ELSEVIER

Biochimica et Biophysica Acta 1410 (1999) 287–298

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

The green fluorescent protein targets secretory proteins to the yeast vacuole

I. Kunze [a], G. Hensel [a], K. Adler [a], J. Bernard [a], B. Neubohn [a], C. Nilsson [a], R. Stoltenburg [a], S.D. Kohlwein [b], G. Kunze [a,*]

[a] *Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK), Corrensstraße 3, D-06466 Gatersleben, Germany*
[b] *SFB Biomembrane Research Center, Institut für Biochemie, Technische Universität Graz, Petersgasse 12, A-8010 Graz, Austria*

Received 13 November 1998; received in revised form 4 January 1999; accepted 7 January 1999

Abstract

The green fluorescent protein (GFP) was used as a marker to study the intracellular transport of vacuolar and secretory proteins in yeast. Therefore, the following gene constructs were expressed in *Saccharomyces cerevisiae* under control of the *GAL*1 promoter: GFP N-terminally fused to the yeast secretory invertase (INV-GFP), the plant vacuolar chitinase (CHN-GFP) and its secretory derivative (CHNΔVTP-GFP), which did not contain the vacuolar targeting peptide (VTP), both chitinase forms (CHN and CHNΔVTP), GFP without any targeting information and two secretory GFP variants with and without the VTP of chitinase (N-GFP-V and N-GFP). Whereas chitinase without VTP is accumulated in the culture medium the other gene products are retained inside the cell up to 48 h of induction. Independently of a known VTP they are transported to the vacuole, so far as they contain a signal peptide for entering the endoplasmic reticulum. This was demonstrated by confocal laser scanning microscopy, immunocytochemical analysis and subcellular fractionation experiments as well. The transport of the GFP fusion proteins is temporary delayed by a transient accumulation in electron-dense structures very likely derived from the ER, because they also contain the ER chaperone Kar2p/Bip. Our results demonstrate that GFP directs secretory proteins without VTP to the yeast vacuole, possibly by the recognition of an unknown vacuolar signal and demonstrates, therefore, a first limitation for the application of GFP as a marker for the secretory pathway in yeast.

Keywords: Green fluorescent protein; Default pathway; Secretion; Vacuole; (*Saccharomyces cerevisiae*)

1. Introduction

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is a vital and convenient reporter molecule to localise proteins, to follow their intracellular movement or to study the dynamics of the subcellular compartments to which these proteins are localised [1–4]. Several mutant variants [5–12] of GFP have been developed with optimised excitation, emission characteristics and codon usage to do justice to requirements of the various expression and detection systems. In this study the re-engineered *GFP* variant, *SGFP-TYG*, was used [13]. In this modified GFP mutations in the chromophore (replacement of serine at position 65 by a threonine) resulted in a more than a 100-fold brighter fluores-

* Corresponding author. Fax: +49 (394) 82-5366; E-mail: kunzeg@ipk-gatersleben.de

0005-2728/99/$ – see front matter
PII: S0005-2728(99)00006-7

cence signal upon excitation at 490 nm and swifter chromophore formation compared to the wild-type GFP [11].

In many examples it has been demonstrated that GFP-tagged proteins were transported to their correct cellular compartments of yeast, plant and mammalian cells including the cytoskeleton [14–16], secretory vesicles en route to secretion [17–19], the endoplasmic reticulum [20,21], the *trans*-Golgi network [17], the endosomal compartment [22], the plasma membrane [23,24], the nucleus [11,25–27] as well as mitochondria [28,29], peroxisomes [30,31] and plastids of plants [11]. However, so far the default pathway for secretory proteins without any retention signals has not been visualised yet using GFP-tagged proteins.

Chitinase (CHN) of class I of tobacco *Nicotiana tabacum* is a vacuolar protein and has been functionally expressed in the yeast *Saccharomyces cerevisiae*. We have previously shown that its N-terminal signal peptide (NSP) and its vacuolar targeting peptide (VTP) are necessary and sufficient to direct the heterologous protein to the yeast vacuole [32]. We have now fused the tobacco CHN, its truncated form without the VTP (CHNΔVTP) as well as invertase (INV) as a typical secretory yeast protein to the N-terminus of the GFP (CHN-GFP, CHNΔVTP-GFP, and INV-GFP, respectively) and compared the intracellular transport of these fusion proteins with two engineered GFP variants equipped with the chitinase NSP alone (N-GFP) and in addition with the VTP from the CHN (N-GFP-V). All GFP-containing gene products, which can enter the endomembrane system, are transported to the vacuole. This vacuolar deposition did not depend on an appropriate targeting signal. The exact translocation mechanisms of the three fusion proteins occur similar to that of the CHN, but differ from that of the secretory GFP variants in a temporary delayed transport to the vacuole.

2. Materials and methods

2.1. Strains

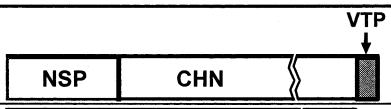
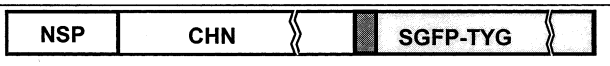
Escherichia coli TOP 10 F′[*mrc A Δ (mmr-hsdRMS-mcrBC) F80 ΔlacZ DM15 DlacX74 deoR recA1 araD139 Δ(ara leu) 7697 galU galK I⁻ rpsL endA1 nupG F′*)] from Invitrogen, San Diego, CA, was used as host strain for bacterial transformation and for plasmid propagation. The strain *S. cerevisiae* Cl3ABYS86 (*leu*2, *ura*3, *his*, *pra*1, *prb*1, *prc*1, *cps*) described by Bröker et al. [33], lacking several of the major vacuolar proteases, was used for heterologous expression of GFP constructs. The strain S288C (MATα, *SUC*2, *mal*1, *gal*2, *CUP*1) was taken for the isolation of chromosomal DNA, which was used as template for the polymerase chain reaction (PCR) to amplify the gene *SUC*2. Transformed strains were grown under selective conditions at 28°C using minimal salt medium (SD medium) with 2% glucose or 2% galactose as carbon source and the appropriate amino acids as described by Rose et al. [34].

2.2. Plasmid constructs and transformation procedures

Different gene constructs, listed in Table 1, were inserted into the yeast-*E. coli* shuttle vector pYES2 (Invitrogen, San Diego, CA), between the *S. cerevisiae GAL*1/10 promoter and *CYC*1 terminator sequences.

