the dilution series with *in vitro* transcribed sense RNA of PAT as described (Denecke *et al.*, 1990).

Plant transformation

The pTDE plasmids were mobilized into *Agrobacterium tumefaciens* C58C1Rif^R (pGV2260; Deblaere *et al.*, 1987). Transformed plants were obtained by cocultivation of protoplasts from *N.tabacum* SR1 with the respective *Agrobacterium* strains. Transformants were selected on medium containing kanamycin (50 μg/ml) according to De Block *et al.* (1987). For each construct, randomly chosen plants were screened for PAT activity and a subpopulation containing a 100-fold variation in PAT protein levels was kept. The percentage of PAT versus total protein was estimated by comparing dilution series of plant extracts with dilution series of purified PAT in Western blot experiments. PAT-KDEL and PAT-HDEL were estimated to represent

~1% of total leaf protein in the plants with the highest transgene expression levels and gave rise to a visible band on Coomassie blue stained gels. None of the analysed PAT secreting plants contained >0.05% of the transgene product. However, the analysis is only semiquantitative and values should therefore be regarded as approximate.

Yeast culture and analysis

The strain DL1 (MATα, *leu2*-3, *leu2*-112, *his3*-11, *his3*-15, *ura3*-251, *ura3*-372, *ura3*-328, *lac2*, see Van Loon *et al.*, 1986) was transformed with the pEMBLyex4 derivatives according to Klebe *et al.* (1983). The resulting transformants were grown in YEB (1% yeast extract, 2% neutralized bacteriological peptone) or YNB (0.67% yeast nitrogen base supplied with 20 μg/ml leucine and 20 μg/ml histidine) with 2% glucose as carbon source. Inductions of the chimeric genes were done in the presence of 2% galactose and in the absence of glucose. Culture medium was concentrated 100-fold by 60% ammonium sulfate precipitation followed by ultrafiltration with the Centricon 10 spin dialysis system (Amicon). Reconstitution experiments with similar amounts of purified PAT demonstrated that PAT was concentrated quantitatively. The amount of cells equivalent to the amount of concentrated culture medium was extracted with PAT extraction buffer (Denecke *et al.*, 1989) supplied with 0.1% Triton X-100. The pellet was resuspended in approximately twice the pellet volume and extracted by vortexing twice with glass beads (0.5 mm diameter) for 2 min. The suspension was removed from the glass beads by washing with extraction buffer and diluted to the same volume as the concentrated culture medium. The suspension was sonicated (20 s, 10 μm amplitude) and cleared by centrifugation at 16 000 *g*.

Enzymatic assays

Spectrophotometric measurement of PAT and GUS activities were performed as described by Denecke *et al.* (1989).

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