Insertion of the chitinase genes *FB7-1* and *FB7-1ΔVTP* into the vector pYES2 were described by Kunze et al. [32]. To fuse the chitinase genes *FB7-1* and *FB7-1ΔVTP* to the *GFP* gene the chitinase genes were flanked directly by *Nco*I/*Bam*HI and *Nco*I cleavage sites. The plasmid pYES2 containing the *FB7-1* gene [35] was the template in the PCR experiments to insert the bold-type sequences, indicated in the following oligonucleotides: (1) 5′-**CCATGGATCC**ATGAGGCTTTGTAAATTCACAGC-3′ (nucleotide positions 1–32; *Nco*I and *Bam*HI restriction sites are in bold type), and (2) 5′-**CCATGG**CCATAGTATCGACTAAAAGTCCATTTCCAAAAGAC-3′ (nucleotide positions 955–985 of the gene *FB7-1*; *Nco*I restriction site is in bold type) for the gene *FB7-1* and the first oligonucleotide together with the oligonucleotide 5′-**CCATGG**CATTTCCAAAAGACCTCTGGTTGCCGC-3′ for the gene *FB7-1ΔVTP* (nucleotide positions 955–964 of the gene *FB7-1*, *Nco*I restriction site in bold type). The *Nco*I fragments containing the complete chitinase gene or the gene without the VTP sequence were inserted into the *Nco*I restriction site situated directly before the *GFP* gene contained in the construct ‘blue-

Table 1
Genes and encoded gene products used in this study

Gene	Gene product	Protein structure	Targeting signal	Reference
FB7-1	CHN		NSP[a],VTP	[35]
FB7-1ΔVTP	CHNΔVTP		NSP[a]	[32]
FB7-1-GFP	CHN-GFP		NSP[a], VTP	this study
FB7-1ΔVTP-GFP	CHNΔVTP-GFP		NSP[a]	this study
SUC2-GFP	INV-GFP		NSP[b]	this study
N-GFP-V	N-GFP-V		NSP[a], VTP	this study
N-GFP	N-GFP		NSP[a]	this study
GFP	GFP		none	[13]

a - NSP of the chitinase; b - NSP of the invertase.

SGFP-TYG-nos KS' [13]. The *Bam*HI fragments containing the gene fusions, *FB7-1-GFP* or *FB7-1ΔVTP-GFP* were inserted into the *Bam*HI restriction site of the plasmid pYES2.

To fuse the invertase gene *SUC2* to the *GFP* gene the *SUC2* gene was firstly flanked by *Xho*I/*Bgl*II and *Sal*I cleavage sites by a PCR. Chromosomal DNA of the strain S288C was used as template with the following oligonucleotides: (1) 5′-**CTCGAGAGATCT**ATGCTTTTGCAAGCTTTCCTT-3′ (nucleotide positions 1–32; *Xho*I and *Bgl*II restriction sites are in bold type) and (2) 5′-**GTCGAC**TTTTACTTCCCTTACTTGGAA-3′ (nucleotide positions 1580–1606 of the gene *SUC2*; *Sal*I restriction site is in bold type). The *SUC2* containing fragment was cloned into the vector pCR2.1. The *GFP* gene was inserted as a *Sal*I/*Sac*I fragment directly behind the invertase gene and the fusion was recloned as *Eco*RI/*Bgl*II fragment in the *Eco*RI/*Bam*HI restricted vector pYES2.

For the cytosolic GFP variant the *GFP* gene, *SGFP-TYG*, was inserted as *Hin*dIII/*Bam*HI fragment into the vector pYES2.

To fuse the sequences for the NSP as well as the C-terminal VTP of the chitinase to the gene *SGFP-TYG* the plasmid pYES2 containing the chitinase gene *FB7-1* [32] was used as template for the PCR with the two oligonucleotides 5′-CG**GTCGAC**GGATTAGAAGCCGCCGAGCGGGTGA-3′ (nucleotide positions 1–32 of the *GAL1* promoter, *Sal*I restriction site in bold type) and 5′-CTACTGCTTTCTGCCTCGGCAGAAC**CCATGG**TCC**TCTAGA**-3′ (nucleotide positions 49–74 of the *CHN* gene, *Nco*I and *Xba*I restriction sites are in bold type). The resulting *Sal*I-*Xba*I fragment containing the *GAL1* promoter and the sequence encoding the NSP of the *CHN* gene, *FB7-1*, was inserted into the construct 'blue-SGFP-TYG-nos KS' [13]. Subsequently, this construct was used as template for a second PCR to flank the NSP-SGFP-TYG-nos region with *Bam*HI restriction sites and with/without the VTP-sequence at the 3′ end. Therefore, the oligonucleo-

tides: 5′-CG**GGATCCCGGG**ATGAGGCTTTGTA-AATTCACAGCT-3′ (nucleotide positions 1–24 of the NSP-sequence, *Bam*HI and *Sma*I restriction sites are in bold type) and 5′-**GATCC**TTACATAG-TATCGACTAAAAGTCGCTTGTACAGCTCGT-CCA-TGCCGTG-3′ (nucleotide positions 711–743 of the *GFP* gene+VTP sequence of the *CHN* gene *FB7-1*, *Bam*HI restriction site in bold type) and 5′-**GGATCC**TTACTTGTACAGCTCGTCCATGCC-GTG-3′ (nucleotide sequence 711–743 of the *GFP* gene, *Bam*HI restriction site in bold type), respectively, were used. All constructs were verified by sequencing. *S. cerevisiae* strain C13ABYS86 was transformed according to Dohmen et al. [36]. Transformation of *E. coli* followed the procedure described by Hanahan [37].

2.3. Cell culture

The yeast transformants were grown in liquid SD medium+2% glucose as sole sources of carbon and energy at 28°C overnight to an OD_{600} between 0.6 and 1.2. Under these conditions the formation of the introduced heterologous gene products was repressed. Cells were inoculated in fresh SD medium+2% galactose at 0.1–0.3 OD_{600} and cultured at 28°C for different times, as indicated. For fluorescence microscopy cells were stained as described below. For Western blot experiments cells were harvested and extracted in TNT buffer (500 mM NaCl, 0.02% Triton X-100, 50 mM Tris-HCl, pH 8.0). Cells were frozen in liquid nitrogen, 3 glass beads (4 mm in diameter) and 300 μl buffer were added to the cell pellet. Subsequently, the cells were vortexed for 3 min and frozen. Vortexing and freezing steps were repeated twice. After centrifugation at $32\,000 \times g$ for 15 min the crude extracts were used for protein determination according to Bradford [38] using BSA as standard and for SDS-PAGE. The culture media were concentrated 100-fold by ultrafiltration using centricell units (Millipore, Bedford). 30 μg protein of the cell extracts and 50 μl of the concentrated culture media were used for SDS-PAGE.

2.4. Staining procedures and confocal laser scanning microscopy

The vacuolar membrane was stained with FM4-64 (Molecular Probes, OR) as described by Vida and Emr [39]. The following conditions were used for confocal microscopy (LSM 410, Zeiss, Jena, Germany): excitation at 488 nm with a krypton-argon laser (FT488/543) and detection using the BP510-525 nm emission filter allowed visualisation of GFP-mediated fluorescence with no significant autofluorescence background. 488 nm excitation and emission filter BP575-640 were used to visualise the red fluorescence of the dye FM4-64. Single images (512×512 pixels) were collected using line-averaging eight times, each for 1 s. Images were exported as TIFF files, and were processed for printing on a Tektronix Phaser II SDX using Adobe Photoshop.

2.5. Antibodies

Production of anti-chitinase antibodies of tobacco (anti-CHN) were described by Kunze et al. [32]. The polyclonal anti-GFP antiserum was obtained from Molecular Probes (Eugene, OR).

2.6. Gel electrophoresis and immunoblot analysis

SDS-PAGE was carried out as described by Laemmli [40] except that gradient gels from 11 to 20% polyacrylamide were used. Immune blots were prepared by the method of Towbin et al. [41]. Proteins were transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes and the membranes incubated for 1 h at room temperature in 0.5% Tween 20 in TBS buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.5). All GFP-containing gene products were detected by incubating the blots with anti-GFP antiserum (Molecular Probes, OR) and chitinases encoded by the genes *FB7-1* and *FB7-1ΔVTP* were identified by the anti-CHN antibody [32]. Goat anti-rabbit IgG or anti-mouse IgG coupled to alkaline phosphatase was used as secondary antibody. The blots were stained by Western Blue Stabilized Substrate (Promega, Heidelberg, Germany).

2.7. Subcellular fractionation experiments

Cells were grown for 20 h on SD medium to an OD_{600} of approximately 0.6. After preparation of sphaeroplasts as described by Maraz and Subik [42]

by lysing enzyme (Sigma-Aldrich, Deisenhofen, Germany) sucrose gradient centrifugations were performed as described by Kunze et al. [32].

2.8. Immunocytochemistry

Cells in 0.1 M phosphate buffer (pH 7.2) were either frozen without prefixation (see Figs. 4 and 6 below) or prefixed (see Fig. 5 below) with 3% formaldehyde and 0.5% glutaraldehyde for 1 h. The cell material was then used for high pressure freezing in a LEICA high pressure freezer HPF (Leica, Bensheim, Germany). Freeze substitution with ethanol+3% GA was carried out in an automated freeze substitution unit AFS (Leica, Bensheim, Germany) at −90°C for 44 h. After transfer into fresh medium the substitution was continued for additional 116 h. Subsequently, the temperature was raised slowly (0.5°C/min) to −35°C and the material washed twice in pure ethanol for 1 h. After resin infiltration the samples were embedded in gelatine capsules and the resin was hardened over a period of 70 h at −35°C by UV-polymerisation. Sections of resin-embedded cell material on grids were labelled as described earlier [32] using anti-CHN and anti-Kar2p/Bip antibodies. The grids were evaluated in a CEM 902A transmission electron microscope (Carl Zeiss, Oberkochen, Germany) and pictures were taken on Kodak SO-163 films (Kodak, Ostfildern, Germany). The EM pictures were scanned from the photographical negatives using an AGFA Duoscan scanner (Agfa-Galvert, Köln, Germany). Pictures were processed using FotoFinish 3.0 (WATC, Atlanta, WV) and Word 6.0 (Microsoft, USA) software.

3. Results

3.1. GFP retains secretory proteins inside yeast cells

The following eight gene constructs (Table 1) were introduced under control of the *GAL*1 promoter into *S. cerevisiae* Cl3ABYS86 [32] to study the suitability of GFP as a marker for the intracellular transport of vacuolar and secretory proteins in yeast: (1) three GFP fusions comprising the yeast secretory invertase, the plant vacuolar chitinase and its secretory derivative C-terminally fused to GFP, (2) both chitinase forms, (3) GFP without any targeting information, and (4) two secretory GFP variants with and without the chitinase VTP. The mutant strain *S. ce-*

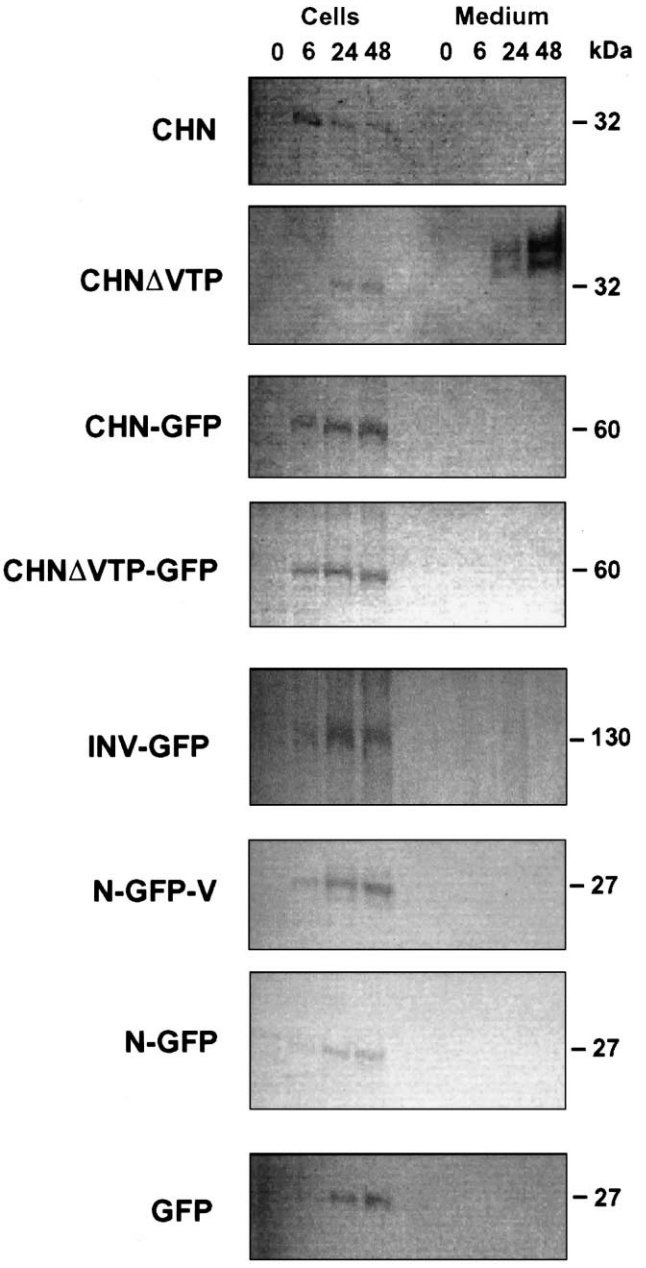


Fig. 1. Detection of CHN, CHNΔVTP, CHN-GFP, CHNΔVTP-GFP, INV-GFP, N-GFP-V, N-GFP and GFP in cell extracts and concentrated media of transformants after 0 (1), 6 (2), 24 (3) and 48 h (4) of incubation in liquid SD medium+2% galactose. 30 μg protein of cell extracts and 50 μl of 100-fold concentrated media were separated by SDS-PAGE and immunoprobed with anti-CHN in case of CHN and CHNΔVTP and anti-GFP antibodies for all GFP derivatives.

revisiae Cl3ABYS86 lacks activity of the vacuolar proteases A, B and Y and should therefore be a suitable host to study intracellular transport and accumulation of heterologous gene products. Firstly, we wanted to know whether the gene products are accumulated inside the cells or secreted into the culture medium. Therefore, stationary phase cells grown on SD medium with 2% glucose were induced by galactose containing SD medium (2%) for 0, 6, 24 and 48 h. At each time cell extracts and 100-fold concentrated culture media were used for Western blot experiments with the anti-chitinase antibody, anti-CHN, to identify the CHN and CHNΔVTP and the anti-GFP antibody for all GFP derivatives (CHN-GFP, CHNΔVTP-GFP, INV-GFP, N-GFP, N-GFP-V) (Fig. 1). In extracts of cells expressing the CHN and the CHNΔVTP one polypeptide of approximately 32 kDa was visible. A high portion of CHNΔVTP was accumulated in the culture medium, where two chitinase polypeptides of 32 and 37 kDa were detected, which was very likely due to a partial glycosylation of the plant enzyme [32]. In contrast, all three GFP fusion proteins and both secretory GFP variants were retained inside the cells. This retention inside the cells occurred obviously independently of a known retention signal.

In extracts of transformant cells expressing the fusion protein CHN-GFP one polypeptide of approximately 60 kDa was detected with the anti-GFP antibody. This indicates that CHN-GFP was not processed at the VTP, because an appropriate cleavage would result in two single proteins of 32 kDa (CHN) and 27 kDa (GFP).

The molecular mass of the GFP encoded by the genes N-GFP and N-GFP-V was 27 kDa which was identical to that of the cytosolic GFP (Fig. 1). That means that the 23 amino acids long NSP of N-GFP and N-GFP-V has been cleaved off, indicating their passage through the endoplasmic reticulum.

3.2. GFP targets secretory proteins to the yeast vacuole

Intracellular GFP-accumulation was firstly visualised by confocal laser scanning fluorescence microscopy. Transformant cells expressing the GFP derivatives were incubated in SD medium+2% galactose for 20 h and subsequently treated with the red fluorescent dye FM4-64 (see Section 2) which stains the tonoplast of yeast [39]. The overlays of red FM4-64 and green GFP-fluorescence clearly show the position of the gene products in relation to the vacuole (Fig. 2). GFP encoded by the genes *N-GFP* and *N-GFP-V* was already completely inside the vacuole after 20 h of incubation whereas the cytosolic GFP variant was distributed homogeneously in the cytoplasm and was completely excluded from the vacuole (Fig. 2). In contrast, all fusion proteins were found inside the vacuole and additionally in concentrated fluorescent spots outside the vacuole.

The complete vacuolar accumulation of the fusion proteins is temporary delayed. After 15 h of incubation in galactose-containing medium only 6–12% of the fluorescent cells expressing the fusion CHN-GFP show exclusive vacuolar fluorescence without detectable fluorescence outside the vacuole, whereas the remaining 88–94% of cells showed fluorescence inside as well as outside the vacuole. The portion of cells with exclusive vacuolar fluorescence increased to 55–65% after 20 h and reached 80–95% after 24 h of induction. The time-dependent accumulation of CHNΔVTP-GFP and INV-GFP was very similar to that of CHN-GFP.

If the synthesis of the fusion proteins was repressed after 20 h of induction by adding glucose to a final concentration of 2% the fluorescence was completely accumulated inside the vacuole during the next 8 h of incubation (not shown).

The vacuolar accumulation of both secretory GFPs with and without the chitinase VTP (N-GFP-V and N-GFP) and of one fusion protein (INV-GFP) was confirmed by subcellular fractionation experiments. Both transformants grown for 20 h in galactose containing medium were gently lysed and layered on top of a sucrose gradient. After centrifugation, 20 fractions were collected from bottom to top of the gradient and enzyme activities as markers for different organelles were measured. In addition, aliquots of fractions with odd numbers were used for Western blot experiments with the anti-GFP antibody to identify GFP (Fig. 3A and B) and INV-GFP (Fig. 3C). Most of the cytochrome *c* reductase activity was measured near the bottom of the gradient indicating that the ER was represented there. The cytosolic enzyme GAP-DH was found near the top of the gradient, in fractions 15–19 of the trans-

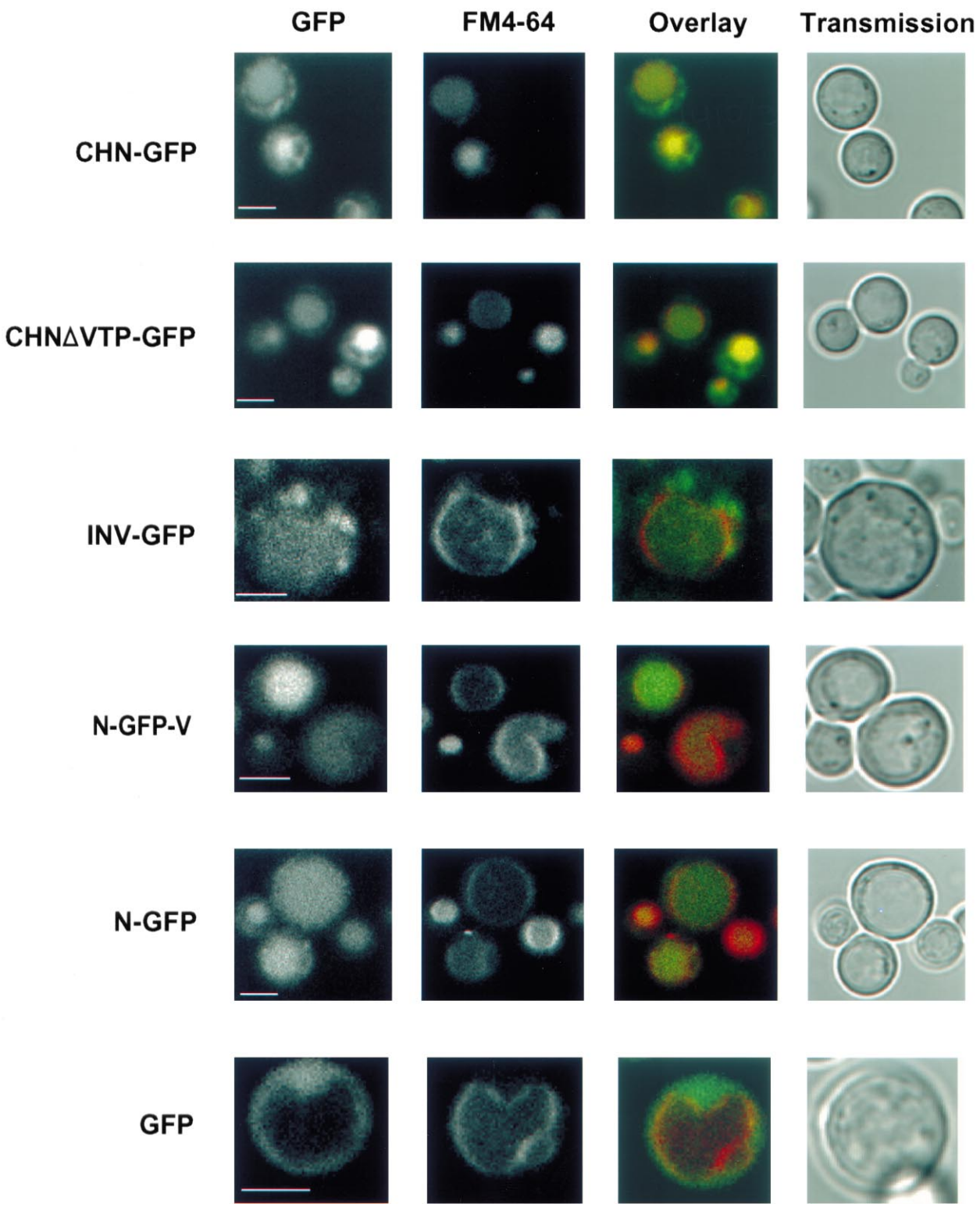


Fig. 2. Confocal images of transformant cells expressing the fusions CHN-GFP, CHNΔVTP-GFP, INV-GFP, the engineered GFP variants N-GFP-V and N-GFP and the cytosolic GFP. Cells were cultured for 20 h in SD medium+2% galactose and subsequently used for staining. The vacuolar membrane was stained by the specific fluorescent dye FM4-64 [39]. Bars represent 10 μm.

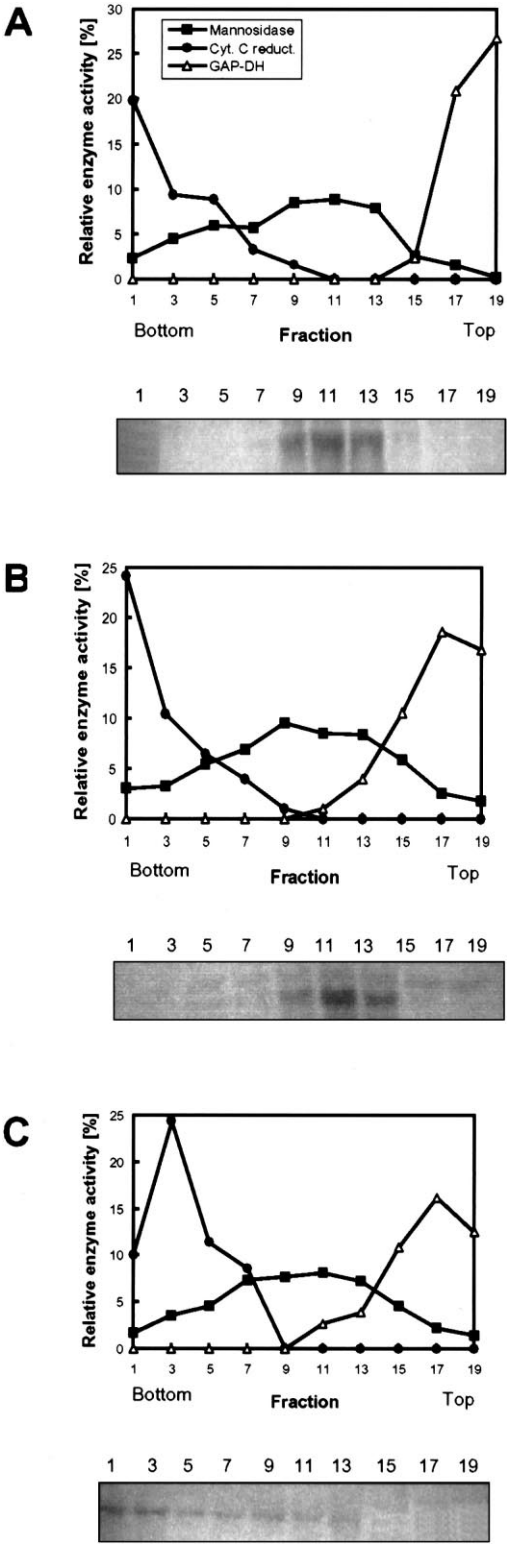


Fig. 3. (A, B, C) Subcellular fractionation of transformants expressing N-GFP-V (A), N-GFP (B) and INV-GFP (C). Enzyme activities were normalised to those measured in an aliquot of the mixture layered on the top of the gradient. After 20 h of cultivation, cells were used for spheroplasting, gently lysed and loaded on the top of a sucrose gradient (15–55%). After centrifugation to equilibrium, fractions (500 μl) were collected from the bottom and assayed for cytochrome *c* reductase (Cyt. *c* reduct.), α-mannosidase, and glyceraldehyde-3-phosphate dehydrogenase (GAP-DH). Aliquots were additionally used for Western blotting with the anti-GFP antibody to identify the gene products.
←

formant expressing N-GFP-V and 13–19 of the transformant expressing N-GFP. The highest activity of the α-mannosidase, which was used as a marker for vacuoles, was measured in fractions 9–13. Only in these fractions GFP was detected. This correlation confirmed our hypothesis of the vacuolar localisation of N-GFP-V and N-GFP.

The fusion protein INV-GFP was found in fractions 1–13. These fractions contained the ER marker cytochrome *c* reductase (fractions 1–7) and the vacuolar marker enzyme α-mannosidase with the highest α-mannosidase activity in fractions 7–13. This distribution over ER- and vacuolar fractions led us to conclude that INV-GFP was partly accumulated in the ER as well as in the vacuole after 20 h of induction.

3.3. CHN and CHN-GFP are transiently accumulated in an ER-dilated compartment

Immunocytochemical experiments using the anti-CHN antibody were performed to analyse the intracellular localisation sites of the proteins CHN-GFP and CHN in more detail (Fig. 4A,B, Fig. 5A,B). In cells of both transformants incubated for 1 day on SD-medium with 2% galactose protein A gold particles were associated with the vacuole (Fig. 4B, Fig. 5A) and additionally with electron-dense structures located in the cytoplasm (Fig. 4A, Fig. 5B). These electron-dense structures also contained the protein Kar2p/Bip (Fig. 6A,B), which is a chaperone of the ER often found in association with misfolded proteins to retain them in the ER [43].

Differential centrifugation experiments using transformant cells expressing the fusion protein CHN-

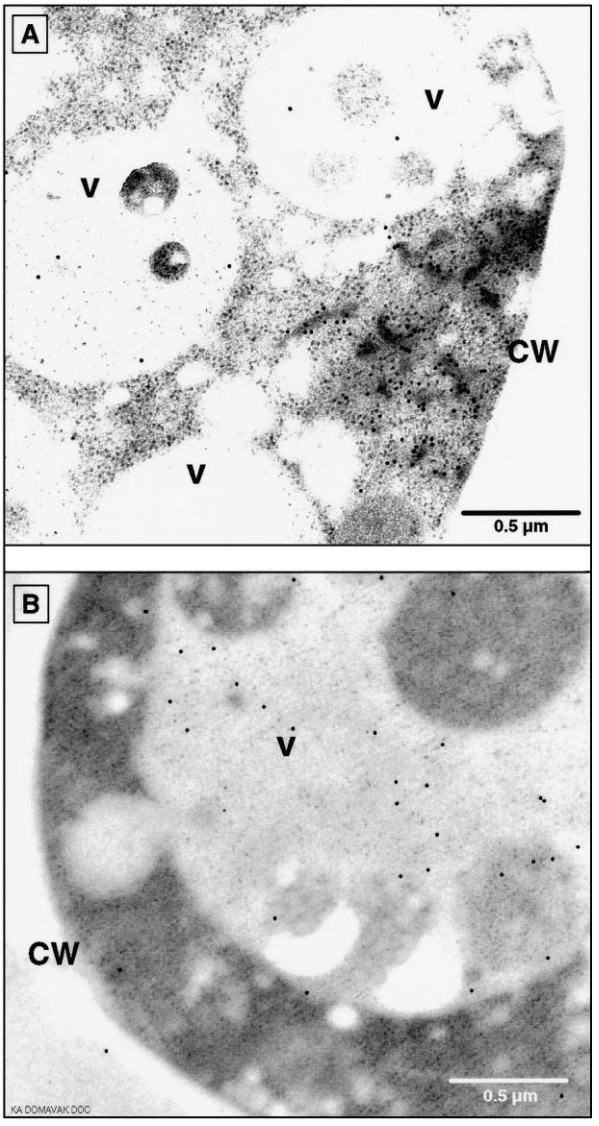


Fig. 4. (A, B) Immunocytochemical localisation of the CHN-GFP fusion protein in two transformant cells (A and B) of *S. cerevisiae* Cl3ABYS86 with anti-CHN antibody after incubation in SD medium+2% galactose for 1 day.

GFP after 15 h of induction confirmed its association to membranes (not shown).

4. Discussion

GFP is worth to be a suitable tag to follow the transport of proteins to their correct cellular compartment. However, analysis of the default pathway of soluble proteins tagged with GFP are missing in yeast and plants and are rare in animal cells [18] and mammalian cells [44]. In insect cells, for example, Laukkanen et al. [18] demonstrated that engineering of a signal peptide to GFP is sufficient to cause secretion of fluorescent GFP into the culture medium. The authors used *Trichoplusia ni* (High Five) and *Spodoptera frugiperda* (Sf21) cells for expression of epitope (Flag)-tagged signal peptide-GFP. Thereby

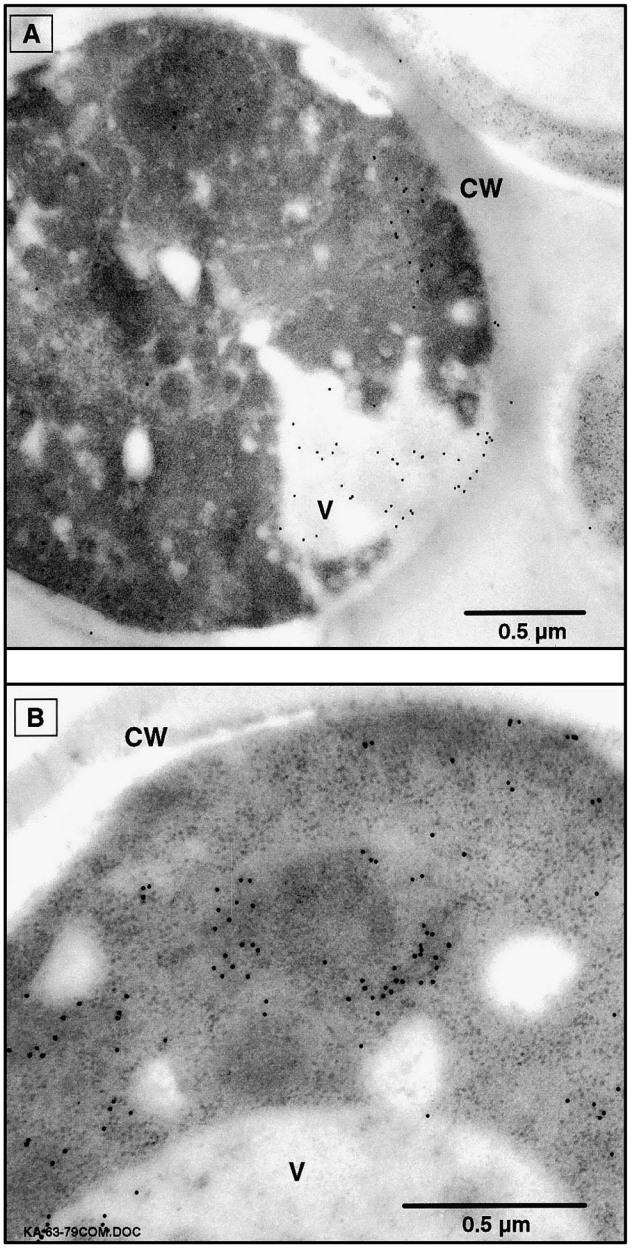


Fig. 5. (A, B) Immunocytochemical localisation of the CHN in two transformant cells (A and B) of *S. cerevisiae* Cl3ABYS86 harbouring the gene *FB7-1* with anti-CHN antibody after incubation in SD medium+2% galactose for 1 day.

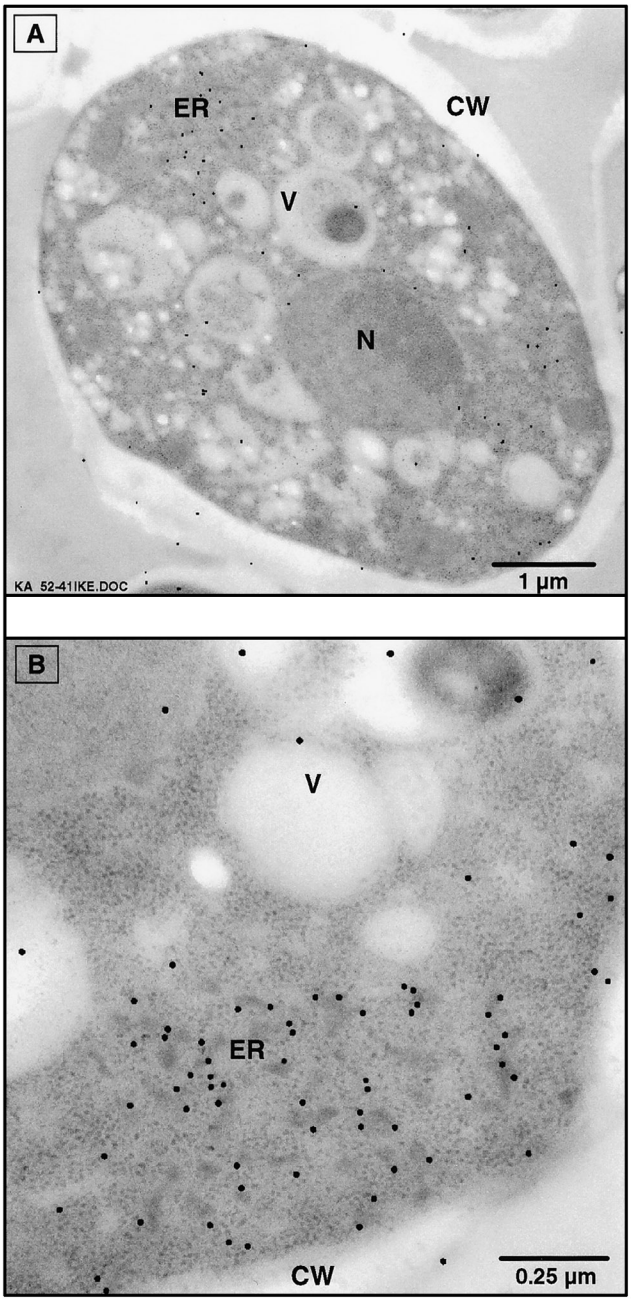


Fig. 6. (A, B) Immunocytochemical localisation of the ER chaperone Kar2p/Bip in two transformant cells (A and B) of *S. cerevisiae* Cl3ABYS86 expressing the fusion protein CHN-GFP incubated in SD medium+2% galactose for 1 day.

the recombinant baculovirus-infected cells became fluorescent and the High Five cells but not the Sf21 cells secreted GFP into the culture medium.

We expressed several GFP constructs in the strain *S. cerevisiae* Cl3ABYS86. Three GFP fusions comprising the yeast secretory invertase, the plant vacuolar chitinase and its secretory derivative C-terminally fused to GFP and two secretory GFPs engineered with the NSP of the plant chitinase with (N-GFP-V) and without the chitinase VTP (N-GFP). All GFP derivatives were retained inside the cells and transported to the yeast vacuole so far as they contained a NSP enabling them to enter the ER. This vacuolar transport occurred independent of the presence of a known VTP. This was surprising since soluble proteins have been shown to require a targeting signal for deposition in the vacuole [45]. Deletion or changes of the targeting sequence from soluble vacuolar proteins resulted in a more or less efficient secretion of the protein to the cell surface, which was demonstrated for homologous proteins, e.g. vacuolar carboxypeptidase Y [46] as well as for heterologous proteins like tobacco chitinase ([32], this study) and phytohaemagglutinin (PHA), the major seed lectin of the common bean, *Phaseolus vulgaris* [47]. Sequences that are involved in the vacuolar sorting bear homology to the carboxypeptidase Y (CPY). This CPY homologous domain was sufficient, but not strictly required, for vacuolar sorting of PHA in yeast, because some mutations in different PHA constructs did not result in the same increase in secretion [47]. There are other reports that document a vacuolar destination of heterologous proteins lacking specific vacuolar targeting domains, e.g. the plant protein sporamin, which, independently of the propeptide, ends up in the vacuole [46]. A fusion between yeast secretory invertase and barley lectin lacking the carboxy-terminal propeptide was retained inside the cells, presumably in the vacuole [48] suggesting that at least in some cases signals other than those recognised in homologous hosts can be used in yeast for vacuolar targeting.

Despite the common vacuolar destination for all GFP-constructs containing a NSP differences in the temporal manner of transport were observed between the engineered GFPs (N-GFP-V and N-GFP) on one hand and the GFP fusions (CHN-GFP, CHNΔVTP-GFP and INV-GFP) on the other hand. The later ones were transported with the same temporal delay as the plant chitinase. This delayed transport of the chitinase and the GFP fusions by a transient accumulation in extravacuolar compartments is not to

explain by overexpression because the secretory GFP variants equipped with the signal peptides of the chitinase (N-GFP-V) is exclusively accumulated inside the vacuole after 20 h of induction. The same compartments also contain the ER chaperone Kar2p/Bip, which is known to associate with newly synthesised proteins until they reach their correct folding. Misfolded proteins often stay associated with Bip [43] and are therefore retained in the ER for degradation [43,46]. In yeast cells 'ER degradation' was described, for example, by Finger et al. [49] for two mutated vacuolar proteins, proteinase yscA (PrA) and carboxypeptidase yscY (CPY) which are degraded rapidly after entering the secretory pathway. However, misfolded soluble proteins can also be transported to the vacuole. Hong et al. [50] have recently shown, that the hybrid protein carrying the wild-type repressor domain of α repressor protein and the secreted protein invertase is mostly secreted to the cell surface, whereas thermodynamically unstable hybrid proteins are transported to the vacuole.

The structure of the electron-dense vesicles accumulating chitinase and chitinase GFP fusions resembles to an ER subcompartment recently described by Umebayashi et al. [51] for retention of misfolded proteins like the analysed prosequence-deleted derivative of RNAP-I, an aspartic proteinase from a filamentous fungus *Rhizopus niveus*. The intact protein is secreted very efficiently in *S. cerevisiae* and it was shown that the prosequence of this protein is important for its correct folding and secretion. The misfolded protein is sorted from the ordinary ER lumen to form aggregates so that the ER function would not be grossly impaired, and the dilated ER may represent an ER subcompartment where the misfolded protein are degraded. In contrast to our results, Umebayashi et al. [51] did not found any vacuolar accumulation for their gene product.

We cannot exclude a partial misfolding of our gene products, but we do not favour this hypothesis, whether to explain the transient accumulation in ER-dilated compartments (1) nor for the targeting of secretory GFP-tagged proteins to the vacuole (2):

1. If the exact translocation mechanism of the fusions CHN-GFP and CHNΔVTP-GFP is determined by the chitinase itself one would expect that its derivative CHNΔVTP encoded by the truncated gene without the VTP sequence (*FB7-1ΔVTP*) should be accumulated in the same way. But this is not the case, it is exported into the medium.
2. GFP was shown to be correctly targeted to many compartments and organelles in eukaryotic cells, misfolding caused by GFP appears therefore unlikely to explain the vacuolar deposition of the GFP constructs, unless the N-terminal signal processing in the ER leads to a partially misfolded, yet still fluorescent GFP. Since the GFP structure is so highly compact and has not permitted any major structural alterations at the C- and N-termini without loss of chromophore formation.

Therefore, we favour instead a model which involves as yet unknown peptide structures of GFP in conjunction with the NSP which can be recognised by the yeast vacuolar sorting machinery and, therefore, result in a deposition to the vacuole.

Taken together, our results demonstrate that GFP directs secretory proteins to the yeast vacuole. This occurs in absence of a specific VTP. Thus, a general use of GFP as a marker for analyzing the secretory pathway in yeast appears limited and has to be considered very carefully in the absence of any further specific targeting information.

Acknowledgements

We thank R. Schekman (University of California, Berkeley, CA) for his helpful discussions and the kind gift of the anti-Kar2p/Bip antibody. We also thank the CSIRO Division of Plant Industry (Canberra, Australia) for the kind gift of the cDNA *FB7-1* of tobacco, J. Sheen (Department of Molecular Biology, Boston, MA) for the GFP gene, *SGFP-TYG* and D.H. Wolf (University of Stuttgart, Stuttgart, Germany) for the *S. cerevisiae* strain Cl3A-BYS86. We gratefully acknowledge the skilful technical assistance of W. Panitz and B. Claus. The experimental work was supported by the Deutschen Forschungsgemeinschaft (SFB363), the Fonds zur Förderung der wissenschaftlichen Forschung in Österreich (SFB F706 to S.D.K.) and by the Fonds of Chemical Industry (G.K.).

References

[1] M. Chalfie, Y. Tu, G. Euskirchen, W.W. Ward, D.C. Prasher, Science 263 (1994) 802–805.
[2] R. Rizzuto, M. Brini, P. Pizzo, M. Murgia, T. Pozzan, Curr. Biol. 5 (1995) 635–642.
[3] H.-H. Gerdes, C. Kaether, FEBS Lett. 389 (1996) 44–47.
[4] R.K. Niedenthal, L. Riles, M. Johnston, J.H. Hegemann, Yeast 12 (1996) 773–786.
[5] R. Heim, D.C. Prasher, R.Y. Tsien, Proc. Natl. Acad. Sci. USA 91 (1994) 12501–12504.
[6] R. Heim, A.B. Cubitt, R.Y. Tsien, Nature 373 (1995) 663–664.
[7] S. Delagrava, R.E. Hawtin, C.M. Silva, M.M. Yang, D.C. Youvan, BioTechnology 13 (1995) 151–154.
[8] T. Ehrig, D.J. O'Kane, F.G. Prendergast, FEBS Lett. 367 (1995) 163–166.
[9] R. Heim, R.Y. Tsien, Curr. Biol. 6 (1996) 178–182.
[10] T.T. Yang, L.Z. Cheng, S.R. Kain, Nucleic Acids Res. 24 (1996) 4592–4593.
[11] W.-L. Chiu, Y. Niwa, W. Zeng, T. Hirano, H. Kobayashi, J. Sheen, Curr. Biol. 6 (1996) 325–330.
[12] S.Z. Pang, D.L. Deboer, Y. Wan, G.B. Ye, J.G. Layton, M.K. Neher, C.L. Armstrong, J.E. Fry, M.A.W. Hinchee, M.E. Fromm, Plant Physiol. 112 (1996) 893–900.
[13] J. Sheen, S. Hwang, Y. Niwa, H. Kobayashi, D.W. Galbraith, Plant J. 8 (1995) 777–784.
[14] T. Doyle, D. Botstein, Proc. Natl. Acad. Sci. USA 93 (1996) 3886–3891.
[15] J.L. Carminati, T. Stearns, J. Cell Biol. 138 (1997) 629–641.
[16] I. Wacker, C. Kaether, A. Krömer, A. Migala, W. Almers, H.-H. Gerdes, J. Cell Sci. 110 (1997) 1453–1463.
[17] C. Kaether, H.-H. Gerdes, FEBS Lett. 369 (1995) 267–271.
[18] M.-L. Laukkanen, C. Oker-Blom, K. Keinänen, Biochem. Biophys. Res. Commun. 226 (1996) 755–761.
[19] C. Kaether, T. Salm, M. Glombik, W. Almers, H.-H. Gerdes, Eur. J. Cell Biol. 74 (1997) 133–142.
[20] P. Boevink, S. Santacruz, C. Hawes, N. Harris, K.J. Oparka, Plant J. 10 (1996) 935–941.
[21] R.Y. Hampton, A. Koning, R. Wright, J. Rine, Proc. Natl. Acad. Sci. USA 93 (1996) 828–833.
[22] L.S. Barak, S.S. Ferguson, J. Zhang, C. Martenson, T. Meyer, M.G. Caron, Mol. Pharmacol. 51 (1997) 177–184.
[23] T.V. Kurzchalia, C. Forker, H. Böhm, Eur. J. Cell Biol. 69 (1996) Abstract 447.
[24] K. Moriyoshi, L.J. Richards, C. Akazawa, D.D.M. O'Leary, S. Nakanishi, Neuron 16 (1996) 255–260.
[25] R. Grebenok, E. Pierson, G.M. Lambert, F.-C. Gong, C.L. Afonso, R. Haldeman-Cahill, J.C. Carrington, D.W. Galbraith, Plant J. 11 (1997) 573–586.
[26] M. Bucci, S.R. Wente, J. Cell Biol. 136 (1997) 1185–1199.
[27] S.C. Ushinsky, H. Bussey, A.A. Ahmed, Y. Wang, J. Friesen, A. Williams, D. Storms, Yeast 13 (1997) 151–161.
[28] F. Degiorgi, M. Brini, C. Bastianutto, R. Marsault, M. Montero, P. Pizzo, R. Rossi, R. Rizzuto, Gene 173 (1996) 113–117.
[29] M. Prescott, A. Lourbakos, M. Bateson, G. Boyle, P. Nagley, R.J. Devenish, FEBS Lett. 411 (1997) 97–101.
[30] J.E. Kalish, G.A. Keller, J.C. Morrell, S.J. Mihalik, B. Smith, J.M. Cregg, S.J. Gould, EMBO J. 15 (1996) 3275–3285.
[31] E.Z. Monosov, T.J. Wenzel, G.H. Luers, J.A. Heyman, S. Subramani, J. Histochem. Cytochem. 44 (1996) 581–589.
[32] I. Kunze, C. Nilsson, K. Adler, R. Manteuffel, C. Horstmann, M. Bröker, G. Kunze, Biochim. Biophys. Acta 1395 (1998) 329–344.
[33] M. Bröker, O. Bäuml, A. Göttig, J. Ochs, M. Bodenbrenner, E. Amann, Appl. Microbiol. Biotechnol. 34 (1991) 756–764.
[34] M.D. Rose, F. Winston, P. Hieter, in: Methods in Yeast Genetics, A Laboratory Course Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1990.
[35] D.R. Meeks-Wagner, E.S. Dennis, K.T.T. Van, W.J. Peacock, Plant Cell 1 (1989) 25–35.
[36] R.J. Dohmen, A.W.M. Strasser, U. Dahlems, C.P. Hollenberg, Yeast 7 (1991) 691–692.
[37] D. Hanahan, J. Mol. Biol. 166 (1983) 557–580.
[38] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[39] T.A. Vida, S.D. Emr, J. Cell Biol. 128 (1995) 779–792.
[40] U.K. Laemmli, Nature 227 (1970) 680–685.
[41] H. Towbin, T. Staehelin, J. Gordon, Proc. Natl. Acad. Sci. USA 76 (1979) 4350–4354.
[42] A. Maraz, J. Subik, Mol. Gen. Genet. 181 (1981) 131–133.
[43] C. Hammond, A. Helenius, Curr. Biol. 7 (1995) 523–529.
[44] N.V. Burke, W. Han, D. Li, K. Takimoto, S.C. Watkins, E.S. Levitan, Neuron 19 (1997) 1095–1102.
[45] B.F. Horazdovsky, S.D. Emr, J. Biol. Chem. 268 (1993) 4953–4962.
[46] K. Matsuoka, K. Nakamura, Plant Cell Physiol. 33 (1992) 453–462.
[47] B.W. Tague, C.D. Dickinson, M.J. Chrispeels, Plant Cell 2 (1990) 533–546.
[48] S. Gal, N.V. Raikhel, Plant J. 6 (1994) 235–240.
[49] A. Finger, M. Knop, D.H. Wolf, Eur. J. Biochem. 218 (1993) 565–574.
[50] E. Hong, A.R. Davidson, C.A. Kaiser, J. Cell Biol. 135 (1996) 623–633.
[51] K. Umebayashi, A. Hirata, R. Fukuda, H. Horiuchi, A. Ohta, M. Takagi, Yeast 13 (1997) 1009–1020.